International Food Research Journal 18: 775-781 (2011)

Optimization of enzymatic protein hydrolysis from silver catfish (*Pangasius* sp.) frame

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Abstract: This study aims to determine the combined effects of hydrolysis time, temperature, pH and ratio of enzyme to substrate on the degree of hydrolysis (DH) of silver catfish frame using Response Surface Methodology. The proximate compositions of silver catfish frame and silver catfish hydrolysate powder were determined as well. The effects of independent factors were described using a three-level factors Face Centered Central Composite design. The suggested hydrolysis conditions for obtaining the optimum DH using Alcalase® were – temperature of 55°C, hydrolysis time of 163 min, pH of substrate at 9.45 and an enzyme concentration of 2.0%. The generated model showed a quadratic fit with experimental data. Proximate analyses revealed that silver catfish frame contained 25.02% protein, 68.21% fat and 7.08% ash. While silver catfish frame hydrolysate powder contained 65.05% protein, 32.92% fat and 0.86% ash. The protein recovery in silver catfish frame hydrolysate was as high as 71.6%.

Keywords: Silver catfish, waste, frame, protein, hydrolysate, *Pangasius* sp.

Introduction

Annual discard of fish waste industry is estimated to be approximately 20 million tones (or 25% of the total production) (Rustad, 2003). The major fish waste from fish processing industry include bone frame, bones, viscera, skin and scales, and they contributed as high as 70% of the original raw materials (Benjakul and Morrissey, 1997). Fish waste is a good source of protein (Arnesen and Gildberg, 2007), but a huge amount of the waste is still being discarded without much effort to recover its protein (Kristinsson and Rasco, 2000; Gildberg, 2002). Besides that, the discarding of fish waste creates the environmental problem as well as disposal problem.

Fish waste can be value added by converting it into fish protein hydrolysate (FPH) by utilizing proteolytic enzymes to hydrolyze the fish protein (Kristinsson and Rasco, 2000; Venugopal, 2006). Enzymes used to produce FPH should be of food grade, and if they are of microbial origin, the producing microorganism has to be non-pathogenic. The enzymatic protein hydrolysis of fish waste will produce soluble and insoluble fractions. The insoluble fraction may be used as animal feed and the soluble fraction is normally dried to produce a stable concentrated protein called fish protein hydrolysate. Alcalase®, a serine bacterial endopeptidase prepared from a strain of *Bacillus licheniformis* has been proven as one of the best enzyme by many researchers to be used in the preparation of fish protein hydrolysate (Kristinsson and Rasco, 2000; Bhaskar *et al*., 2007). Several studies has been reported on the optimization of enzymatic fish protein hydrolysis such as in Catla viscera (Bhaskar *et al*., 2008), pacific whiting solid waste (Nilsang *et al*., 2005), threadfin bream (Normah *et al*., 2005) and grass carp skin (Wasswa *et al*., 2008).

Silver catfish (*Pangasius* sp.) is a popular freshwater fish used as dish in Malaysia and accounts for 36.7% of total freshwater aquaculture production (Abbas *et al*., 2006). The edible portion of silver catfish is only 50%, implicating that another 50% is their waste. Silver catfish waste is not suitable to be used for fish feed due to its high fat content. Thus, study on the potential use of silver catfish waste is needed. Until now, no information has been reported on the optimization of enzymatic protein hydrolysis from silver catfish frame.

RSM is a statistical model frequently used for the optimization of complex systems and uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate problems (Madamba, 2002). Based on the experimental data, RSM could tell us the optimum conditions to obtain the desired responses, as well as the mathematical model in explaining the relationship between the experimental variables and its responses.

The aim of this study is to optimize the enzymatic

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protein hydrolysis from silver catfish frame in terms of hydrolysis time, hydrolysis temperature, hydrolysis pH and concentration of enzyme (ratio of Alcalase® to substrate) to achieve the maximum degree of hydrolysis (DH).

Materials and Methods

Raw materials

About 100 kg of silver catfish (*Pangasius* sp.) were purchased from local supplier and were brought alive to the laboratory. Two batches of fish were used in this research with 100 kg per batch. Alcalase® 2.4 L in liquid form (2.4 AU/g) was purchased from Novo Industry (Denmark). All other chemicals used were of analytical grades.

Methods

Since there was lack of silver catfish waste available from the local industry, it was prepared in the laboratory. Silver catfish was purchased fresh from local market. Silver catfish frame was prepared by filleting the silver catfish. Next, the head of the silver catfish was cut, leaving the fish frame only. The fish frame included bones, fins, tails of and some remaining flesh of fish attached to the frame. Next, the frame was homogenized by mincing it using Waring blender (model HGB2WTS3) at high speed for about 60 seconds with addition of water at ratio of 1 kg frame to 400 ml water to aid the mincing process. After that the wastes were stored in freezer at – 20°C until further use.

