

# Optimization of the Enzymatic Hydrolysis of Poultry Slaughterhouse Wastes using Alcalase Enzyme for the Preparation of Protein Hydrolysates

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## Abstract

**Background and objective:** Continuous increases in poultry production have led to increases in slaughterhouse wastes and needs for control of these organic matter-rich wastes. Previously, burning and burial methods were used to control the wastes that contaminated the environment and spread diseases. Nowadays, hydrolysis of the waste proteins is an effective way to produce new added-value products and to control wastes. The aim of this study was to achieve optimal conditions for the enzymatic hydrolysis of the industrial wastes from poultry slaughterhouses.

**Material and methods:** To achieve the highest hydrolysis efficiency, an experimental design was used based on the surface response methodology to assess optimum conditions of the hydrolysis reaction parameters. Factors, including temperature (50-70°C), pH (6.5-8.5) and enzyme to substrate ratio (1.5-5.5% (v w<sup>-1</sup>)), were investigated at five levels using central composite design. Degree of the hydrolysis was considered as response.

**Results and conclusion:** Statistical analysis showed that the regression coefficient (R<sup>2</sup>) for the model included 0.9592, which indicated a high accuracy of the model to predict the reaction conditions with variables. The highest degree of hydrolysis was achieved as 12.78% at an enzyme/substrate ratio of 3.5%, pH of 7.5 and temperature of 60°C. Under optimized conditions, hydrolyzed proteins included molecular weights less than 14.5 kDa. Results have shown that enzymatic hydrolysis of the whole poultry slaughterhouse wastes is possible using alcalase as a protease enzyme.

**Conflict of interest:** The authors declare no conflict of interest.

## Article Information

### Article history:

Received 20 Dec 2019  
Revised 15 March 2020  
Accepted 02 May 2020

### Keywords:

- Alcalase
- Degree of hydrolysis
- Protein hydrolysis
- Poultry slaughterhouse waste
- Response surface methodology

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## How to cite this article

Mirzaei Teshnizi Z, Robotjazi SM, Mohammadian Mosaabadi J, Optimization of the Enzymatic Hydrolysis of Poultry Slaughterhouse Wastes using Alcalase Enzyme for the Preparation of Protein Hydrolysates. *Appl Food Biotechnol* 2020; 7(3): 153-160. <http://dx.doi.org/10.22037/afb.v7i3.28417>

## 1. Introduction

Global production and marketing of the poultry meats have increased rapidly over the last 50 years. The chicken slaughterhouses produce much wastes, including feathers, legs, heads, bones, viscera and bloods. These wastes are often used as livestock feeds, soil fertilizers and pet foods [1,2]. These wastes include numerous biomolecules such as proteins that can be processed to valuable products by recycling. These compounds can be converted into products that can be useful in pharmacy, microbiology, nutrition, medicine and cosmetics [1-4]. Hydrolysis of proteins to prepare amino acids and peptides smaller than the raw materials can be carried out using chemical or enzymatical

methods [5]. Enzymatic hydrolysis is an effective, controllable and repeatable method for recycling biomolecules such as inaccessible protein sources of the wastes into protein hydrolysates with high degrees of hydrolysis. In addition to help waste recycling, this method produces value-added products. Studies have shown that controlled hydrolysis releases bioactive peptides and enhances functional properties such as antioxidant, antihypertensive and emulsifying properties as well as fat adsorption and solubility in hydrolyzed products. Antioxidant, antihypertensive and antibacterial activities of the protein hydrolysates are affected by the protein source, enzyme

