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Optimization of Enzymatic Extraction of ACE Inhibitory Peptide from Rohu (*Labeo rohita*) Fish Waste using RSM

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ABSTRACT

Background: Hypertension is one of the cardiovascular disease that kills people silently across the globe. It can be controlled, in one of the way, by ACE inhibitory peptide extracted from aquatic resources.

Methods: Rohu (*Labeo rohita*) fish wastes were quantified for their anatomical yield; analyzed for their proximate composition and optimized the enzymatic extraction parameters for ACE inhibitory peptides. Response surface methodology with Box-Benhken Design (RSM-BBD) was used to optimize alcalase concentration (0.5-2% v/w), hydrolysis temperature (45-60°C), hydrolysis time (60-240 min.) and solid: liquid (S/L) ratio (0.2-1) to obtain rohu fish waste peptides.

Result: More waste generated in smaller (49.4%) than medium and bigger (34.5%) fish. Quantum of edible flesh (59.06%) was followed by head (23.9%), trimmings (5.18%), scales (4.19%) and swim bladder (0.65%). However, protein content was highest in swim bladder (34.1%) followed by scales (22.9%), trimmings (18.7%) and head (17.1%). Alcalase concentration (1.08%, v/w), temperature (52.10°C), hydrolysis time (129.18 min) and S/L ratio (0.8:1) were found optimum for extraction ACE inhibitory peptide with DH, ACE inhibition and PY of 19.27%, 54.98% and 51.37% respectively. Results showed the potential of extracted ACE inhibitory peptide as ingredients in functional food.

Key words: ACE inhibitory peptide, Alcalase, Anatomical yield, Enzymatic ecxtraction, Optimization, Proximate composition, Rohu fish waste.

INTRODUCTION

Recently there is an increase in cardiovascular disease (CVD) possibly because of reduced physical activities, stressful lifestyle, obesity *etc*. One of the major CVDs is hypertension. Globally 1.13 billion people are suffering from hypertension, a condition when systolic blood pressure (SBP) reaches at least 140 mm Hg or/and diastolic blood pressure (DBP) to 90 mm Hg or both while the normal SBP/DBP is <120/<80 (Poulter *et al*., 2015). BP is regulated by the renin-angiotensin system (RAS) with the critical role of angiotensin-I converting enzyme (ACE) (Ngo *et al*., 2016). ACE (EC 3.4.15.1) is a membrane bound enzyme found at pulmonary and renal endothelium surfaces. ACE converts Angiotensin I into Angiotensin II removing a dipeptide (His-Leu) from C-terminus (Gao *et al*., 2018) and there by resulting in high BP. Therefore, any ACE inhibitory biomolecule will reduce BP and there by minimizing hypertension. Pharmaceutical drugs (*e.g*. enalapril, captopril, lisnopril, ramipril, *etc*.) target ACE inhibition for lowering BP but these drugs have associated side-effects like cough, angiodema, chest pain *etc*. (Coleman and Cox, 2012). Therefore, scientists are trying to identify the peptides having potential therapeutic benefits like ACE inhibitory activity from various foods including fish (Yathisha *et al*., 2018).

India is the 2nd largest producer of freshwater fish in the world. This higher production provides more fish for processing. However, global fish processing dispose >60% of fish biomass as skin, head viscera, trimmings, liver, frames, bones, *etc*. (Halim *et al*., 2016) imposing a cost burden on the industry for its disposal. Though it is used traditionally to produce low value products like fish silage, fish meal and sauce (Halim *et al*., 2016), it can be utilized for high value products with health benefits. The FPH, bioactive peptides including antihypertensive (or ACE-inhibitory) peptides can be extracted from considerable amount of protein in fish waste and novel bioactive peptides can be identified to provide with next generation functional products (He *et al*., 2013).

