HYDROLYSIS OF *Pangasius hypophthalmus* BY- PRODUCTS TO PEPTIDE FORM

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ABSTRACT

Currently, the by-products of *Pangasius hypophthalmus* in the seafood processing factories in the Mekong Delta region is very abundant but not yet taking full advantages. The utilization of these by-products and processing them into high value added products will bring high economic efficiency for producers as well as reducing the amount of by-products caused environmental pollution.

This study surveyed and optimized conditions for hydrolysis of *Pangasius hypophthalmus* by-products by enzyme Alcalase 2.4 L for the purpose of collecting the most proteins that have low molecular weights (peptide form); and thereafter, we will continue to study their applied features (solubility, emulsion forming, calcium binding…) in Food Technology.

The optimal conditions for hydrolysis of *Pangasius hypophthalmus* by-products were resulted as follows: ratio of enzyme/substrate (E/S) is 0.15% (equivalent 0.5IU/g substrate); temperature is 58^0C; pH 6.5; time of hydrolysis is 120 min.; rate of water added is 30%. The highest yield in hydrolysis (% Peptide Form Yield - PFY) of *Pangasius hypophthalmus* by-products was 19.20%.

1. INTRODUCTION

The ratio of *Pangasius hypophthalmus* by-products with body weight are up to 60%. These by-products include head, bones, skin, organs ... should be handled or further processing. In Vietnam, these ones have been used to produce fishmeal, fish oil but most of them are raw products of low economic efficiency (Vu Hoa Binh, 2007)

The Enzymes that are used to hydrolyse fish products, obtained hydrolyzate can be endoprotease such as: papain, Alcalase®, Neutrase®, Flavourzyme®, Protamex®, etc (Adinarayana. K, Ellaiah. P, Prasad. DS. 2003). The most common enzyme is Alcalase (Wasswa, J., Tang, J., Gu, XH, & Yuan, XQ. 2007; Nilsang, S., Lertsiri, S., Suphantharika, M., & Assavanig, A. 2005). Enzyme protamex also be used but the yield is lower than the one that using Alcalase

(Ovissipour, M., Abedian, AM, Motamedzadegan, 2008; A., Rasco, B., Safari, R., & Shahiri, H. 2009).

The use of proteolytic enzymes to get fish protein hydrolysate (FPH) can be carried out in controlled conditions to obtain the peptide with new properties are used as ingredients of food (Hordur G. Kristinsson và Barbara A. Rasco 2000). Many enzymes have been applied to increase FPH. Some protein with enzyme hydrolysis improved their functional properties: increased solubility, foaming and emulsifying properties and increased application in food industry (L. Picot, S. Bordenave. 2006).

The use of Flavozyme will get high effective for Cod, Carp , Shark, or of surimi from fish. Using Protamex has less effectiveness with fish by-products (Onodenalore, AC, & Shahidi, F. 2010 ; Ensoy, U., N. and Candogan Kolsarici k. 2009). Fish Protein Hydrolysate (FPH) obtained by hydrolysing contains peptides with different molecular weights are applied in the Medicare, such as anti-cancer activity, anti-metastatic cancer, protects intestinal activity of antioxidants (N. Krasaechol and R. Sanguandeekul 2008; Rasa Slizyte, Egidijus Dauksas, Eva Falch, Ivar Storrø, Turid Rustad. 2005)

Some applied research of hydrolyzed protein products in some foods that using the commercial enzyme to improve the technological properties and nutritional value of product received from (Ragnar Johannsson, Ludmila A. Pavlova, Biscalchin-Gryschek, Sf, Oettere M. Gallo and CR. 2003 ; Kristinsson, HG, & Rasco, BA. 2000; Gbogouri, GA, Linder, M., Fanni, J., & Parmentier, M. 2004). The using of Alcalase for hydrolyzing of some catfish byproducts, the yield can reach 32.4% (Kian Goh Heng Soon and Jeap Eong, 2008; Batista, I., Ramos, C., Coutinho , J., Bandarra, NM, & Nunes, ML. 2010).