specificity, proteolysis condition, degree of hydrolysis and characteristics of the released peptides such as their molecular weight, amino acid sequence and hydrophobicity [6,7]. Protein hydrolysates are widely used; for example, in nutrition for patients who are unable to digest intact proteins, in microbiology as a source of nitrogen and carbon in growth media and in animal foods [2]. Hydrolysis of the chicken wastes has been investigated in previous studies including autolysis of viscera poultries [8], enzymatic hydrolysis of chicken meats [9], hydrolysis of wet poultry industry residues [10], enzymatic hydrolysis poultry heads and legs [11]. Other studies have been carried out on chicken feather hydrolysis using chemical and enzymatic treatments [12-14]. Several studies have used enzymatic methods to produce protein hydrolysates [2,15]. Enzymatic methods are carried out under mild controlled conditions and maintaining nutritional values. Hence, it provides better and distinct chemical and nutritional properties for the hydrolyzed proteins [16,17]. Enzymatic hydrolysis of the chicken wastes investigated using diverse enzymes such as alcalase, Protamex, flavourzyme, liquipanol and pepsin. Alcalase is characterized as a more appropriate enzyme choice than other enzymes for the production of protein hydrolysates with high degrees of hydrolysis and recovery [9,10,15]. Generally, alkaline proteases such as alcalase demonstrate higher activities and degrees of hydrolysis than neutral and acid proteases [18]. Optimizing parameters involved in hydrolysis, researchers have used experimental design methods such as response surface methodology [10,11,19]. In the current study, production of the protein hydrolysates from powdered dried poultry wastes such as legs, skins, heads, bones, viscera and bloods was carried out with no separations using enzymatic hydrolysis with alcalase. Effects of variables such as enzyme to substrate ratio, temperature and pH were investigated on hydrolysis and optimization of the processes using response surface methodology to achieve the highest degree of hydrolysis.

## 2. Materials and methods

Poultry slaughterhouse wastes were prepared from ETKA, Iran. First, industrial poultry wastes, including heads, legs, bones, viscera and feathers, were collected. A heating step of 110°C was used for 15 min to inactivate internal enzymes followed by addition of a toxin binder (0.1% Captex T2) to the wastes. Wastes were dried eventually using tray dryer at 45-55°C for 24 h. Dried brown powders were transferred to the laboratory in plastic bags for enzymatic hydrolysis and stored at -20°C until use. Total protein content was assessed as 450 mg g<sup>-1</sup> of the waste powder using Kjeldahl method. Alcalase 2.4 L FG, (EC No. 3.4.21.62) with an activity of 2.4 AU-A g<sup>-1</sup> was purchased from Novozymes, Denmark, and stored at 4°C until use.

### 2.1. Preparation of the raw materials for hydrolysis

Dried powders of the chicken wastes were mixed with distilled water to a ratio of 1:4 (waste: distilled water) and homogenized using conventional rotor homogenizer with a stainless steel probe of 7 mm (Heidolph DIAX 100, Germany) at 1500 rpm for 5 min and the suspensions were used for the optimization experiments. Samples were centrifuged at 5000 ×g for 25 min, supernatants were separated and plates were discarded. Quantities of the extracted proteins were assessed using Biuret method.

### 2.2. Optimization of the experiments

Experiments were designed to optimize the hydrolysis conditions using response surface methodology and central composite design model. This method accelerates research process by decreasing number of experiments and costs. In this study, three independent variables, including enzyme to substrate ratio, temperature and pH, were investigated using central composite model of each variable with five levels of distance (-α, -1, 0, +1, +α) [9]. This model proposed 20 experiments totally, including six replicas in the center point. The degrees of hydrolysis was considered as the output variable (Table 1). Each experiment included 35 ml of the suspensions of chicken wastes in water, incubated at 50-70°C, pH of 6.5-5.8 and enzyme to substrate ratio of 1.5-5.5%. The pH of reaction was continuously checked every 5 min within the first 20 min. This was continued every 10 min and then every 20 min (after 1 h). The pH decreases were compensated to maintain the initial pH by adding 2 N sodium hydroxide. Hydrolysis process continued until pH did not change and sodium hydroxide was not utilized. The quantity of NaOH used to maintain the pH was recorded to calculate degree of hydrolysis. After complement of the hydrolysis process, samples were fixed using water bath at 85°C for 20 min to inactivate the enzyme. Hydrolyzed samples were cooled down to ambient temperature; then, samples were centrifuged to remove solid particles at 6010 ×g for 20 min. Soluble protein layers were separated and the residuals were discarded.