Indian major carps (IMC) *viz*. catla, rohu and mrigal contribute 70-75% of total freshwater fish production in India, followed by silver carp, grass carp, common carp and catfish (Kudre *et al*., 2017). Among IMC, rohu is one of commonly

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cultured freshwater fish. High growth potential, nutrition, delicious attributes and high market value make rohu an economically important fish. It is sold extensively in the Indian fish market generating approx. 0.9 MT/annum of rohu fish waste (RFW) in India (Kudre *et al*., 2017). Thus, the utilization of RFW for production of ACE inhibitory peptide would greatly benefit in reducing fish waste disposal. Extracted peptides can also be a suitable ingredient in functional food reducing hypertension without any side effects. Therefore, the objective of this study were to calculate anatomical yield of generated fish waste, to estimate proximate composition and to optimize the independent variables of extraction of ACE inhibitory peptide.

MATERIALS AND METHODS

Rohu fish was procured in chilled condition from local fish market to ICAR-CIPHET, Ludhiana. Non-edible tissues (fins, scales, swim bladder and head) separated from edible flesh were mixed in natural proportion to get a homogenized RFW. Analytical grades chemicals, organic solvents and enzymes procured from Sigma, St. Louis, USA; Himedia, Mumbai, India and MP Biomedicals Mumbai, India were used. Importantly, Alcalase® (Protease from *Bacillus licheniformis*), Angiotensin-I Converting Enzyme (ACE), 2,4,6-Trinitrobenzene Sulphonic Acid (TNBS) and N[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) were obtained from Sigma. Sodium Phosphate Dibasic Heptahydrate, Sodium Phosphate Monobasic Monohydrate, Bovine Serum Albumin, Bradford Reagent, Tricholoroacetic Acid and L-Leucine were procured from Hi-media and Captopril and Hydrochloric acid were received from MP Biomedicals. Experiments were performed during 2018-2020.

Anatomical yield was determined based on total length (TL) and total weight (TW) of fish (Table 1). The percentage of fish head (FH), fins (FN), scales (SC) and swim bladder (SB) were calculated. Moisture, fat, ash and protein of rohu wastes were analyzed individually and after mixing them in natural proportion (RFWP) (AOAC, 2020).

Homogenized RFW added with a buffer (PBS, 50 mM, pH 7.5) and alcalase enzyme was incubated for 30 min followed by 240 min hydrolysis and subsequent enzyme inactivation at 90°C for 15 min and centrifugation (5000Xg, 20 min). The supernatant filtered was named rohu fish hydrolysate (RFH). Its DH was determined by Adler-Nissen (1979) method. RFH (0.25 ml) added with 2 ml PBS (0.2125 M, pH 8.2) and 2 ml TNBS (0.1%) was incubated in the dark at 50°C (1 h). The reaction was terminated using HCl (0.1 M, 5 ml) and the absorbance was measured at 340 nm. DH expressed Q-amino acids in terms of L-Leucine (Eq. 1).

$$DH\ (\%) = \frac{(Lt\text{-}Lo)}{(Lmax\text{-}Lo)} \times 100 \quad \text{.....(1)}$$

Where,

Lt= Amount of α-amino acids released at time 't'.

Lo= Amount of α-amino acids released in acid-solubilized substrate.

Lmax= Amount of maximum α-amino acids found.

Ultrafiltration

Rohu fish hydrolysate (RFH) obtained at the end of enzymatic hydrolysis was filtered twice with filter paper (hi-media, 12.5 cm dia; retention: 10 µm; grade 631 equivalent to grade 4) and then by a syringe filter (hi-media, PES, 0.45 µm) and subsequently again through a syringe filter (hi-media, PES 0.2 µm pore size). The filtrate was then passed through a MWCO (10 kDa and subsequently 3 kDa). The filtrate (<3 kDa) named RFW peptides (RFWP) was used to assess the ACE inhibitory activity.

