Optimization for autolysis assisted production of fish protein hydrolysate from underutilized fish *Pellona ditchela*

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Abstract—Response surface methodology (RSM), based on Central composite design was used to optimize the hydrolyzing conditions, for the preparation of soluble protein hydrolysate from underutilized fish *Pellona ditchela*. Most favorable interfacing external factors, such as time, temperature and pH on Degree of hydrolysis were determined from the model equations of RSM. Hydrolyzing time of 90min, pH 5 and temperature of 50^{o}C was found to be the ideal condition to attain higher DH of 40.2% in acidic condition. Proximate analysis revealed that protein hydrolysate had relatively high protein (74.0 ± 0.2 %) and low lipid (0.77±0.1%) content. The chemical score of the hydrolysate indicates that it fulfils adult human nutritional requirements. The amino acid composition of the protein hydrolysate verified to have the potential for application as an ingredient for poultry diet and adult human requirement. Protein hydrolysate from *Pellona ditchela* prepared, involving mild inorganic acid is cost effective and eco-friendly approach and the product obtained can potentially serve as a good source of desirable peptides and amino acids.

Index Terms— *Pellona ditchela*, Hydrolysate, Degree of hydrolysis, RSM, Chemical score.

1 INTRODUCTION

Exploitation of aquatic environment is major cause for the destruction of the fish population. FAO (2010) reports estimates that by 2020 about 38% of total fish produced expected to export. As the fish production is increasing each year, the discarding rate also increases. About 38.5 million tones of species, globally discarded as by-catch due to low economic value. These unusable or unwanted sub-sets off discards, known as 'by-catch', is subsequently thrown back to the sea in dead or dying condition (Harrington *et al.,* 2006). Issue due to by-catch has major adverse ecological impacts on ocean conservation and resource management of fisheries. (Lewison *et al.,* 2004).

Pellona ditchela is known as Pellona ditchelee, is a species of long finned herring native to the coast mangrove, swamps and estuaries of Indian ocean and Western Pacific generally in tropical waters. *Pellona ditchela* is reported as a common bycatch (Zymudheen *et al.,* 2004) mostly seen in Indo-West Pacific South-Africa. It feeds on small planktonic organisms especially diatoms (Siddique *et al.,* 2007). *Pellona ditchela* referred to a non-target species and commercial discards with less consumer preference and low economic value. *Pellona ditchela* noted to be major discards of the catch, which leads to threaten factor creating environmental issues. Discards reduction is a key to any transition to a sustainable and resilient fishery and a keystone of the ecosystem approach. Novel processing methods needed to convert seafood by-products into more profitable, marketable products. Many of these protein-rich seafood byproducts have wide range of dynamic properties and potentially used in foods as binders, emulsifiers, and gelling agents.

Fish and fishery products represent a very valuable source of protein and essential micronutrients for balanced nutrition and good health. Fish silage or liquefied fish protein is a simple way to convert fish bycatch and fish processing by-products into very nutritious feedstuff. Value of waste fish can be improved by converting it into fish protein hydrolysate (FPH) by utilizing proteolytic enzymes to hydrolyze the fish protein (Kristinsson and Rascso, 2000; Venugopal, 2006). The production of fish protein hydrolysate using endogenous enzyme performed traditionally, such hydrolysates are produced using the endogenous proteolytic enzymes present in muscles or fish viscera (Kristinsson and Resco, 2000). The endogenous enzymes trigger the breaking down of biomolecules to smaller peptides through autolysis process. Such process may either run at neutral or slightly alkaline pH, exploiting the serine protease of intestine in alkaline or the carboxyl protease of gastric juice in acidic pH (Pastoriza *et al.,* 2004). Despite its availability in large quantities and it's low cost, fish waste used as an excellent source of nutrients. When acidified, the viscera was stored and autolyze to yield a liquid of soluble peptide and essential amino acids (Ashraf, 2012).

The optimization process by conservative method is monotonous, incomplete and requires a lot of time to complete a single experiment. The Response Surfaces Methodology (RSM) seems to be a very popular and effective method in the optimization and food process monitoring (Wangtueai and Noomhorm, 2009). It is the modern, statistically derived experimental design. This relates product treatment to the outputs and establishes a regression equation to describe interrelations between input parameters and product properties and very cost effective (Cho *et al.,* 2004).