Currently, the by-products of *Pangasius hypophthalmus* are being studied by Vietnamese scientists to produce value added products like biodiesel, collagen, gelatin, enzymes... (Vu Hoa Binh, 2007). In other countries, protein hydrolysate and peptides with different molecular weights from by-products of fish processing have been studied and improved many important applications in the food industry (Guerard, F., Guimas, L., & Binet, A. 2002; Hoyle, N. T., & Merritt, J. H. 2004).

This article focuses on researching the hydrolysis of protein in these by-products by enzyme Alcalase 2.4 L to obtain the maximum proteins in low molecular weights.

The PFY with molecular weight would be used as a food additive for improving the foaming and emulsification, calcium binding abilities. We will research these abilities in up-coming studies.

2. MATERIALS AND METHODS

2.1. Materials

By-products (the spine and head) of *Pangasius hypophthalmus* were received from Can Tho Fish Join Stock Company (CAFICO – Mekong Delta, Vietnam), then refrigerated, transported to the laboratory and stored at - 20^0C until use.

Enzymes Alcalase 2.4L were purchased from EAC Co., Ltd. (sole-exclusive agent for Novozyme in HCM city, Vietnam). This enzyme is a Endopeptidase which was produced by submerged fermentation of selected bacterial strains *Bacillus licheniformis*. Alcalase 2.4L is affected

easily by heat. Its optimum temperature for hydrolysis is at 60^0C, pH at 7.0 and retaining its activity for at least 24 months from date of manufacture in the circumstance of storing at temperatures below 10^0C. The activity of Alcalase 2.4L is 500 Anson unit/ml, this one is equivalent to 0.5IU enzyme/gram substrate.

All chemical reagents used for the experiments were of analytical grade.

2.2. Methods

Hydrolysis process:

Raw materials were cut in size of 5 mm, added water, heated to studying temperature, cooled to proper temperature, added enzyme Alcalase 2.4L. Adjusted the pH, temperature, E/S ratio, hydrolysis time appropriately. After the hydrolysis process, filtering to get the protein hydrolysate.

The experimental data (with factors: E/S ratio; time of hydrolysis; water added) were treated and processed by ANOVA analytical method.

The experimental data (with factors: hydrolysis temperature, pH) were treated by optimization process following the method of planned experiments. The significance of the regression-equation coefficients are tested by *Student Standard*. The compatibility of the regression equation with the experiment is tested by *Fisher Standard.*

The analytical methods:

- ✓ Total crude Protein (Nx6.25) in raw materials was determined by *Kjeldahl method* (AOAC 2002)
- ✓ Degree of hydrolysis (DH) was determined by *pH-stat* method. DH is calculated as a percentage of the peptide bonds that were broken off to the total number of peptide bonds (total nitrogen-N), and in each case calculated using the base volume, according to the formula:

$$DH = \frac{V_B \times c_B}{m_P} \times \frac{1}{\alpha} \times \frac{1}{N_{total}} \times 100$$

- ❖ V_B is the volume (liter) of base used (NaOH) to keep the pH constant during the reaction.
- ❖ C_B is the concentration molar
- ❖ M_P is the total protein (NX6, 25)
- ❖ α is the degree of dissociation of the α-NH2 released during hydrolysis
- ✓ Determination of protease activity by the *Anson* method.
- ✓ Formaldehyde N in hydrolysate (free amino acid, ammonia…) in the hydrolysate is determined by *formaldehyde titration* method (Sorensen method).

$$\text{Formaldehyde N in hydrolysate (\%) (Amino Acid + } NH_3\text{)} = \frac{\text{Formaldehyde N in hydrolysate (determined by formaldehyde titration method (Sorensen method).}}{\text{Total protein (Nx6.25) in raw materials (determined using Kjeldahl method (AOAC 2002)}} \times 100$$

Peptide Form Yield - PFY (%) is determined by:

% Peptide Form Yield (PFY) = D.H. (%) – Formaldehyde N in hydrolysate (%)

3. RESULTS AND DISCUSSION

3.1. Determining E/S ratio

Hydrolysis five samples (each sample 100 g) by-products of *Pangasius hypophthalmus* by enzyme Alcalase 2.4L: temperature 60^0C, pH = 7.0, hydrolysis time 90 min. (according to the manual of enzyme Alcalase 2.4L for proteolytic conditions for catfish).