ACE inhibitory assay

A modified method of Murray *et al*. (2004) and Theodore and Kristinsson (2007) using FAPGG (a synthetic substrate) was used for ACE inhibitory assay. RFWP (100 µL, 0.2% protein w/v), ACE enzyme (15 mU, 50 µL) and substrate (1 mL, 0.5 mM FAPGG) were mixed together, incubated at 25°C (60 min) and the absorbance was measured at 340 nm. FAPGG without ACE enzyme served as a control. Captopril was used as standard. Hydrolysis of FAPGG by ACE resulted in a decrease in absorbance. ACE inhibition calculated as (Eq. 2).

$$ACE\ Inhibition\ (\%) = 1 - \frac{Slope\ of\ sample\ cursve}{Slope\ of\ control\ cursve} \times 100 \quad \text{.....(2)}$$

Estimation of peptide yield (PY)

PY was determined as per Wu *et al*. (2019). RFWP (5 ml) dissolved in TCA (10%, 1ml) was kept at room temperature (20 min.) and then centrifuged (10000xg, 10 min). Biuret method was used to find peptide content in supernatant taking bovine serum albumin as standard. PY was calculated (Eq. 3) considering conversion coefficient of nitrogen to protein as 6.25.

$$PY\ (\%) = \frac{TCA\text{- }Soluble\ peptide\ content}{(Total\ protein\ nitrogrn \times 6.25)} \times 100 \quad \text{.....(3)}$$

Statistical analysis

Single factor experiments were executed at different alcalase concentrations, hydrolysis temperatures, time and S/L ratios to find ACE-inhibitory peptide. Design-Expert ver.12 was used for design of experiments, optimization of independent variables and statistical calculations. Response surface methodology applied with Box Behnken Design (RSM-BBD) (32 runs) optimized 4 independent variables for highest DH, ACE inhibition and PY. All experiments were done in triplicate and expressed as the mean±standard deviation (n=3).

RESULTS AND DISCUSSION

Anatomical yield and proximate composition of RFW

The anatomical yield of RFW (FH, FN, SC and SB) varied significantly ($p<0.05$) among small, medium and large rohu fish (Table 1). Small fish generated more waste (49.45%) than that of medium (34.51%) and large (34.54) fish. Lower

yield of head (27.62, 32.43 and 22.11% in common carp, bighead carp and grass carp respectively) (Skałecki *et al*., 2015) than found in our study might be due to species difference.

Moisture, fat, crude protein and ash differed significantly ($p<0.05$) among all wastes (Table 2). Though SB constitutes a small fraction (1.42-1.91% by wet weight) of total generated waste, it can be mostly preferred for peptide extraction because of its highest protein content (>34%) among fish wastes. Protein content in scales of farmed grass carp (*Ctenopharyngodon idella*) (21.74%) (Naqvi *et al*., 2014) was closer to that found in rohu scales (22.9%). More protein (4% in FH), fat (11% in FH) and ash (17% in SC) were found in rohu in our study than grass carp (Naqvi *et al*., 2014). In contrast comparatively less moisture (3% in FH and 1% in SC), ash (11% in FH and 2.5% in SC) and protein (1% in SC) were also observed in rohu (in our study) than grass carp (Naqvi *et al*., 2014).

Alcalase concentration

With increase in alcalase concentration from 0.5 to 2% (v/w) there was a significant ($p<0.05$) increase in DH, ACE inhibition and PY (Fig 1A). However, ACE inhibition and PY decreased beyond 2.5% (v/w) alcalase concentration but DH continued to increase. In salmon (*Salmon salar*) skin hydrolyzed by alcalase, See *et al* (2011) found 2.5% enzyme as optimum for highest DH (77.03%). In eel (*Monopterus* sp.) protein hydrolysate with alcalase (1.5-2%) showed DH ranging 36-69% (Baharuddin *et al*., 2016). In grass carp (*Ctenopharyngodon idella)* skin hydrolysate obtained with increasing alcalase enzyme to substrate ratio (0.12 to 1.08) showed increasing DH (5.02 to 14.9%) (Wasswa *et al*., 2007). In our study ACE inhibition reduced from 61.1 to 56.6% as alcalase concentration increased from 2 to 2.5% (v/w). Similar result is reported in lizard fish (*Saurida elongata*) muscle protein hydrolysate wherein ACE inhibitory activity decreased from 78 to 70% with increase in E/S from 1:1 to 1.5:1 (Wu *et al*., 2012). Optimum PY (amount of peptide/s (g) produced per 1 g crude protein) in pig bone collagen peptide was 36% with alcalase (6000/ u/g powder) at 6% substrate concentration (Wu *et al*., 2019).