**Table 1.** Three independent variables at five levels used in design of the experiments by central composite design.

The parameters	levels				
	-α	-1	0	+1	+α
Enzyme-to-substrate ratio	1.5	2.5	3.5	4.5	5.5
Temperature	50	55	60	65	70
pH	6.5	7	7.5	8	8.5

### 2.3. Assessment of the degree of hydrolysis

The degrees of hydrolysis of reaction was calculated using pH-stat method. Quantity of the utilized NaOH was directly linked to the number of hydrolyzed peptide bonds and used to estimate the degrees of hydrolysis. The recent relationship was presented by Alder Nissen in the Eq 1:

$$DH = \frac{B \times N_B}{M_P \times h_{tot} \times \alpha} \times 100 \quad \text{Eq.1}$$

Where, B was the volume of utilized NaOH (ml),  $N_B$  was the normality of NaOH,  $M_P$  was the mass of protein which was often calculated as nitrogen content and used to assess the protein content by a factor of 6.25,  $h_{tot}$  was the number of peptide bonds in the substrate ( $7.7 \text{ mEq g}^{-1}$  protein) and  $\alpha$  was the average degree of dissociation of  $\alpha\text{-NH}_2$  groups as described in the Eq. 2 [9]:

$$\alpha = \frac{1}{1 + 10^{pK - pH}} \quad \text{Eq. 2}$$

The pK strongly depended on temperature and could be achieved by Eq. 3:

$$pK = 7.8 + \frac{298 - T}{298 \times T} \times 2400 \quad \text{Eq. 3}$$

Where, T was temperature in Kelvin degree.

#### 2.4. Assessment of the protein recovery

Proportions of the extracted proteins linked to the substrates were calculated according to the Eq. 4:

$$PR(\%) = \frac{MP_s}{MP} \times 100 \quad \text{Eq. 4}$$

Where, PR was the protein recovery (%),  $MP_s$  was the mass of protein in the supernatant (g) and MP was the mass of protein in the initial substrate (g) [9]. Protein contents in supernatants and original substrates were assessed using Biuret and Kjeldahl methods, respectively.

### 3. Results and discussion

#### 3.1. Analysis of variance

In this study, experiments were carried out using response surface methodology. For each experiment, the pH-stat method was used to assess the degrees of hydrolysis. Effects of temperature ( $X_1$ ), pH ( $X_2$ ) and enzyme to substrate (E/S) ratio ( $X_3$ ) on the degrees of hydrolysis were investigated using central composite design (Table 2). Regression analysis was used to fit a full response surface model for the responses investigated, including all linear ( $X_1, X_2, X_3$ ), interaction ( $X_1X_2, X_1X_3, X_2X_3$ ) and second-order terms ( $X_{11}, X_{22}, X_{33}$ ). Model coefficients estimated for degrees of hydrolysis and results of the analysis of variance (ANOVA) showed that confidence level of the statistical model included 99.999% ( $P < 0.0001$ ) (Table 3). Comparing

p-values within independent variables, significant differences were seen in linear results for temperatures ( $P < 0.0001$ ). Results of the interaction did not show significant differences. However, results of the second-order model showed significant differences, which parameters of pH ( $P \leq 0.0001$ ) and temperature ( $P = 0.0002$ ) were higher than the parameter of enzyme to substrate ratio ( $P = 0.0038$ ). Accordingly, the software suggested a second-order model. Second-order polynomial models were suggested to achieve degrees of hydrolysis based on the coded variables (Eq. 1).

$$1/\text{Sqrt}(DH) = 0.37 + 0.025 * A - 5.267E-003 * B - 5.357E-003 * C + 6.164E-003 * AB - 2.414E-003 + 2.820E-003 * BC + 0.013 * A^2 - 0.019 * B^2 - 8.355E-003 * C^2$$

DH= degrees of hydrolysis

After carrying out experiments designed by response surface methodology in this study, total determination coefficient in ANOVA included  $R^2 = 0.9592$  for degrees of hydrolysis. The  $R^2$  value showed that how much variation was explained by the model. In this experiment,  $R^2$  of the model was greater than 90%, intimating a very good explanation of the variance in data. To assess appropriateness of the model, the parameter of lack of fit was used (Table 3). The lack of fit of the proposed model was not significant ( $P < 0.05$ ), which showed that the model included a good fit based on the achieved data. The lack of fit compared the remaining error with the pure error.

