EVALUATION OF HYDROLYSATE AND OPTIMIZATION OF ENZYME ACTIVITY FOR CONSERVATION OF FISH WASTE AND CHEEZE WHEY USING PROTEASE

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ABSTRACT

The study was undertaken with an objective to conserve fish waste, cheese whey to nutritional product and reducing environment contaminations from their disposal. Fish waste was diluted in the ratio of 1:2 with water, buffer and cheese whey separately. Dilution of fish waste with cheese whey reduced the awful smell and improved the substrate chemical value. Protease was used for hydrolysis of fish-cheese whey mix. Protein yield and degree of hydrolysis was higher when protease was 2%. Protease concentrations of 1.5 to 2.0 % were found to be optimum range of concentration based on protein recovery, average and marginal degree of hydrolysis. Dry matter content of the residue was 13 % and hydrolysate was 8%. All the proximate constituents in hydrolysate were about 50 % of the corresponding constituents in the residue. Current investigation concludes with an inference of identifying further economic and readily available enzymatic source for the hydrolysis of fish waste and cheese whey and to conduct a comparative analysis with the data of present study.

Keyword: *Cheese whey, Enzymes, Fish waste, Hydrolysis, Optimum range, Protein yield, Protease.*

1. INTRODUCTION

Indian fisheries and dairy are two important agri-economic sectors playing crucial role in health, nutrition, economy and, stabilising the national food security. Fishery and dairy industries are two important sectors in agriculture in India. Both the sectors are growing at the rate of over 4.5 and 3.7 %, respectively in the past decade. Both fish processing and cheese manufacturing industry are suffering in the disposal of fish wastage produced during processing (dressing percentage of fish is 50 % only) and whey produced in bulk during cheese making (9 kg whey from 1 kg of cheese manufactured), respectively. Both fish waste and cheese whey are highly putrescible. Both have a high biological oxygen demand and classified as certified waste. Contrasting feature of both the wastes is their solid concentration. Whey has only 3 to 6 % solids while fish waste may contain 70 to 80 % solids. Treatment for disposal of these wastes can be expensive and laborious. Best choice is conserving these wastes into value added products. Enzymatic hydrolysis of FW and CW and harvesting valuable nutrients, particularly proteins as human and livestock supplements is widely attracted technology developed in the last three decades. Harvesting of protein by hydrolysis method is mainly achieved by the use of acids, alkalis and enzymes, where the first two are chemical

methods and later one is enzymatic method, enzymatic method is accomplished by using either exogenous, endogenous or combination of both the methods [1]. In recent years, protein hydrolysates harvested from FW or CW have attracted much attention of food biotechnologists due to availability of large quantities of raw material for the process, presence of high protein content with good amino acid balance and bioactive peptides. Enzymatic method for hydrolysis is used widely due to better and reduced processing steps compared to chemical methods (acids and alkalis). Majority of the commercial enzymes used for hydrolysis are of microbial origin. They are also costly because of processing cost involved in the manufacturing which makes this method economically not feasible. This is particularly true when the amount of substrate to be hydrolysed is considerably large in quantity and, fall under the category of by-product. Hence it is necessary to develop a cost cutting strategy to process FW and CW using alternative catalysts to harvest protein hydrolysates.

2. MATERIALS AND METHODS

2.1 Fish waste (FW)

Raw, minced and fresh FW of sardines (*Sardinella longiceps*) were collected from the local fish market of Bangalore. Weight and pH of the minced FW were recorded (M/s Eutech instruments, pH Digital Tutor, Malaysia).

2.2 Cheese whey (CW)

CW were collected from the cottage cheese manufactures; M/s Akshaya dairy food, Koramangala, Bangalore. CW were disposed at free of cost by the producer. The volume and pH (M/s Eutech instruments, pH Digital Tutor, Malaysia) of the CW were recorded.

2.3 Protease Enzyme

Industrial protease for an Enzyme activity of 13,00,000 IU/g were procured from M/s Varsha Multitech, Bangalore and it was stored at -4°C.

2.4 Proximate composition of substrates.

The proximate constituents of all substrates and product were estimated according to AOAC [2]

2.5 Energy Value of the diet

Energy value of the diet was predicted from the amounts of digested nutrients viz., CP, EE and TCHO using the East German system for cattle [3]. Empirical formulae used for different energy parameters were as below:

- Gross energy (GE; kcal) = $4Y_1 + 9Y_2 + 4Y_3$
- Digestible energy (DE; kcal) = $5.79X_1 + 8.15X_2 + 4.24X_3$

Where,

Y_1 = CP (g) X_1 = Digested CP (g)
Y_2 = EE (g) X_2 = Digested EE (g)
Y_3 = TCHO (g) X_3 = Digested TCHO (g)

The above value used for the DE was digestible fraction of X_1, X_2 and X_3. All values were Mcal/Kg.