Protein hydrolysates have a wide range of applications in a various industries, including, human nutrition, animal nutrition, pharmaceuticals, cosmetics and fertilizers. Pro-

tein hydrolysate generated from fish proteins as bioactive compounds is good source for nutritional supplements as it can be easily absorbed and utilized for diverse metabolic activities (Nesse *et al.,* 2011). It is also useful as a nitrogen source in the growth media for microorganisms (Duarte de Holanda & Netto, 2006; Quitain *et al.,* 2001; Kristinsson & Rasco, 2000). Due to the high protein content, protein hydrolysates, extracted from marine by-products, have become well-accepted in the food industry (Cordova-Murueta *et al.,* 2007).

However, until now no information reported regarding the preparation and characterization of protein hydrolysate in *Pellona ditchela*. Hence, the objective of this study is designed to investigate the optimum condition of protein hydrolysis from underutilized fish *Pellona ditchela*, to achieve maximum degree of hydrolysis in acidic condition (sulfuric: formic acid) through RSM design. Furthermore, the chemical composition and amino acid content of the protein hydrolysate obtained in optimized hydrolysis condition can be analyzed to identify the nutritional value of the product. The final fish protein hydrolysate from *Pellona ditchela* will serve as value added product and will improve the economical value of this discarded fish. Hence the autolysis process in optimized condition obtained through RSM design will improve a nutritionally rich product and the bio-product obtained can serve potential way to enhance the economic value of underutilized fish.

2. Materials and Methodology

2.1 Sample Collection

Pellona ditchela obtained from locality near to the coastal area of Marina for low commercial value. The fish samples packed in the sterile polyethylene bag, kept in ice and transported to laboratory.

2.2 Physical Analysis and Sample preparation

The samples were collected in ice-cold condition and subjected to physical analysis including the measurement of weight, length of the species, the texture of the skin; color and opacity of the corneal condition and gills were checked to understand the freshness of the sample. After analysis of physical condition to evaluate the freshness, fish samples were rinsed and filleted down. The fillets were packed in polyethylene bags and freeze-stored at -20°C until used. The homogenate was obtained by mincing filleted tissues with water (in the ratio of 1:3) using high-speed blender for 2mins and preserved at -20^{0}c until further analysis.

2.3 Preparation of hydrolysate

The frozen homogenate was thawed and divided into 15 portions corresponding to time, pH and temperature respectively for the preparation of hydrolysate to undertake optimization study. The acidic condition maintained in the presence of sulfuric acid: formic acid. The pH values ranging from (3-6.6) were adjusted with 1N H_2S0_4 and 1N NaOH and the autolysis was carried out by maintaining at temperature of (33^{0}C, 40^{0}C, 50^{0}C, 60^{0}C & 66^{0}C,) in orbital shaking at 125rpm, samples were withdrawn at different time interval for analysis (0, 73, 80, 90, 100, 106 mins). After each incubation period, the auto-hydrolysates are exposed to 80^{0}C for 15min to inactivate the endogenous enzymes. The heated hydrolysates centrifuged at 6000rpm for 15min to obtain the corresponding supernatants (Ashraf, 2012). The supernatant was subjected to freeze-drying and the product obtained termed as fish protein hydrolysate was stored at -20^{0}C until further analysis.

2.4 Experimental of Response Surface Methodology (RSM)

The hydrolysis conditions for *Pellona ditchela* were optimized using response surface methodology (RSM) with a Central composite design. Three different independent variables includes reaction temperature (X1, °C), reaction time (X2, minute), and pH (X3) were employed and the factor levels were coded as −1 (low), 0 (central point) and +1 (high) the different factors and corresponding levels are presented in Table 1. The range for each independent variable was predetermined based on results of preliminary study (data not shown). Degree of hydrolysis (DH) selected as dependent variables. The central composite design composed of 20 treatments which includes three factorial points, six axial points (α=1.68) and six replicates of the central point. The design of experiments and dependent variable values was presented in Table 1

Table 1: Shows independent factors and corresponding coded and actual levels used in optimization experiment

Factors	Factors	Levels of factor		
		-1	0	1
X1	Time(min)	80	90	100
X2	pH	4	5	6
X3	Temperature (°C)	40	50	60

Randomized experimental runs were carried out with the purpose of minimizing the effect of unexpected variability in the observed responses (Wangtueai and Noomhorm, 2009).