**Table 2.** Actual levels of the independent variables with observed values for the response variable and degree of hydrolysis.

Run	$X_1$	$X_2$	$X_3$	Y	PR
1	60	7.5	3.5	7.45	62.11
2	60	7.5	5.5	9.69	65.91
3	65	7	2.5	7.1	60.75
4	65	8	4.5	7.22	74.06
5	65	7	4.5	6.95	48.27
6	65	8	2.5	6.35	55.87
7	60	7.5	3.5	7.06	59.40
8	55	8	4.5	8.9	50.17
9	55	7	2.5	8.01	65.64
10	70	7.5	3.5	4.44	39.58
11	60	7.5	3.5	7.45	61.57
12	60	7.5	3.5	7.06	59.13
13	55	7	4.5	9.35	78.67
14	60	7.5	1.5	8.02	68.63
15	55	8	2.5	10.39	86.24
16	60	8.5	3.5	12.78	87.63
17	60	6.5	3.5	10.3	58.85
18	60	7.5	3.5	7.66	65.64
19	50	7.5	3.5	7.31	60.84
20	60	7.5	3.5	7.06	64.56

$X_1$ , temperature;  $X_2$ , time;  $X_3$ , enzyme concentration;  $X_4$ , pH of substrate; Y, degree of hydrolysis; PR, protein recovery

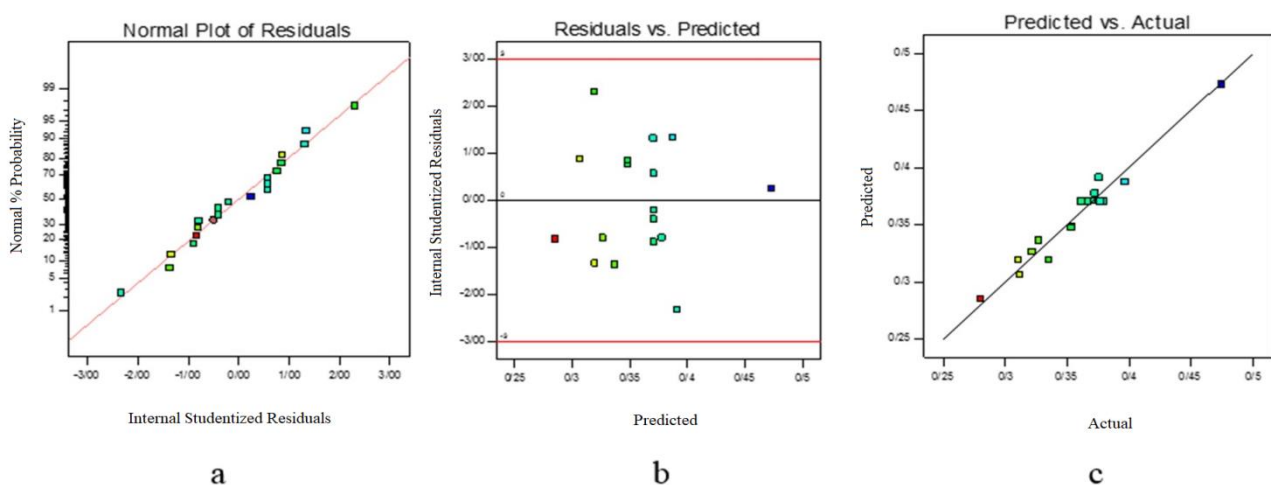
**Table 3.** Analysis of variance table for the degrees of hydrolysis as affected by independent variables during optimization experiments