Hydrolysis temperature

DH, ACE inhibition and PY increased significantly ($p<0.05$) with increase in hydrolysis temperature (Fig 1B). Higher percentage of all these reduced dependent variables were noticed between 50-60°C which further reduced beyond 60°C. This may be due to degradation of enzyme at higher temperature. Similar increase in DH (36 to 69%) with increasing temperature (40 to 60°C) observed in eel (*Monopterus* sp.) protein hydrolysate (Baharuddin *et al*., 2016). In our study a DH reduced from 19.9 to 15.9% beyond 65°C. Similar decrease (71 to 61%) in DH reported in alcalase hydrolysate of salmon (*Salmon salar*) skin (See *et al*. (2011) when temperature increased from 55.3°C to 70°C. The result was consistent with the optimal temperature (55°C) of alcalase enzyme. Highest ACE inhibition (62.2% at 60°C) did not increase at >60°C might be due to inactivation of enzyme at higher temperature. Alcalase hydrolysate of Basa fish skin showed 33.3% ACE inhibition at 52°C (Zhang *et al*., 2016). Minced striped snakehead hydrolyzed with alcalase (3% g/mL) showed 70% ACE inhibition at 55°C (Ma, *et al*., 2021). Our study showed PY 51.8% at 60°C.

Hydrolysis time

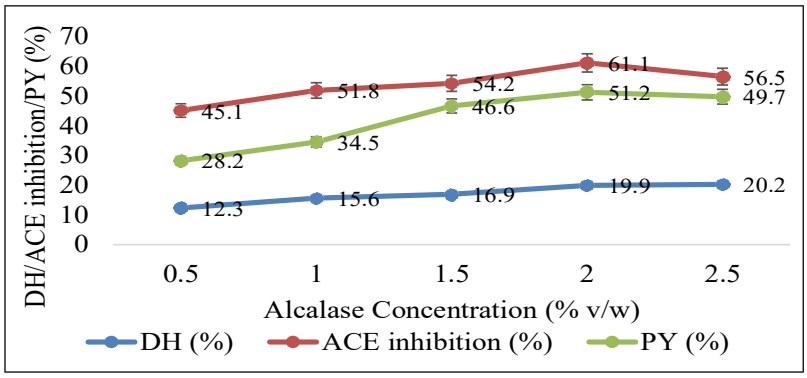
There was a significant ($p<0.05$) increase in DH, ACE inhibition and PY with increase in hydrolysis time (Fig 1C). A rapid hydrolysis of RFWP at 60 min of hydrolysis indicated that large number of peptide bonds were cleaved. Later on, DH decreased significantly ($p<0.05$) until 180 min. and further reduced beyond 180 min. Similar to our study, DH increased from 5.02 to 14.9% in grass carp (*Ctenopharyngodon idella*) skin hydrolysate when hydrolysis time increased from 75 to 120 min. (Wasswa *et al*., 2007) and 36, 48 and 69% in eel

Table 1: Anatomical yield of wastes generated from rohu (n: 20).

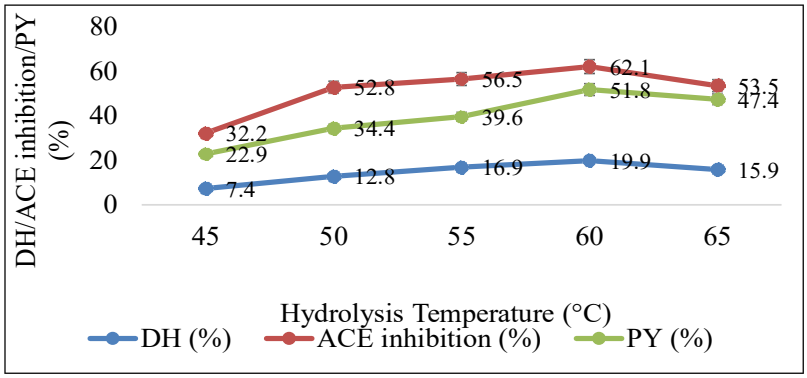
		Small rohu	Medium rohu	Large rohu
Total length (cm)		20-25	25-30	30-35
Total weight (gm)		100-250	250-400	400-550
Anatomical yield (g)	Head weight	31.33±4.16	57.33±3.06	96.00±14.14
	Trimmings weight	7.13±0.79	13.74±0.95	17.95±1.73
	Scales weight	4.38±0.36	13.20±0.85	20.60±2.37
	Swim bladder weight	0.79±0.31	1.78±0.04	3.18±0.04
	Viscera and other wastes	11.36±5.10	17.94±0.77	29.58±5.12
Total yield of waste (%)	Total yield (g)(%)	55.00±10.15 (49.4)	104.00±5.29 (34.4)	167.3±13.15 (34.5)