Before the protein hydrolysis was carried out, the proximate analysis of the silver catfish frame was carried out (AOAC, 2002). Calculation of raw materials to be used in hydrolysis process was based on crude protein content. The calculation is necessary because the mass of raw materials and enzyme depend on the protein content of silver catfish frame. The calculation of raw materials to be used in hydrolysis process was based on modified calculation from Hordur and Barbara (2000). Preparation of fish hydrolysate was carried out according to Bhaskar *et al.* (2006) with some modification.

Optimization of the hydrolysis conditions were accomplished by employing the response surface methodology (RSM) with a central composite design (CCD). Four different independent variables which were temperature (A,°C), time (B, minutes), enzyme to substrate concentration (C, %v/w) and pH (D) were employed at three equidistant levels (−1, 0 and +1). The hydrolysis processes were carried on based on the parameters shown in Table 1.

About 82.5 g of Patin frame was added with 60.5 g of distilled water (including the volume of 1 N NaOH used to adjust to required pH) and heated at 85°C for 20 minutes prior to hydrolysis. After heat treatment, 20 g of Alcalase enzyme solution (enzyme was diluted to the final volume of 20 g with distilled water) was added to the slurry and the hydrolysis process was carried out using an autotitrator (Metrohm model 799 GPT Titrino). The volume of 1 N NaOH used resulted from the hydrolysis process was used to calculate the degree of hydrolysis in each run. Each run after the specified hydrolysis time was terminated by heating the sample at 85°C for 20 minutes. The optimized design was further validated through different combinations of parameters, with DH as the response variable, to evaluate the usefulness of the design. Degree of hydrolysis (DH; %) was determined as the response variable (Y). Degree of hydrolysis was determined using pH-stat method according to Adler-Nissen (1986). Calculation of degree of hydrolysis was carried out according to Adler-Nissen (1986) as follows:

$$DH = B \times N_b \times 1/\alpha \times 1/MP \times 1/h_{tot} \times 100\%$$

where B is base consumption (in ml), N_b is normality of the base, α is average degree of dissociation of the α-NH groups, MP is mass of protein (in g) and h_{tot} is total number of peptide bonds in the protein substrate.

A hydrolysate that gave the highest degree of hydrolysate was freeze dried and analyzed for its proximate analyses. Proximate analyses for both silver catfish frame and silver catfish frame hydrolysate were carried out using AOAC method (AOAC, 2002). Recovery of protein, fat and ash content were calculated as well by comparing their total content in hydrolysate powder as compared to their total content in initial silver catfish frame. For example, recovery of protein in silver catfish hydrolysate powder was calculated as follows:

$$\frac{\text{(\% protein in hydrolysate powder) x (mass of hydrolysate powder) x 100\%}}{\text{(\% protein in frame) x (mass of initial frame)}}$$

Statistical analysis

The optimization data were analyzed using Design-Expert 6.0.10 software (Stat-Ease 2003). For proximate analysis, all analyses were carried out in triplicates.

Table 1. Ranges of parameters used in the RSM design

Factor	Level		
	-1	0	+1
Temperature (°C), A	40	50	60
Time (min), B	60	120	180
Enzyme (%), C	1	1.5	2
pH, D	7.5	8.5	9.5

Table 2. Proximate composition and yield of silver catfish frame and its hydrolysate powder (dry basis)

	Crude Fat (%)	Crude Protein (%)	Ash (%)	Yield (g)
Silver catfish frame	68.21± 0.63[a]	25.02 ± 0.27[b]	7.08 ± 0.36[a]	82.50*
Silver catfish frame hydrolysate powder	32.92 ± 0.42[b]	65.05± 1.68[a]	0.86 ± 0.92[b]	9.44
Recovery (%)	17.10	71.60	1.8	

*Yield of silver catfish frame is actually the original mass of silver catfish frame used in the hydrolysis, while yield of hydrolysate powder is the mass of hydrolysate powder produced from the hydrolysis run.

Results and Discussion

Proximate composition

The proximate analysis was carried out on raw materials (silver catfish frame) and the final product (freeze-dried silver catfish frame hydrolysate powder). Freeze-dried silver catfish frame hydrolysate powder was prepared using the hydrolysis conditions that gave the highest DH based on experimental data, i.e. at pH 9.5, temperature of 60°C, 1.5% enzyme and hydrolysis time of 180 minutes. Table 2 shows the proximate composition and yield of silver catfish frame and its hydrolysate powder. The yield for silver catfish frame is the weight of raw materials used, while the yield for silver catfish frame hydrolysate powder refers to the total weight of the freeze-dried powder recovered from the soluble fraction of hydrolysate.