The rates of E/S: sample 1 was control sample - no additional enzyme Alcalase 2.4L, sample 2, 3, 4, 5 added enzyme Alcalase 2.4L at 0.05%; 0.10%; 0.15%, 0.20% respectively basing on the volume of *Pangasius hypophthalmus* by-product materials.

Experimental results were treated, processed by ANOVA analytical method and presented in Table 1 and Figure 1.

Table 1. The effect of the enzyme/substrate ratio to Peptide Form Yield - PFY

E/S ratio (%)	Peptide Form Yield - PFY (%)
0,00	$2,69^{a(*)}$
0,05	$10,48^{b(*)}$
0,10	$15,04^{c(*)}$
0,15	$17,90^{d(*)}$
0,20	$18,68^{d(*)}$

(*): the small exponential character: a, b, c, d showing the difference was significant or not significant in ANOVA analytical method. In case of the same character, the difference of E/S rates is not significant; and the difference character shows the significant difference of E/S rates)

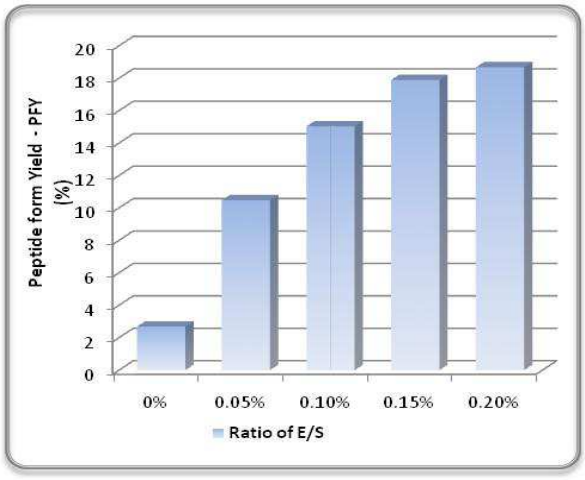

Figure 1. The effect of the enzyme / substrate ratio to Peptide Form Yield - PFY

PFY at the rates of E/S: 0%; 0.05%; 0.10% are significantly different with a reliability of 95% (p>0.05). The PFY at these rates are low.

PFY at the rates of E/S 0.1 and 0.15% are significantly different with a reliability of 95% (p>0.05) compared with the rate 0%; 0.05%; 0.10%.

PFY at the rate 0.20%, the hydrolysis efficiency is highest but the difference was not significant with the reliability of 95% (p> 0.05) compared with 0.15%. So we could choose any E/S rate of 0.15% or 0.20%.

Therefore, to ensure economic efficiency (low rate of enzyme) while maintaining the highest PFY, we chose the rate of E/S for hydrolysis of *Pangasius hypophthalmus* by-products at 0.15%. This result is consistent with research by Hoyle, N. T., & Merritt, J. H (2004) with the herring fish *(Clupea harengus).* [12]

Enzyme Alcalase 2.4L is 500 Anson unit/ml. So after the calculating, the dose used is 0.5IU enzyme/gram substrate

3.2. Determination of hydrolysis temperature

Hydrolysis samples (each sample 100 g) by-products of *Pangasius hypophthalmus* by enzyme Alcalase 2.4L: temperature at 60^0C, pH = 7.0, hydrolysis time 90 min. (according to the manual of enzyme Alcalase 2.4L for proteolytic conditions of catfish).

The E/S rate at the optimum 0.15% as the result of previous experiment.

Each sample is repeated and placed at different temperatures, respectively, as follows: 45^0C, 50^0C, 55^0C, 60^0C, 65^0C, 70^0C.

PYF results are temporary shown in Figure 2. The temperature is much impacted on hydrolysis and PFY, so these data will be processed by optimization process following the method of planned experiments later (see 3.6 below for further information).