2.6 Homogenization of raw material.

The raw materials for the hydrolysis were homogenized with different diluents as discussed by Ovissipour et al., 2009 [4]. The pH of the FW was 5.8. Homogenization of Fish with water: FW was homogenized with water in the ratio of 1:2 and homogenized for 10 minutes and the pH of the mixtures was 6.2. Homogenization with Phosphate Buffer: (Sodium phosphate buffer) FW was homogenized with freshly prepared sodium phosphate buffer in the ratio of 1:2 and conditions followed for homogenization was as same as previous homogenization. Homogenization with CW: FW was homogenized with CW as a dilution medium in the ratio of 1:2. The *p*H of the mixtures was maintained at 6.4.

2.7 Hydrolysis of substrate

Homogenized FW, 100 ml was taken as substrate for the hydrolysis in a 250 ml conical flask. Hydrolysis was carried out with protease as follows; Six different concentrations of protease enzyme such as 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 % were added to the homogenate, mixed thoroughly and incubated in horizontal shaker water bath for 24 hours at 40 °C at 200 rpm.

3. RESULT AND DISCUSSION

3.1 Substrate composition and dilution medium

Dry matter (DM) content of FW (**Table 1**) was 25% and CW was 5.4%. FW was homogenised with water in the ratio of 1:2 in order to improve the enzymatic activity. In a simple chemical reaction, a linear relationship between the rate of formation of the product, and the concentration of substrate exists. However, a hyperbolic relation exists between the rate of reaction and the concentration of the substrates in an enzymatic hydrolysis. At a low concentration of the substrate, the rate of reaction increases steeply. On the other hand, when concentration of substrate increases, the enzyme activity reaches a platue and it is called V_{max}. In practical purposes, half V_{max} is selected to achieve increased rate of reaction. The concentration of the substrate at that point is called K_m. Enzymes with a high K_m have low affinity for its substrate. It requires a greater concentration of substrate to achieve V_{max}. On review of literature, FW to diluents ratio used was 1:2 [4], [5]. Hence, FW was homogenised with DW [6], [7] and, sodium phosphate buffer [8] in the ratio of 1:2 in our study. Apart from both the diluents, CW was also used as diluent. Diluting the FW with the CW had advantage of conservation of another waste product and also economises the water and/or reduces the cost of buffers.

After addition of diluents to the FW, the pH was increased to 6.2, 6.7 and 6.4 for waetr, sodium phosphate buffer and CW, respectively compared to initial pH of 5.8. When FW was homogenized with 3 different diluents, there was a change in the odour of the content. Awful smell of the content was reduced more when FW was diluted and homogenised with CW because CW was a supernatant of rennet hydrolysed milk solids and contain hydrolysed peptides. Peptides may have characteristic odour and they are even reported to be source of social communication in many animal species [9]. Probably peptides in CW protein as well as natural odour of the fermented CW might suppress the awful odour compared to the water or sodium phosphate buffer as diluents. TA (**Table 1**) content in FW, CW, fish-whey mix (FWM) was 6.5, 2.4 and 4.4 %, respectively ($P< 0.01$). Hence hydrolysable OM was higher in CW (98 %), than FW (94 %).

Table 1: Proximate composition of substrates used for hydrolysis

Parameter	Replicate	Substrate		
		FW	**CW**	**FWM**
Dry Matter **(SEM=0.246)**	1	24.080	5.332	12.207
	2	25.086	5.469	12.106
	3	25.641	5.385	12.688
	Mean	**24.936^{c}**	**5.395^{a}**	**12.334^{b}**
	± S.E	0.457**	0.040**	0.179**
Organic Matter **(SEM=0.175)**	1	93.534	97.731	95.825
	2	93.772	97.261	95.724
	3	93.207	97.776	95.300
	Mean	**93.504^{a}**	**97.589^{c}**	**95.616^{b}**
	±S.E	0.164**	0.165**	0.161**
Crude Protein **(SEM=0.364)**	1	55.300	8.484	56.535
	2	54.370	8.335	56.649
	3	55.374	7.117	56.847
	Mean	**55.015^{b}**	**7.979^{a}**	**56.677^{c}**
	±S.E	0.323**	0.433**	0.091**
Crude Fat **(SEM=0.209)**	1	30.958	1.893	19.396
	2	31.596	1.208	19.851