2.4.1. Statistical Analysis

The response surface methodology (RSM) was statistically analyzed by Design-Expert, Version 8.0.7.1 software (Stat-ease Inc., Minneapolis, Minn., U.S.A.). The multiple regressions analysis was performed, as three parameters were varied, 10β-coefficients had to be estimated which included coefficients for the three main effects, three quadratic effects, three interactions and one constant (See *et al.,* 2011). It was assumed that the estimated behavioral model of dependent variables was described by a second order polynomial equation. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

$$Y1 = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \alpha_{ij} X_i X_j + \sum_{i=1}^{n} \beta_i X_i^2 + \varepsilon$$

Where, α0 is the constant, αi is the linear effect of the input factor Xi, αij is the linear effect by linear interaction effect between the input factors Xi and Xj, βi is the quadratic effect of

input factor Xi and ε is the error (Benyounis *et al.*, 2005). The optimization experiments for autolysis assisted hydrolysis and the responsiveness for the corresponding independent factors are exhibit is Table 2.

3 DEGREE OF HYDROLYSIS

As demonstrated in Degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. It was estimated according to the TNBS method (Alder

Table2: Show coded level combinations of three variables and DH as response variable

Run no.	X1	X2	X3	DH % (Y)
1	-1	-1	-1	29.15
2	1	-1	-1	15.24
3	0	0	0	40.2
4	0	0	0	40.2
5	1	1	-1	39.9
6	0	1	0	35.87
7	0	0	0	40.2
8	-1	1	1	19.2
9	0	0	0	40.2
10	0	0	-1	8.37
11	0	0	0	40.2
12	0	0	1	9.1
13	0	-1	0	18.9
14	-1	0	0	27.53
15	1	-1	1	10.76
16	1	0	0	27.36
17	-1	-1	-1	9.8
18	1	1	1	17.58
19	0	0	0	40.2
20	-1	1	0	18.4

X1- Time, X2 - pH, X3 - Temparature, Y - Degree of hydrolysis (DH)

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Nissen, 1979) using L-Leucine as standard the extent of hydrolysis in the fish hydrolysate was calculated.

3.1. Proximate composition

The proximal composition of control and hydrolysate sample with maximum DH was determined according to AOAC (2005) methods. The total crude protein content was analyzed using Kjeldahl method (AOAC, 2005). The moisture content was determined according to oven method (AOAC, 2005). Lipid content of samples was estimated by Soxhlet extraction. Ash content was determined by charring the predried sample in crucible at 600°C until a white ash was formed (AOAC, 2005). Estimation of protein was done with modified Bradford's Method and the standard curve was plotted using standard protein BSA, the unknown protein concentration is plotted in standard curve.

4. ANALYSIS OF AMINO ACID PROFILE USING HPLC

Hydrolyzed samples with more DH value were derivatised prior to HPLC analysis. The total amino acids were analyzed by HPLC using C18 column at the flow rate of 0.5 ml/min with 338 nm Wavelength of detection (VWD) and reaction temperature 40°C.

5. RESULTS AND DISCUSSIONS

A central composite design was employed to determine the interaction of three factors (time, temperature and pH) in order to establish optimum condition for maximizing the degree of hydrolysis (DH%). The effect hydrolyzing time (X_1), pH (X_2) and temperature (X_3) on the degree of hydrolysis (DH) (Y) was determined using Response Surface methodology (RSM). Based on Central Composite Design (CCD) 20 sets of hydrolysis experiments were conducted and the experimental data obtained is shown in Table 3.

Table 3: Show the ANOVA for 2nd order polynomial order, regression co-efficent and corresponding p values in optimization experiment

Source	Degree of Freedom	Mean Square	*F* value	*P* value Prob> F
Model	9	325.72	274.63	<0.0001 (significant)
Residual	10	1.19		
Lack of fit	5	2.37		
Pure error	5	0		
Total	19			
R^2				0.996
Model	**Degree of freedom**	**Estimate**	**P value**	
Intercept	1	40.2		
X_1-*Time*	1	0.49	0.1272	
X_2-*pH*	1	4.3	<0.0001	
X_3-*Tempe*	1	-0.4	0.2076	
$X_1 X_2$	1	4.1	<0.0001	
X_1 X3	1	-5.87	<0.0001	
$X_2 X_3$	1	-4.55	<0.0001	
$X1^2$	1	-4.51	<0.0001	
$X2^2$	1	-4.54	<0.0001	
$X3^2$	1	-11.13	<0.0001	

The responsiveness of Y was evaluated through quadratic model and the response surface regression equation deployed was

$$Y= +40.20+0.49\ X_1+4.30X_2-0.40\ X_3+4.10\ X_1X2-5.87X_1X_3-4.55\ X_2X_3\ -4.51\ X_1^2-4.54\ X_2^2-11.1\ X_3^2$$

Where, Y, X_1, X_2 and X_3 are DH, time (mins), pH and temperature (°C) respectively.