Factors	Sum of squares	Degrees of freedom	Mean squares	F-ratio	p-value
Model	0.030	9	3.279	26.13	<0.0001
Independent variables					
Temperature (A)	0.010	1	0.010	82.61	<0.0001
pH (B)	4.438	1	4.438	3.54	0.0994
E/S ratio(C)	4.592	1	4.592	3/66	0.0848
Interaction					
AB	3.040	1	3.040	2.42	0.1506
AC	4.663	1	4.663	0.37	0.5557
BC	6.361	1	6.361	0.51	0.4927
Quadratic					
A <sup>2</sup>	4.188	1	4.188	33.37	0.0002
B <sup>2</sup>	8.826	1	8.826	70.34	<0.0001
C <sup>2</sup>	1.755	1	1.755	13.99	0.0038
Residual	1.255	10	1.255		
Lack of fit	1.033	5	2.067	4.67	0.0580
Pure error	2.213	5	4.426		
Correction total	0.031	19			

### 3.2. Interpretation of the remaining graphs

Statistical adequacy of the model can be verified through a review of charts (normal distribution, predicted values versus response values and arbitrary plot). The normal plot is one of the most probable continuous distributions in probability theory. If the residual values of a normal distribution are around a straight line, this suggests that the test point is reasonably consistent with the value predicted by the model. Results of this study showed that the residual values were around a straight line of a normal distribution, consistent with the values predicted by the model (Fig. 1a). Plot of the predicted values against response values (Fig. 1b)

assesses the assumption of constant variance. This plot specifies between +3 and -3 standard deviations. If data is outside of this range, the model is outrunning. This plot includes a random dispersion, which demonstrates fixed spectra across the graph with no observed outrunning in the graph. The parity plot (Fig. 1c) provides a relatively acceptable level in this experiment. Review of this chart is necessary to compare the predicted response values against the actual values to ensure that the model is adequately conformed to the experimental system. Moreover, this diagram helps identify a group of not predicted values by the model.



**Figure 1.** Remaining graphs for verified adequacy of the model. **Fig. 1a:** The normal plot, the internally studentized residues in Fig. 1a were extended logically close to the diagonal line, showing accuracy of the model to explain relationships between the control factors and the degrees of hydrolysis; **Fig. 1 b:** Plot of the predicted values against response values, the random distribution of residuals between -3 and +3; and **Fig. 1c:** The parity plot, comparison of the predicted response values with the actual values showed the adequate compatibility of the model with the laboratory system