	3	31.587	1.676	19.791
	Mean	**31.380^{c}**	**1.592^{a}**	**19.679^{b}**
	±S.E	0.211**	0.202**	0.143**
Total Carbohydrates **(SEM=0.528)**	1	7.276	87.354	19.894
	2	7.806	87.717	19.223
	3	6.246	88.983	18.662
	Mean	**7.110^{a}**	**88.018^{c}**	**19.260^{b}**
	±S.E	0.458**	0.494**	0.356**
Total Ash **(SEM=0.175)**	1	6.466	2.269	4.175
	2	6.228	2.739	4.276
	3	6.793	2.224	4.700
	Mean	**6.496^{c}**	**2.411^{a}**	**4.384^{b}**
	±S.E	0.164**	0.165**	0.161**

Mean values bearing ^{a,b,c} superscripts for a parameter in a row differ significantly. * P< 0.05; **P < 0.01

Dilution of FW with CW was improved hydrolysable OM by 3% than other water or sodium phosphate buffer as diluent. FW had a very high fat content of 31.4 % (P< 0.01). Slizyte et al, 2005 [10] has shown that the fish raw material containing the highest amount of fat yielded the lowest percent of solubilised protein. Contrary to FW, CW had low fat content of 1.6 %. Fat in the diluted fish substrate with CW was only 20 %. Fat content varies with the whole fish and its different organs in the FW. Ovissipour et al, 2009 [4] reported a low lipid content of 1.34 % in the visceral waste of beluga sturgeon *Huso huso*. Amiza et al, 2011 [6] reported 68.21 % fat in the silver cat fish frame. Total carbohydrates (TCHO) in CW were 88 % which is mainly lactose. In contrast to CW, TCHO in FW was 7 % only. Dilution of the later with the CW resulted in 19 % of TCHO. Although higher amount of fat gives the lowest percentage of solubilise protein, de-fatting the raw material result in a very high ash (TA) content of 25 % in hydrolysates [11], [10]. The diluted FW with CW marginally improved protein content, as compared to water or sodium phosphate buffers as diluents. Thus, dilution of FW with CW had biphasic advantage of reducing the awful smell and improved the substrates chemical activity by increasing the protein and reducing the lipids.

3.2 Substrate hydrolysis and optimization: Protease

PY from the protease hydrolysis varied significantly (P< 0.01) with increasing concentration of enzyme (**Table 2**). It was more when protease concentration was 2%. PY was increased (40 to 60 %) with increasing concentration of enzyme from 0.5 to 2 % but, decreased by about 5 and 15 %, respectively when concentration was 2.5 and 3.0 %. Amiza et al.,2011 [6] reported a protein recovery from silver catfish (*Pangasius* sp.) was as high as 71.6 % from its frame and the hydrolysate in powder form contained 65.05 % protein against the 25.05 % in the unhydrolysed substrate. Optimum concentration of enzyme for substrate hydrolysis was reported to be 0.15 % [12] to 2.5 % [8]. Higher PY observed with enzyme concentration of 2.0 % was in agreement to the Amiza et al., 2011[6] where silver catfish frame was the substrate. The degree of hydrolysis (DH) at different concentration of protease such as 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 % was 59, 57, 76, 93, 86 and 71 %, respectively (**Chart: 1**). Both PY and DH were highest when enzyme concentration was 2.0 %. Although DH was higher when protease concentration was 2.0 %, ADH (P< 0.01) and MDH (P< 0.01) was highest when the concentration was 1.5 %. However, MDH was not significantly (P = 0.32) different between 1.5 and 2.0 % of concentration unlike ADH. Optimum protease concentration thus, was 1.5 %. However, since PY and DH at 2.0 % concentration was greater than 1.5 % concentration as well as marginal yields between both the concentrations were comparable, later concentration was opined to be more appropriate for better productivity (**Chart: 2**). If concentration of enzyme was increased from 1.5 % to 2.0 % of substrates, an additional cost of Rs. 500 incurred was justifiable with the significantly higher PY, DH and MDH.

Table: 2 Optimization of protease activity

Concentration of Protease (%)	Replicate	PY	DH	ADH	MDH
0.5	1	40.590	59.691	119.382	0.000
	2	40.340	59.324	118.647	0.000
	3	40.260	59.206	118.412	0.000
	Mean	**40.397^{b}**	**59.407^{b}**	**118.814^{f}**	**0.000^{d}**
1.0	1	39.240	57.706	57.706	-3.971
	2	39.290	57.779	57.779	-3.088
	3	39.460	58.029	58.029	-2.353
	Mean	**39.330^{a}**	**57.838^{a}**	**57.838^{e}**	**-3.137^{c}**
1.5	1	51.690	76.015	50.676	36.618
	2	51.370	75.544	50.363	35.529
	3	51.440	75.647	50.431	35.235
	Mean	**51.500^{d}**	**75.735^{d}**	**50.490^{d}**	**35.794^{e}**
2.0	1	63.210	92.956	46.478	33.882
	2	63.450	93.309	46.654	35.529
	3	63.330	93.132	46.566	34.971
	Mean	**63.330^{f}**	**93.132^{f}**	**46.566^{c}**	**34.794^{e}**
2.5	1	58.390	85.868	34.347	-14.176
	2	58.320	85.765	34.306	-15.088
	3	59.050	86.838	34.735	-12.588
	Mean	**58.587^{e}**	**86.157^{e}**	**34.463^{b}**	**-13.951^{b}**
3.0	1	48.480	71.294	23.765	-29.147
	2	48.400	71.176	23.725	-29.176
	3	48.050	70.662	23.554	-32.353
	Mean	**48.310^{c}**	**71.044^{c}**	**23.681^{a}**	**-30.225^{a}**
	SEM	0.139**	0.215**	0.154**	0.676**
#Within concs.	**Sig.**	0.01	0.01	0.01	0.01