5.1. Analysis of Variance - Conditions of optimum responses

The competence of the model is justified through

analysis of variance (ANOVA), as shown in Table 4. The statistical significance of the quadratic polynomial model equation was evaluated through analysis of factor (F test) and ANOVA. The F-value implies that the second order polynomial model is significant to the analysed data and the coefficient of variation (CV) is 4.21% which signifies the extent of accuracy of the experiment. In general, lower the value of CV, higher the reliability of the experiment, here a lower value of CV indicated a better precision and reliability of data (Box *et al.*, 1978).

Table 4: Shows the actual levels of independent variables used in optimizing the hydrolysis conditions for autolysis process in protein hydrolysate along with the observed and predicted values for the response variable (DH, Y).

Run no	X1	X2	X3	Y^a	Y^b
1	-1	-1	-1	29.15	29.36
2	1	-1	-1	15.24	13.83
3	0	0	0	40.2	40.2
4	0	0	0	40.2	40.2
5	1	1	-1	39.9	39.72
6	0	1	0	35.87	34.59
7	0	0	0	40.2	40.2
8	-1	1	1	19.2	20.64
9	0	0	0	40.2	40.2
10	0	0	-1	8.37	9.38
11	0	0	0	40.2	40.2
12	0	0	1	9.1	8.05
13	0	-1	0	18.9	20.14
14	-1	0	0	27.53	26.61
15	1	-1	1	10.76	10.39
16	1	0	0	27.39	28.26
17	-1	-1	-1	9.8	9.32
18	1	1	1	17.58	18.09
19	0	0	0	40.2	40.2
20	-1	1	0	18.4	18.8

Y^a - Observed Value, Y^b - Predicted Value

The precision of a model was checked by the regression coefficient (R2) and calculated as 0.9960, which indicated that the quadratic model can explain a high percentage of the variability *i.e.* 9960%. It can also suggest that, this quadratic model appropriately represent the real association among the chosen hydrolysis parameters. While fitting the model, various statistical analysis techniques were used to judge the experimental error, the suitability of the model, and the statistical significance of the terms in the model. The regression coefficients and corresponding P values are given in Table 4.

The mutual interaction between the optimum factors was implied through P values which act as tool to check the coefficient. For instance lower the P value, higher the significance of the corresponding coefficient. The interaction between the independent factors has been described through regression model and interactive effects illustrated in 3D response surface plots. From the model, optimum condition required for maximum DH was identified as, time (X_1) of 90mins, pH(X_2) 5 and temperature (X_3) 50^0C. Models presented showing figure 1 the effect of two factors, while the other factors were held in zilch effect. In the present study, DH value 40.1% was obtained from the model equation and the graph obtained displays in bell-shaped pattern in the response surface graph. The DH of the present analysis is in bell-shaped pattern, the comparable to outcome reported by Amiza *et al.*, 2012, the effects of pH and temperature on the DH, Sami Saidi *et al.*, 2013. From figure 1(a) it is clearly evident that the DH increased with increase in pH and the effect of pH and temperature on DH is in bell-shaped pattern clearly visualize in figure (c).