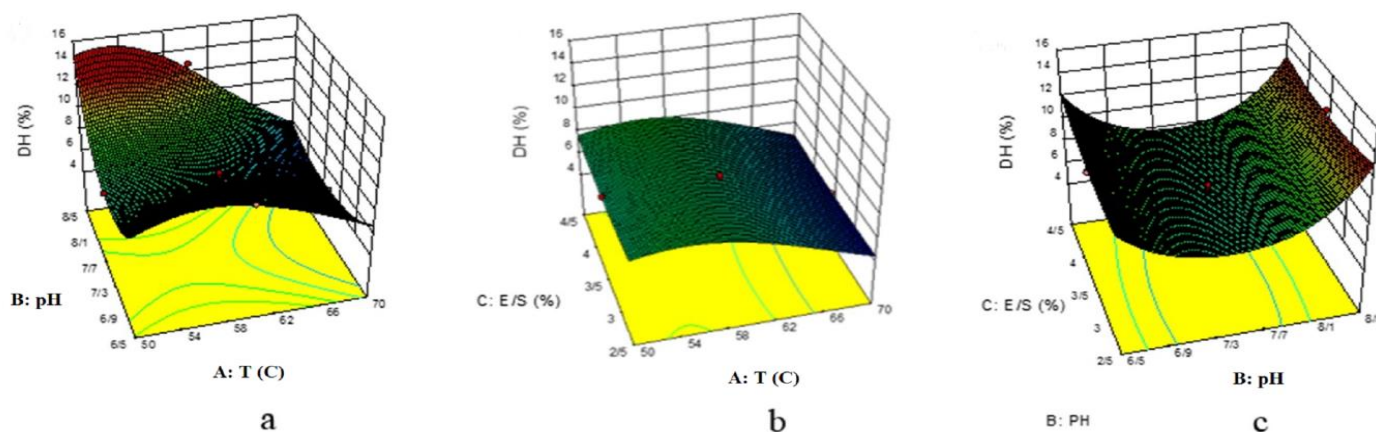


### 3.3. Optimization of multiple responses

At 3D response levels, a variable was fixed at the central level and effects of the changes on two other variables on the response variable degrees of hydrolysis were investigated (Fig. 2). Graphs showed that protein hydrolysis was affected by changes in temperature, pH and enzyme to substrate ratio. In Fig. 2a, ratio of the enzyme to substrate was fixed at 3.5%. Changes in temperature and pH and their simultaneous effects on degrees of hydrolysis were studied. Result demonstrate that degrees of hydrolysis increased at 50-65°C with increasing pH from 7.5 to 8.5. The highest degrees of hydrolysis of 12.78% was achieved at 60°C and pH 8.5. In shown in Fig. 2b, pH was constant at point 8 and effects of the enzyme to substrate ratio and temperature and their simultaneous effects on degrees of hydrolysis were studied. Results showed that the degrees of hydrolysis increased with increased temperature (50-60°C) and enzyme to substrate ratio (2.5-3.5%). Further increases in enzyme to substrate ratio over 3.5-5.5% included less significant effects on degrees of hydrolysis. Decreases in degrees of hydrolysis were seen with further increases in enzyme to substrate ratio at 60-70°C (Figs. 2a, 2b). Fig. 2c shows effects of pH and E/S ratio on degrees of hydrolysis when the temperature was constant at 60°C. The degrees of hydrolysis increased with increasing pH from 7 to 8.5 (Figs. 2a, 2c).

This increase in degrees of hydrolysis was due to the presence of alcalase in its optimum pH [18]. Interactions of the variables in hydrolysis of chicken wastes showed that increased temperature, pH and E/S to certain levels

increased the degrees of hydrolysis, decreasing slightly at higher levels of the variables. A possible reason for decreases in the degrees of hydrolysis with increases in temperature and pH (greater than 60°C and pH 8.5) has been attributed to increases in enzyme denaturation and hence decreases in its biological activity. A similar behavior has been observed for enzymatic hydrolysis reactions of the protein sources such as chicken meats Kurozawa et al, dogfish muscles Diniz et al, catlas Bhaskar et al, salmons Liaset et al and shark muscles Martin et al. The low levels of hydrolysis at pH less than 7.5 could be attributed to decreased enzyme binding to poultry waste proteins and decreased enzyme ability to maintain catalytic activity at specific pH values [9,20-22]. In this study, a mild decrease was seen in the degrees of hydrolysis at E/S above 3.5%. These results were similar to published results of other studies [20,21,23]. Martin et al. showed that a nonlinear trend was seen between the enzyme concentration and degrees of hydrolysis. The higher the E/S ratio, the smaller the increased degrees of hydrolysis [22]. This might be attributed to auto-digestion of the enzyme, product inhibition or over-saturation of the substrate [15,20,22,23]. The highest degrees of hydrolysis was achieved as 12.78% at an enzyme to substrate ratio of 3.5%, pH of 8.5 and temperature of 60°C. The protein recovery was achieved as 87.6% under these conditions and the protein recovery and degrees of hydrolysis were impressed by the enzyme to substrate ratio, pH and temperature.