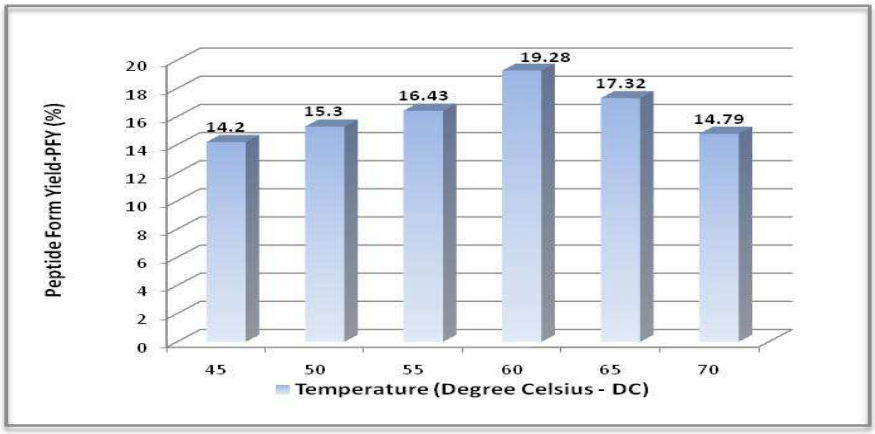

Figure 2. The effect of hydrolysis temperature on PFY

We found that the highest PFY achieved at a temperature of 60^0C (19.28%). Hydrolysis efficiency and PFY increased from 45 to 60 degrees C, then decreased gradually. As we increase the temperature gradually, enzyme Alcalase 2.4 was denatured causing hydrolysis efficiency reduced. So the optimum temperature is 60^0C.

The result in these experiments is similar to the one in researches of Guerard, F., Guimas, L., & Binet, A. (2002) with the substrate is tuna; L. Picot, S. Bordenave, S. Didelot, I. Fruitier-Arnaudin, F. Sannier, G. Thorkelsson, J.P. Bergé, F. Guérard, A. Chabeaud, J.M. Piot (2006) with the substrate is Sardine; Onodenalore, A. C., & Shahidi, F. (2010) with the substrate is Shark. [11], [15], [18]

3.3. Determination the pH of hydrolysis

Hydrolysis samples (each sample 100 g) by-products of *Pangasius hypophthalmus* by enzyme Alcalase 2.4L: hydrolysis time 90 min. (according to the manual of enzyme Alcalase 2.4L for proteolysis conditions of catfish).

The amount of enzyme added at the optimum rate of E/S is 0.15%, the hydrolysis temperature is 60^0C as the results of previous experiments.

Each sample is repeated and adjusted the pH at different value, respectively: 5.5; 6.0; 6.5; 7.0; 7.5; 8.0 and 8.5.

PYF results are temporary shown in figure 3. The same with temperature, pH is much impacted on hydrolysis and PFY, so these data will be processed by optimization process following the method of planned experiments later (see 3.6 below for further information)

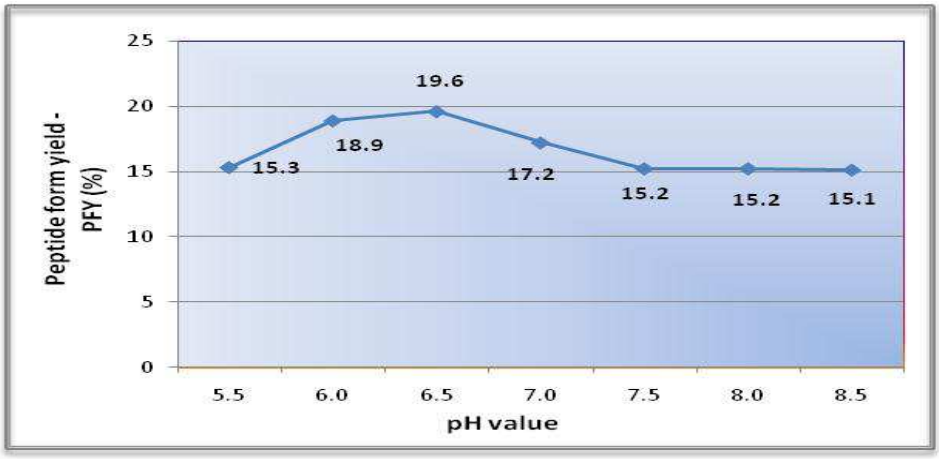

Figure 3. Effect of hydrolysis pH on PFY

Basing on figure 3, we found that: The value of pH above 6.5, the higher pH values we adjusted, the lower rate hydrolysis efficiency and PFY we got. This result proved that weak acid pH range is suitable for enzyme Alcalase 2.4L to hydrolyse the by-products of *Pangasius hypophthalmus*.