The moisture content of silver catfish frame was 63.2% and for 0.64% silver catfish frame hydrolysate powder. Table 2 shows that in the initial raw materials, protein, fat and ash content were 68.21%, 25.02% and 7.08%, respectively. The protein content in silver catfish frame is in the range of protein content reported by other researchers. According to Murray and Burt (2001), the amount of protein in fish muscle is usually between 5 to 20% but values 5% or as high as 28% are occasionally found in some species. The fat content in silver catfish frame was very high, and no defatting was carried out prior to hydrolysis. Although previous study has shown that raw materials containing the higher amount of fat gave the lowest percentage of solubilised protein (Slizyte *et al*., 2005), defatting was not carried out in this study, because another previous study had showed that defatting of raw materials will produced hydrolysates with very high ash content (24.56%) (Sathivel *et al*., 2003; Slizyte *et al*., 2005).

For silver catfish frame hydrolysate powder, the major component was protein (65.05%), followed by fat (32.92%) and finally ash (0.86%). The value of protein content of silver catfish frame in this study was higher than that of spray-dried Tilapia flesh hydrolysate (37.7- 49.6%) (Azizah *et al*., 2001), Catla viscera hydrolysate (14.25%) (Bhaskar *et al*., 2008) but lower than those of sardine, mackerel and white croacker hydrolysate (82.7 – 85.1%) (Arvanitoyannis and Kassaveti, 2008). In order to reduce the fat content of silver catfish hydrolysate and increase its stability to rancidity, defatting should be carried out, prior to hydrolysis. Comparing the fat and ash content for both samples, it was found that the enzymatic hydrolysis process had reduced the original fat content by 2-fold and the original ash content by 8-fold. Benjakul and Morrissey (1997) suggested that the high protein content in fish hydrolysate was due to the solubilisation of protein during hydrolysis, removal of insoluble and undigested non-protein substances and the partial removal of lipid after hydrolysis. Besides that, since only the soluble fraction was freeze-dried, the remaining frame was excluded, thus leading to low ash content.

In order to compare the compositions of both silver catfish frame and produced hydrolysate, the total weight of crude fat, crude protein and ash content and their recovery in the final hydrolysate were calculated as well. Table 2 shows that silver catfish hydrolysate

powder had recovered 71.6% of the original protein content in the raw material. Recovery of protein in silver catfish hydrolysate was in agreement with the values reported by other researchers. Bhaskar *et al*. (2008) reported a protein recovery of 63.13% for Catla visceral hydrolysate, while Sathivel *et al*. (2003) reported a protein recovery of 77-87% for herring hydrolysate.

Optimization of enzymatic protein hydrolysis

The observed values for degree of the hydrolysis (DH) at different combinations of the independent variables are shown in Table 3. Overall, 30 experiments with six replicates in the center of design space were carried out. Centerpoint runs interspersed among the experimental setting runs for two purposes i.e. to provide a measure of process stability and inherent variability and to check for curvature.

Table 3 shows that the range of the degree of hydrolysis was from 6.25% to 21.38%. The interactions between experimental factors can be interpreted by 3-D surface as shown in Figure 1, Figure 2 and Figure 3. The value of degree of hydrolysis for silver catfish frame in this study was higher than DH given by Grass carp skin, 1.1-15.2% (Wasswa *et al*., 2008) but lower than degree of hydrolysis of Catla viscera (34.23-49.65%) (Bhaskar *et al*., 2007). The degree of hydrolysis maybe different because of the difference in the part of fish used in hydrolysis, difference in fish species and difference in enzyme used.

Figure 1 shows the effects of temperature and hydrolysis time on the DH of silver catfish frame hydrolysis. For both factors, DH increased with the increase in temperature and hydrolysis time. DH reached the maximum level near 60^{o}C and 180 minutes. The effect of temperature on DH is normally in bell-shaped pattern. Below optimum temperature, the DH will increase because hydrolysis increased with temperature. However, above optimum temperature, the DH will decrease due to denaturation and inactivation of enzymes at higher temperature.

Previous study on the hydrolysis of fish using Alcalase® reported the optimum temperature of 55^{0}C for Catla visceral waste (Bhaskar *et al*., 2005) and 60^{0}C for threadfin bream and grass carp skin (Normah *et al*., 2008).