**Figure 2.** The 3D surface plot for the degrees of hydrolysis as a function of pH, temperature and enzyme to substrate ratio during hydrolysis with alcalase. **Fig. 2a:** Interactions of temperature and pH on D; **Fig. 2b:** Interactions of temperature and enzyme to substrate ratio on degrees of hydrolysis; and **Fig. 2c:** Interactions of pH and enzyme to substrate ratio on degrees of hydrolysis. DH= degrees of hydrolysis

### 3.4. Optimization and model validation

Protein hydrolysis was investigated under the optimal conditions proposed by response surface methodology. Optimization section of the response surface methodology was used, which provided changes in parameters as desired. The maximum degrees of hydrolysis was selected for the assessment of the experiments. The model suggested experiment conditions, including an enzyme to substrate ratio of 2.5%, pH of 8 and temperature of 55°C. Under these conditions, predicted degrees of hydrolysis by the model included 9.830%. To assess validity of the model, suggested experiment by the model was carried out in three replicates, the mean degrees of hydrolysis value was 10.06%. The degrees of hydrolysis from the experimental assay was similar to the predicted result by the response surface methodology. Results verified that the used model was valid and effective for predicting the degrees of hydrolysis.

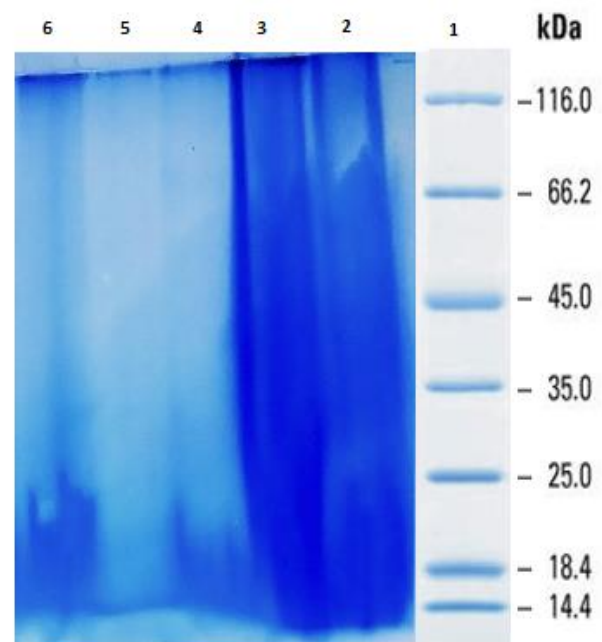
### 3.5 Analysis of the hydrolyzed proteins

Size of the hydrolyzed proteins was investigated using SDS-PAGE electrophoresis of 12.5% acrylamide gel. Investigation of non-hydrolyzed samples and primary powder using SDS-PAGE showed that the samples containing a range of proteins with various molecular weights could produce smears on the gel (Fig. 3, Lines 2 and 3). The substrate protein content was broken down into low molecular weight proteins using enzymatic hydrolysis. Samples with various degrees of hydrolysis values of 4.44 and 12.78% and the hydrolyzed sample in central point conditions with degrees of hydrolysis of 7.45 were assessed. Results in Fig. 3 demonstrated that size of the molecular weight proteins decreased as the degrees of hydrolysis increased. The SDS-PAGE studies showed that size of the molecular weight proteins decreased as the degrees of hydrolysis increased. Size of the peptides for the hydrolyzed sample with degrees of hydrolysis of 4.44% was nearly 25 kD. Molecular weight of the peptides achieved for the hydrolyzed samples at the center point condition with degrees of hydrolysis of 7.8% was 18.4 kD. The smallest peptides with a molecular weight less than 14.45 kD were linked to the hydrolyzed sample under optimized conditions with degrees of hydrolysis degrees of hydrolysis of 12.78%. These results showed that the hydrolysis process was well carried out using alcalase enzyme and the hydrolyzed protein contained a combination of low molecular weight peptides and amino acids that potentially could be used for industrial applications such as animal food supplements.

## 4. Conclusion

In this study, whole poultry slaughterhouse wastes, including feathers, heads, legs and other chicken wastes, were used in form of a primary powder to produce protein hydrolysates. The optimal conditions for enzymatic hydrolysis were achieved using response surface

methodology. Under the optimal conditions, a hydrolysis degree of 12.78% and protein recovery of 87.6% were achieved. Size of the hydrolyzed proteins was less than 14.5 kDa. The Protein hydrolysates included greater digestibility and absorbable due to decreased peptide size and increased nutritional value and bioavailability. In this study, whole wastes of the chicken poultry were used for the production of hydrolysate proteins while the process was applicable at industrial scales. Therefore, this method is suggested for scale-up and industrial use for the management of poultry wastes.