At weak acid pH value (from 5.5 to 6.5), while increasing the pH, the PFY increased. The PFY peaked in the pH range from 6.0 to 6.5

So the pH value from 6.0 to 6.5 is the most efficient choice for enzyme Alcalase 2.4L. Apart from that, pH = 6.5 was almost equal to the natural pH value of the *Pangasius hypophthalmus* by-product. So we should save the cost of adjusting pH during the hydrolysing.

The result in these experiments is similar to the one in researches of Goh Kian Heng and Jeap Soon Eong (2008) with the substrate is Catfish; Gholam Reza Shaviklo (2003) with the substrate is flower bud fish; Gbogouri, G. A., Linder, M., Fanni, J., & Parmentier, M. (2004) with the substrate is Sardine. [8], [15], [18]

3.4. Determination the time of hydrolysis

Hydrolysis five samples (each sample 100 g) by-products of *Pangasius hypophthalmus* by enzyme Alcalase 2.4L in condition: E/S 0.15%, temperature at 60^0C, pH = 6.0 – 6.5.

Examining the hydrolysis in a period of 210 minutes, after time of 30, 60, 90, 120, 150, 180, 210 min., analyzing the samples and determining the protein hydrolysis efficiency as well as PFY

Experimental results were processed by ANOVA analytical method and presented in Table 4 and Figure 4.

Table 4. Effect of hydrolysis time to Peptide Form Yield - PFY

Time (min.)	Peptide Form Yield - PFY (%)
30	12,87^b
60	17,12^c
90	19,38^d
120	21,82^a
150	22,10^a
180	22,10^a
210	22,20^a

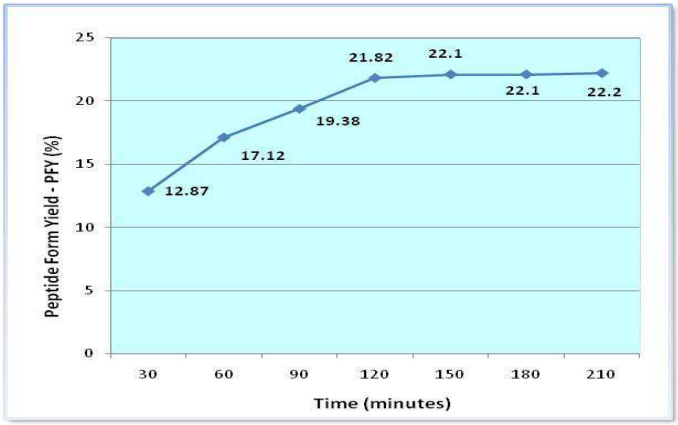

Figure 4. Effect of hydrolysis time to Peptide Form Yield – PFY

When prolonged the hydrolysis time, the PFY shall be higher, especially in the first period (from 30 min. to 120 min.), but not forever. When the time increased to 150 min., the hydrolysis efficiency and PFY will gradually reach the stable stage.

After 150 min., the amount of substrate gradually reduced and the hydrolysis products produced during the hydrolysis will inhibit the hydrolysis, which reduces the reaction rate.

As showed on the figure 4, In the first period, hydrolysis efficient and PFY were increased rapidly: PFY (60 min., 90 min., 120 min.) increased respectively: 1.33; 1.51; 1.7 times (compared to PFY at 30 minutes). But that growth almost has not increased after 150 minutes.

Depending on table 4, the highest PFY reached at 210 minutes. It has significant difference with a reliability of 95% ($p>0.05$) of the time 30 min., 60 min. and 90 min. But the ones at 30 min., 60 min. and 90 min. are low (12.87%, 17.12%, 19.38% respectively)

The highest PFY reached at 210 minutes, but no significant difference with a reliability of 95% ($p>0.05$) of the time 120, 150 and 180 minutes. So we could choose any hydrolysis time of 120min., 150min., or 150min. Apart from that the amount of PFY at 210 min. and the ones at 120 min, 150 min., 180 min. are not very much different (21.82%, 22.1%, 22.1%, 22.2% respectively)

Therefore, in order to save hydrolysis time as well as other expenditures (electricity, human…) while ensuring high efficiency hydrolysis and high PFY, we select the appropriate hydrolysis time is from 120 to 150 min.