Figure 2 shows the effects of temperature and enzyme to substrate ratio on the DH of silver catfish frame hydrolysis. As there are more enzymes molecules present in higher enzyme to substrate ratio, there will be more chances for the hydrolysis to occur. The effect of enzyme to substrate ratio on DH is usually a linear relationship. For enzyme to substrate ratio factor, DH increased with increase in enzyme to substrate ratio (2%). The finding of this study is consistent with the theory. DH was almost constant around 2% enzyme to substrate ratio. This result was similar to the optimum Alcalase® concentration for threadfin bream (Normah *et al*., 2005). However, Wasswa *et al*. (2008) and Bhaskar *et al*. (2007) reported a lower enzyme to substrate ratio which were 1.05% and 1.25% for grass carp skin and Catla viscera, respectively.

Figure 3 shows the effects of pH and temperature on the DH of silver catfish frame hydrolysis. For pH factor, DH increased with increase in pH. The effect of pH and temperature on DH is normally in bell-shaped pattern. The finding of this study is consistent with the theory. This finding is not surprising, as Alcalase® has the optimum pH of 6-10 (Novo Industry, Denmark).

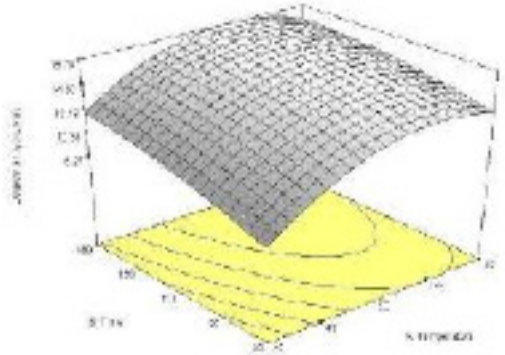

Figure 1. DH as a function of time and temperature during protein hydrolysis of silver catfish frame with Alcalase®

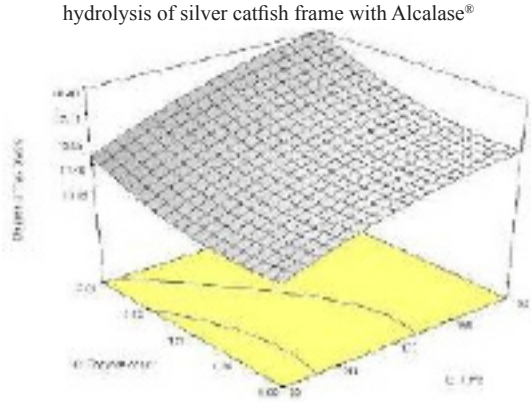

Figure 2. DH as a function of enzyme concentration and time during protein hydrolysis of silver catfish frame with Alcalase®

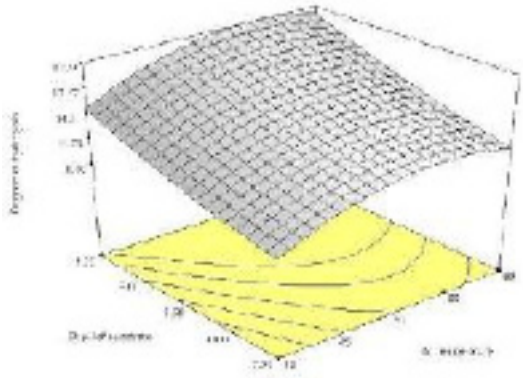

Figure 3. DH as a function of pH and temperature during hydrolysis of silver catfish frame with Alcalase®

Table 3. Actual levels of independent variables used in optimizing the hydrolysis conditions using Alcalase® and its observed values for degree of hydrolysis

Run	Temperature (A)	Time (B)	Enzyme:substrate (C)	pH (D)	DH (Y)
1	40	60	1.0	7.5	6.25
2	60	60	1.0	7.5	9.61
3	40	180	1.0	7.5	8.53
4	60	180	1.0	7.5	11.14
5	40	60	2.0	7.5	7.47
6	60	60	2.0	7.5	11.41
7	40	180	2.0	7.5	10.40
8	60	180	2.0	7.5	14.77
9	40	60	1.0	9.5	11.25
10	60	60	1.0	9.5	18.18
11	40	180	1.0	9.5	16.00
12	60	180	1.0	9.5	19.36
13	40	60	2.0	9.5	13.90
14	60	60	2.0	9.5	19.35
15	40	180	2.0	9.5	18.94
16	60	180	2.0	9.5	21.38
17	40	120	1.5	8.5	11.01
18	60	120	1.5	8.5	15.79
19	50	60	1.5	8.5	13.22
20	50	180	1.5	8.5	17.75
21	50	120	1.0	8.5	15.91
22	50	120	2.0	8.5	17.61
23	50	120	1.5	7.5	13.26
24	50	120	1.5	9.5	20.03
25	50	120	1.5	8.5	14.44
26	50	120	1.5	8.5	14.34
27	50	120	1.5	8.5	14.62
28	50	120	1.5	8.5	14.42
29	50	120	1.5	8.5	15.58
30	50	120	1.5	8.5	15.79