**Figure 3.** Investigation of the protein hydrolysis by alkaline enzyme under optimized conditions. Line 1, marker; Line2, extracted protein; Line3, primary powder; Line 4, hydrolyzed protein with degrees of hydrolysis of 7.45%; Line 5, hydrolyzed protein with degrees of hydrolysis of 12.78%; and Line 6, hydrolyzed protein with degrees of hydrolysis of 4.44%

## 5. Acknowledgements

The authors sincerely thank ETKA for its help to this study.

## 6. Conflict of interest

The authors declare no conflict of interest.

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doi: 10.1002/jsfa.2740380310

## بهینه سازی آبکافت آنزیمی پسماند کشتارگاه طیور با استفاده از آنزیم آلکالاز به منظور تهیه پروتئین آبکافت شده

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### چکیده

**سابقه و هدف:** افزایش مداوم تولید طیور به افزایش پسماند کشتارگاهی و نیاز به کنترل این پسماندهای غنی از مواد آلی منجر شده است. در گذشته، از روش های سوزاندن و دفن کردن برای کنترل پسماندهای آلوده کننده محیط زیست و گسترش بیماری ها استفاده شده است. امروزه، آبکافت<sup>۱</sup> پروتئین ها در پسماندها راهی مؤثر برای تولید محصولاتی جدید با ارزش افزوده و همچنین کنترل پسماندها می باشد. هدف از این تحقیق دستیابی به شرایط بهینه آبکافت آنزیمی پسماند صنعتی کشتارگاهی طیور است

**مواد و روش ها:** برای دستیابی به بالاترین راندمان آبکافت، از طراحی آزمایش براساس روش سطح پاسخ برای تعیین شرایط بهینه پارامترهای واکنش آبکافت استفاده شد. فاکتورهای دما (۷۰-۵۰ درجه سلسیوس)، pH (۶/۵-۸/۵) و نسبت آنزیم به پیش ماده<sup>۲</sup> (۷<sup>-۱</sup> v) در پنج سطح با استفاده از طرح مرکب مرکزی مورد بررسی قرار گرفتند. درجه آبکافت به عنوان پاسخ در نظر گرفته شد.

**یافته ها و نتیجه گیری:** تجزیه و تحلیل آماری نشان داد که ضریب رگرسیون ( $R^2$ ) برای مدل ۰/۹۵۹۲ می باشد که نشان دهنده دقت بالای مدل برای پیش بینی شرایط واکنش به کمک متغیرهاست. بالاترین درجه آبکافت ۱۲/۷۸٪ در نسبت آنزیم به پیش ماده ۳/۵، pH ۷/۵ و درجه حرارت ۶۰ درجه سلسیوس به دست آمد. تحت شرایط بهینه شده، پروتئین های آبکافت شده وزن مولکولی کمتر از ۱۴/۵ کیلو دالتون داشتند. نتایج نشان داده است که آبکافت آنزیمی تمام پسماند کشتارگاهی طیور با استفاده از آلکالاز به عنوان آنزیم پروتئاز امکان پذیر است.

**تعارض منافع:** نویسندگان اعلام می کنند که هیچ نوع تعارض منافی مرتبط با انتشار این مقاله ندارند.

### تاریخچه مقاله

دریافت ۲۰ دسامبر ۲۰۱۹  
داوری ۱۵ مارس ۲۰۲۰  
پذیرش ۲ می ۲۰۲۰

### واژگان کلیدی

- آلکالاز
- درجه آبکافت
- پسماند کشتارگاهی
- آبکافت پروتئین
- روش سطح پاسخ

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تلفن: ۹۸-۲۱-۲۲۹۸۵۸۹۵+

نمابر: ۹۸-۲۱-۲۲۹۷۴۶۰۴+

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<sup>1</sup> Hydrolysis

<sup>2</sup> Substrate