The result in these experiments is similar to the one in researches of Batista, I., Ramos, C., Coutinho, J., Bandarra, N. M., & Nunes, M. L. (2010) with the substrate is black scabbardfish *(Aphanopus carbo)*; Vũ Hòa Bình (2007) with the substrate is *Pangasius hypophthalmus*; Wasswa, J., Tang, J., Gu, X. H., & Yuan, X. Q. (2007) with the substrate is Grass Carp [4], [21], [22]

3.5. Determining the rate of additional water

Hydrolysing five samples (each sample 100 g) by-products of *Pangasius hypophthalmus* by enzyme Alcalase 2.4L in condition: E/S 0.15%, temperature at 60^0C, pH = 6.0 – 6.5, time 120 min.

The rate of additional water: Sample 1 was control sample - no additional water, sample 2, 3, 4, 5 added water 10%; 20%; 30%, 40% respectively basing on the volume of *Pangasius hypophthalmus* by-product.

After 120 minutes of hydrolysis, determining the PFY.

Experimental results were processed by ANOVA analytical method and presented in Table 5 and Figure 5.

Table 5. Effect of rate of water added for PFY

Additional Water	Peptide Form Yield - PFY (%)
0%	$18,14^{a}$
10%	$19,32^{a}$
20%	$22,52^{ad}$
30%	$22,69^{bd}$
40%	$23,06^{cd}$

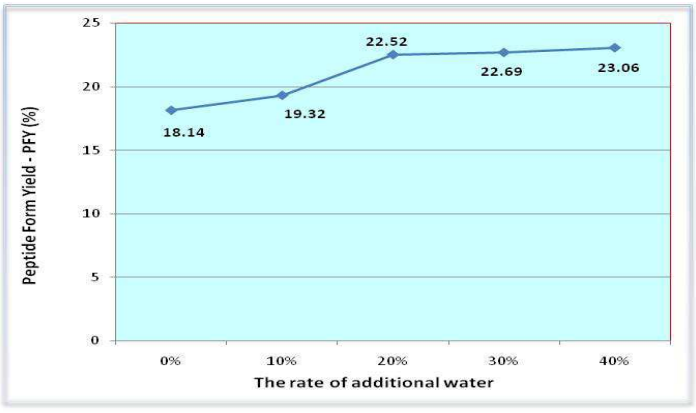

Figure 5. Effect of rate of additional water on PFY

As results showed in table 5, at the time of 120 minutes, hydrolysis efficient and PFY with the additional water of 40% is the highest, but no significant difference (p>5%) compared with added water 20%, 30%. So we could choose any additional water rate of 20%, 30% or 40%. Meanwhile, the PFY at 40% water added is significant difference (P>5%) compared of the additional 10% and 0%.

Therefore, in order to save other expenditures for concentration of PFY in later period while ensuring high efficiency hydrolysis and high PFY, we select the appropriate additional water of 20% basing on the volume of *Pangasius hypophthalmus* by-product.

3.6. Optimization of hydrolysis parameters (pH, temperature) by the method of planning experiments

Two factors pH and temperature are selected to implement the planned experiment because they are very much influential to both the hydrolysis efficient and PFY.

The constant factors in the hydrolysis (as results of experiments: ***3.1, 3.4, 3.5***) include:

- ✓ The rate of enzyme/substrate (E/S) : 0.15% (0.5IU/g substrate)
- ✓ Hydrolysis Time : 120 minutes.
- ✓ The additional water : 20%

We use orthogonal plan level 2:

Optimal number of experiments: N = 9 (according to the formula $N = 2^{k+1} + k-1$, with k = 2)

In particular, we proceeded an experimental in centre of plan. In order to check the coefficient significance the regression equation, we proceeded three more additional experiments in centre of plan.

Two factors need to examine are:

- ✓ X1: pH: below of plan 5.5; center of plan 7.0; above of plan 8.5.
- ✓ X2: Temperatures: below of plan 55^0C, center of plan: 62.5^0C, above of plan 70^0C.