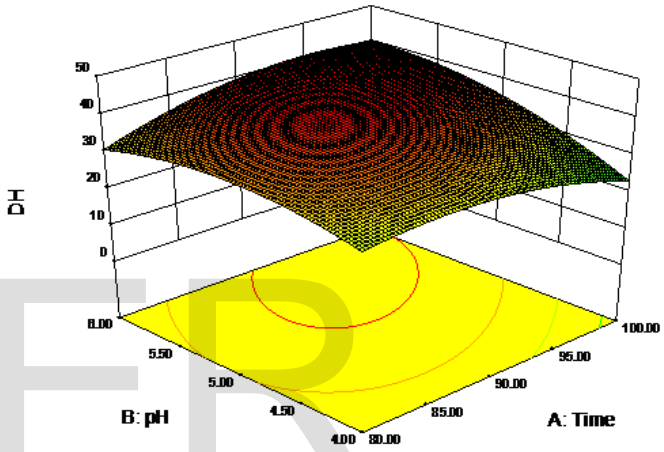

Figure 1: a) Effect of pH and Time on DH

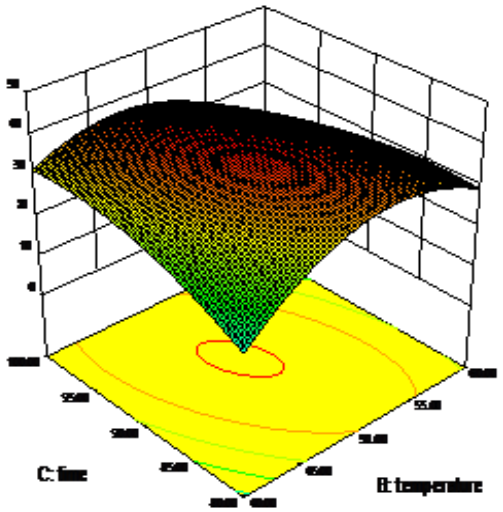

Figure 1: b) Effect of Time and Temperature on DH

The results showed a certain correlation between the mass distribution of soluble peptides and the DH. The results obtained showed these hydrolysis reactions with different conditions provided in general a high proportion of low mo-

lecular weight peptides and free amino acids potentially upgradable in food supplements.

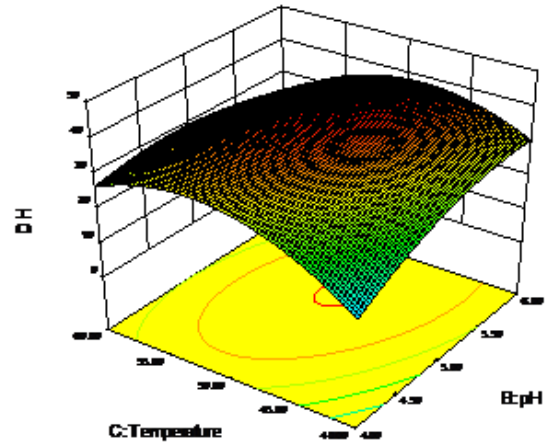

Figure 1: c) Effect of Temperature and pH on DH

5.2. Proximate analysis

The proximate composition on fish protein hydrolysate obtained in optimized condition and unhydrolysed samples of *P. ditchela* mentioned in Table 5.

Table 5: Proximate composition of protein hyrolysate and control samples of *Pellona ditchela*

Composition (%)	Control	FPH
Protein	59.0 ± 0.3	74.0 ± 0.2
Lipid	22.5 ± 0.1	0.77 ± 0.1
Moisture	3.8 ± 0.1	3.65 ± 0.02
Ash	14.7 ± 0.1	21.25 ± 0.1

Hydrolysate sample in optimum condition contains high protein content 74.0±0.2% whereas, in unhydrolsed sample the percent of protein was comparatively low *i.e.* 59.0 ± 0.3 %. In contrast to protein, the lipid content of the prepared hydrolysate exhibits a converse range when compared to unhydrolysed control sample mainly due to the interference of acid in hydrolysis. In acidic hydrolysis, free lipids are cross-linked to proteins and then break down protein particles, so that most lipids are accessible to solvent. The Lipid residues in product must be lower than 0.5% to prevent alteration of the lipid fraction during storage (Spinelli *et al.*, 1972) the reduction in lipid content in protein hydrolysate is due to the exclusion in centrifugation and mostly due to lipid oxidation. This can improve the product stability and quality (Shahidi *et al.*, 1995; Diniz and Martin 1997; Kristinsson and Rasco 2000b: Ali *et al.*, 2010).

5.3. Amino acid composition

The amino acid composition of fish protein hydrolysate and compute chemical score of samples of *P.dictchella* are presented in the Table 6.

Table 6: Shows the comparison of the amino acid composition of *Pellona ditchela* hydrolysate (g/100g) obtained by autolysis with FAO/WHO and NRC reference protein

Amino acid	FPH	RP1 (Adult)[a]	RP2 (Poultry)[b]	Chemical Score RP1	Chemical score RP2
Arginine	1.22		0.7		1.74
Threonine	1.61	0.9	0.47	1.79	3.4
Tyrosine	0.22	-	-	-	-
Valine	1.5	1.3	0.7	1.15	2.14
Methionine	1.81	1.7	0.3	1.1	6.03
Phenylalanine	1.42	3.8	0.83[c]	0.37	1.71
Isoleucine	1.78	1.3	0.65	1.37	2.71
Leucine	2.57	1.9	0.82	1.34	3.13
Lysine	1.99	1.6	0.69	1.24	2.88
Histidine	1.92	1.6	0.17	1.2	11.29
Alanine	1.11				
Aspartic acid	1.1				
Glutamic acid	2.04				
Serine	1.38				
Glycine	1.39				

a Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990)
b Essential amino acid requirements of poultry according to NRC (NRC, 1993)