Optimization of the enzymatic protein hydrolysis using RSM

Table 4 shows the ANOVA results for the effects of the four independent variables during optimization experiments on the DH. The ANOVA of the regression model demonstrates that the model is highly significant at 99% confidence level ($P< 0.0001$). The model fitted the experimental data with an acceptable determination coefficient ($R^2 = 0.9706$).

The quadratic model equation for DH of silver catfish frame proteins as a function of four variables in terms of coded factors was $Y = 15.44+ 2.07A + 1.53B + 1.06C + 3.64D - 2.61A^2 - 0.52B^2 + 0.75C^2 + 0.64D^2 -0.43AB + 0.24AC + 0.23BC + 0.18BD$. The equation shows that the largest value of estimated regression coefficient was for pH (3.64), indicating that it was the most important linear variableinfluencing the DH values. Previous study also reported a quadratic model for enzymatic hydrolysis of fish waste (Nilsang *et al*., 2004; Bhaskar *et al*., 2008). Table 5 shows the desirability profiles for optimum DH suggested by the Design-Expert software. If the desirability value is closer to 1, this means that the conditions suggested were most suitable to obtain the optimum DH. The suggested hydrolysis conditions were temperature of 55°C, hydrolysis time of 163 min, pH of substrate at 9.45 and an enzyme concentration of 2.0%. The optimum conditions were quite similar to those reported by Bhaskar *et al*. (2008) for Catla visceral waste protein hydrolysis using neutral protease which was a hydrolysis temperature of 55 °C, time of 165 min and an enzyme concentration of 1.25%.

Table 4. ANOVA table for DH as affected by independent variables during optimization experiments

Source	SS	DF	MS	F Value	Prob > F
Model	415.41	12	34.62	46.72	< 0.0001
A	77.05	1	77.05	103.97	< 0.0001
B	42.41	1	42.41	57.24	< 0.0001
C	20.06	1	20.06	27.07	< 0.0001
D	238.71	1	238.71	322.14	< 0.0001
A^2	17.63	1	17.63	23.79	0.0001
B^2	0.71	1	0.71	0.96	0.3414
C^2	1.46	1	1.46	1.97	0.1780
D^2	1.05	1	1.05	1.42	0.2503
AB	2.98	1	2.98	4.02	0.0613
AD	0.95	1	0.95	1.28	0.2731
BC	0.82	1	0.82	1.11	0.3078
BD	0.53	1	0.53	0.71	0.4114
Residual	12.6	17	0.74		
Lack of Fit	10.52	12	0.88	2.11	0.2118
Pure Error	2.08	5	0.42		
Cor Total	428.01	29			

R^2 = 0.9706; SS- sum of square; DF- degree of freedom; MS- mean square

Table 5. Suggested conditions of silver catfish frame hydrolysis using Alcalase®

No.	Temperature	Time	Enzyme concentration	pH	DH	Desirability
1	55	163	1.99	9.45	22.73	1
2	50	172	1.92	9.45	22.16	1
3	51	175	1.81	9.49	22.03	1
4	55	176	1.77	9.49	22.05	1
5	50	173	2.00	9.40	22.26	1
6	54	157	1.89	9.49	22.31	1
7	54	176	1.99	9.43	22.76	1
8	56	180	1.70	9.50	21.73	0.984222
9	53	180	1.31	9.50	20.87	0.933266
10	55	180	1.00	9.50	20.63	0.919468

Conclusions

The study shows that degree of hydrolysis of silver catfish frame hydrolysis by Alcalase was significantly influenced by time, temperature, pH of the substrate and the enzyme concentration. The suggested hydrolysis conditions for obtaining the optimum DH using Alcalase® were – temperature of 55^0C, hydrolysis time of 163 min, pH of substrate at 9.45 and an enzyme concentration of 2.0%. The protein recovery in silver catfish frame hydrolysate was as high as 71.6% of the original protein in silver catfish frame. The hydrolysate powder had significantly higher protein content and lower fat and ash content compared to the original raw material. These data could be adopted to produce silver catfish protein hydrolysate at industrial scale.

Acknowledgement

The authors gratefully acknowledged the financial support from Malaysian Department of Fisheries to carry out this research.

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