The target function: The highest PFY (protein of different molecular weight as mentioned in the abstract of this article)

Planning experiments Matrix is presented in Table 6

Table 6. Matrix and experimental results of the planning process of hydrolysis of by-products of Pangasius hypophthalmus

Run No.	x1	x2	Y (%)
1	1	-1	14.5
2	-1	0	21.8
3	-1	-1	22.2
4	0	1	17.8

5	1	1	9.2
6	1	0	19.1
7	-1	-1	10.0
8	1	1	9.3
9	0	0	18.7
10	0	0	18.5
11	0	0	18.6
12	0	0	18.7
Regression equation: $Y=18.32-0.28X_1 - 4.09X_2+ 2.69X_1X_2 - 0.52X_1^2 - 2.84X_2^2$ (1)			

Note: (-): below of plan, (0): center of plan, (+): above of plan

After solving the planning experiments problem and calculating the coefficients regression. The significance of the coefficients are tested following *Student Standard* with p = 0.05, degrees of freedom: *df* = 3-1 = 2; we obtained regression equation (1)

Checking the compatibility of regression equation following the *Fisher Standard* F = 4.732; $F_{0,95}$ (9-2;3-1) = 19.2 (2)

According to (2), F <$F_{0,95}$ (9-2,3-1), so the regression equation (1) is compatible with planning experiment.

Regression equation (1) with the axes X1, X2 are shown in Figure 6

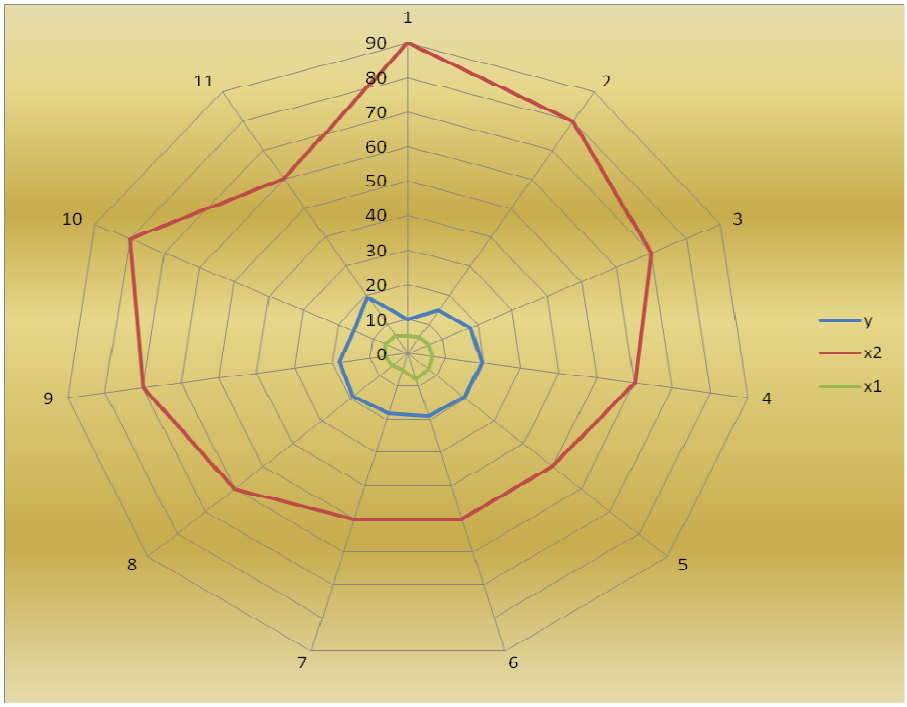

Figure 6. PFY in optimizing the experimental planning problem

We found that the two factors (pH and temperature) affect the PFY in a very complex rule.

We can determine the optimum pH and temperature for hydrolysis the by-products of *Pangasius hypophthalmus* by solving the regression equation (1).

Basing on the results obtained in Figure 6, we select the optimal conditions for hydrolysis by-products of *Pangasius hypophthalmus* at pH = 6.5 and the temperature at 58^0C

4. CONCLUSION

To hydrolyse the by-products (the spine and head) of *Pangasius hypophthalmus* using enzyme Alcalase 2.4 L has the highest PFY (19.20%) in following conditions:

Rate of enzyme/substrate (E/S): 0.15% (equal to 0.5IU/g substrate)

pH: 6.5;

Temperature: 58^0C

Hydrolysis time: 120 minutes, and

Ratio of additional water: 20%

We are continuing to study the technological features (emylsification, calcium binding, foaming…) of the protein hydrolysates have different molecular weights and the application of PFY to the food industry.

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