The hydrolysates obtained from, *P.ditchela* exhibits considerably high level of total amino acids. In the present experiment data, the hydrolysate endure rich in essential amino acid such as theronine, methionine, isoluecine and Phenyl alanine this indicate the hydrolysate is nutritionally rich products (figure 2). From the quantitative point of view, the ratio of essential amino acids to non-essential amino acids was increased in *P.ditchela* tissue after hydrolysis process. Indeed protein hydrolysates developed by acid autolysis was rich essential amino acid, which includes arginine, tyrosine, and but lower in aspartic acid, serine and histidine. This finding was close to the report of Klompong *et al.*, (2009), which showed that yellow stripe trevally (Selaroides leptolepis) protein hydrolysates had a higher ratio of essential amino acids to non-essential amino acids. Shahidi and others (1995) reported that the composition of protein hydrolysates depended on the type of enzyme used.

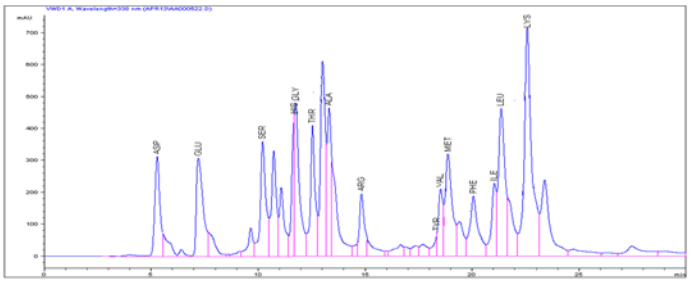

Figure 2: Chromatogram of protein hydrolysate obtained from *Pellona ditchela*

Shahidi *et al.*, 1995 also reported autolytic method can be suggested for recovery of proteins either from underutilized or fish processing waste. Moreover, due to simplicity of the operation and no enzyme costs involved the process still used for the preparation of protein hydrolysate. The protein level in the hydrolysate is influenced by the hydrolysis time, pH and temperature, in the present paper, the optimum condition used for tissue autolysis was determined as 90mins,

pH5 and temperature 50^0Cand is evident from the response surface Figure 1.

The amino acid composition in this study revealed that the amino acid profiles of the *P.dictehla* hydrolysates generally higher in EAA compared to the suggested pattern of requirement by FAO/WHO for the adult humans. The results of the *P.dictehla* chemical score showed that the composition in EAA of fish hydrolysates exhibits higher than the amount required the FAO/WHO and NRC standards, the hydrolysates fulfill human requirements and poultry feed. Based on the results in spite of minor deficiencies in certain essential amino acids the protein hydrolysate does no lose its nutritional value, consequently it can be considered as a balanced ingredient in food. This is an interesting result, since the nutritional composition, especially the essential amino acid content is a determining factor in human and poultry feeding. The outcome of this study indicates that the protein hydrolysate from *P.dictehla* may potentially serve as a good source of desirable peptides and amino acids.

Nutritive value of a protein can be estimated by its chemical score which aids in comparing the levels of essential amino acids between the test and the standard. In the present experiment chemical scores are computed based on the reference of FAO/WHO (1990) for adults. In fact, proteolysis led to the liberation of additional amino acids compared to raw material and to the destruction or complexation of some amino acids. It is quite acceptable that conducting the hydrolytic reactions at ambient temperatures, without adding exogenous enzymes can be effective and financially interesting alternative for protein recovery and dry matter solubilisation

5.4. Conclusion

Raw materials or wastes, if not utilized may cause environmental, health and economical problems. Hence, optimal utilization of the raw material is of prime importance to cope for the increasing demand products. Influence of external parameters such as, time, pH and temperature on protein recovery, observed during hydrolysis reaction. From RSM model, the optimum condition required for maximum degree of hydrolysis (40.01%) was identified as, time (X_1) of 90 mins, pH5 (X_2) and temperature (X_3) 50^0 C. The hydrolysate prepared in optimized condition with maximum DH, exhibits amino acid profile with high content of EAA for human requirement. Thus, hydrolysate obtained from the low value fish *P.dictehla* can serve as a nutritional source for various industrial applications such as, feed and food purpose. Indeed the outcome of such research can upgrade the value of the discarded fish and provide incentive for commercial developments leading to cost-effective production of potential by-product in large-scale.

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