#Mean protein content of fish substrate was 68%
a,b,c,d,e,f

Values bearing different superscripts differed significantly: *P < 0.05, **P < 0.01
 # Greenhouse-Grisser was taken for the significance where P < 0.01. Mauchly's test: Null hypothesis rejected because P < 0.01.

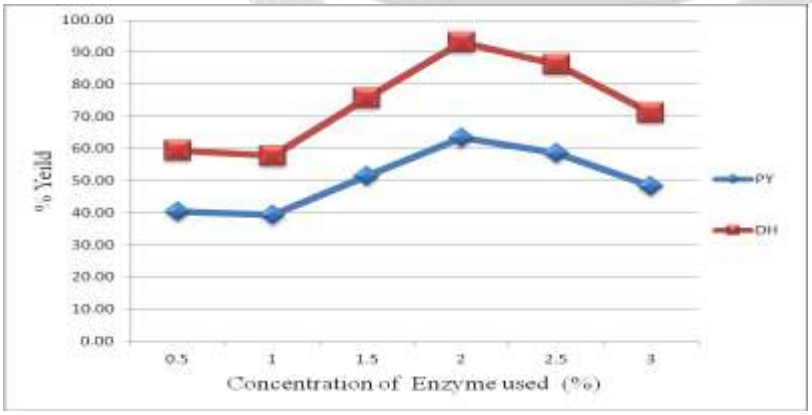

Chart: 1 Protein yield and Degree of hydrolysis obtained by Protease Hydrolysis

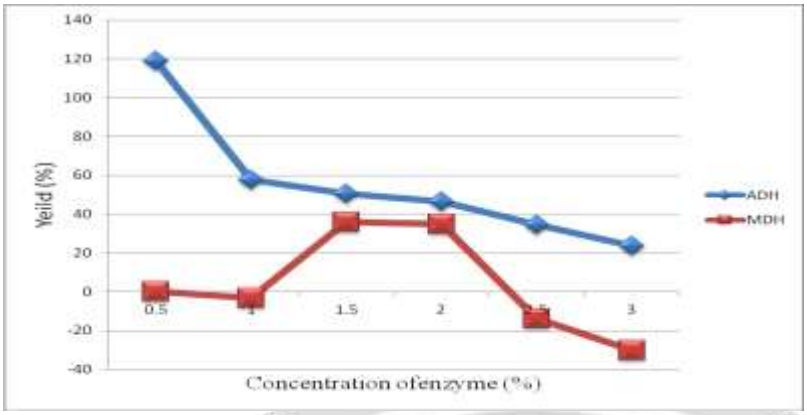

Chart: 2 Average and Marginal degree of hydrolysis obtained by Protease Hydrolysis.

4. CONCLUSIONS

Hydrolysis of fish and fish product were widely done for extracting of the protein present in it. Optimization of the catalyst is required for the better protein yield, especially when the substrate used is a mixture of two different industrial by product. Product formed from such hydrolysis is having a good chemical value.

FW was diluted in 1:2 ratio with water, buffer and cheese whey (CW). Awful smell of the content was reduced more when FW was diluted and homogenised with CW. Dilution of FW with CW was improved hydrolysable OM by 3% than other DW or sodium phosphate buffer as diluent. Dilution of FW with CW had biphasic advantage of reducing the awful smell and improved the substrate value by increasing the protein and diluting the lipid content of the FW which otherwise may reduce protein recovery as for the literature.

Average degree of hydrolysis (ADH) and marginal degree of hydrolysis (MDH) was significantly higher ($P< 0.01$) when the protease concentration was 1.5 %. However, MDH was not significantly ($P = 0.32$) different between 1.5 or 2.0 % concentration unlike ADH. Optimum protease concentration thus, was 1.5 %. However, since PY and DH at 2.0 % concentration was greater than 1.5 % and cost incurred was also lesser than benefit accrued upon, protease concentration found to be productivity and economical point of view ranged between 1.5 to 2.0 % concentration. Moreover it is required to investigate further on cheaper sources of enzymes and their optimization.

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