Table 2: Proximate composition of different wastes generated from rohu.

Waste type	Moisture (%)	Fat (%)	Ash (%)	Protein (%)
Head	58.68±0.40	7.07±2.02	15.23±0.07	17.09±1.15
Trimmings/fins	62.16±0.50	5.72±0.30	11.75±0.21	18.7±0.46
Scale	63.78±0.13	2.93±0.08	8.78±2.04	22.9±2.1
Swim bladder	60.32±0.00	2.37±0.00	1.23±0.28	34.08±0.50



A



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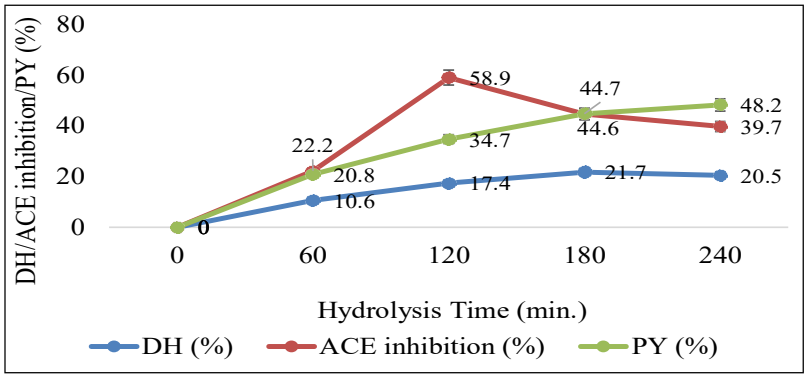
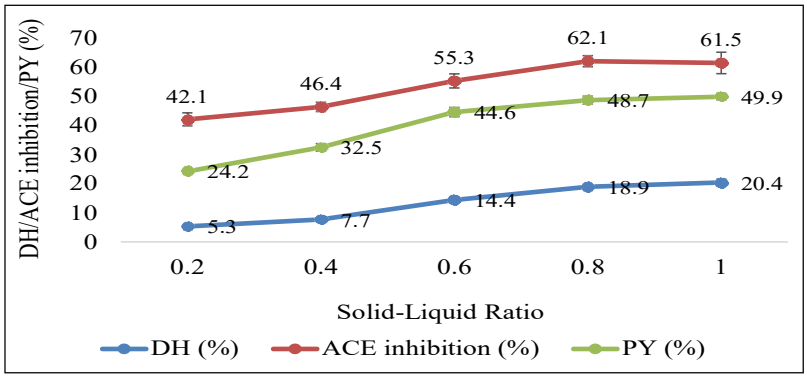


C



D

Fig 1: Effect of alcalase concentration (A); Hydrolysis temperature (B); Hydrolysis time (C) and Solid/liquid ratio (D) on Degree of hydrolysis (DH), ACE inhibition and peptide yield (PY) of ACE-Inhibitory peptide extracted from RFW.

(*Monopterus* sp.) protein hydrolysate at 120, 180 and 300 min. of hydrolysis, respectively (Baharuddin *et al*., 2016). PY (48.2%) found maximum at 240 min when DH and ACE inhibition showed reducing trend. This may be due to fact that more hydrolysis period allowed higher cleavage of protein leading to higher PY. However, optimum PY (36%) of pig bone collagen peptide found at 5 h of hydrolysis (Wu *et al*., 2019).

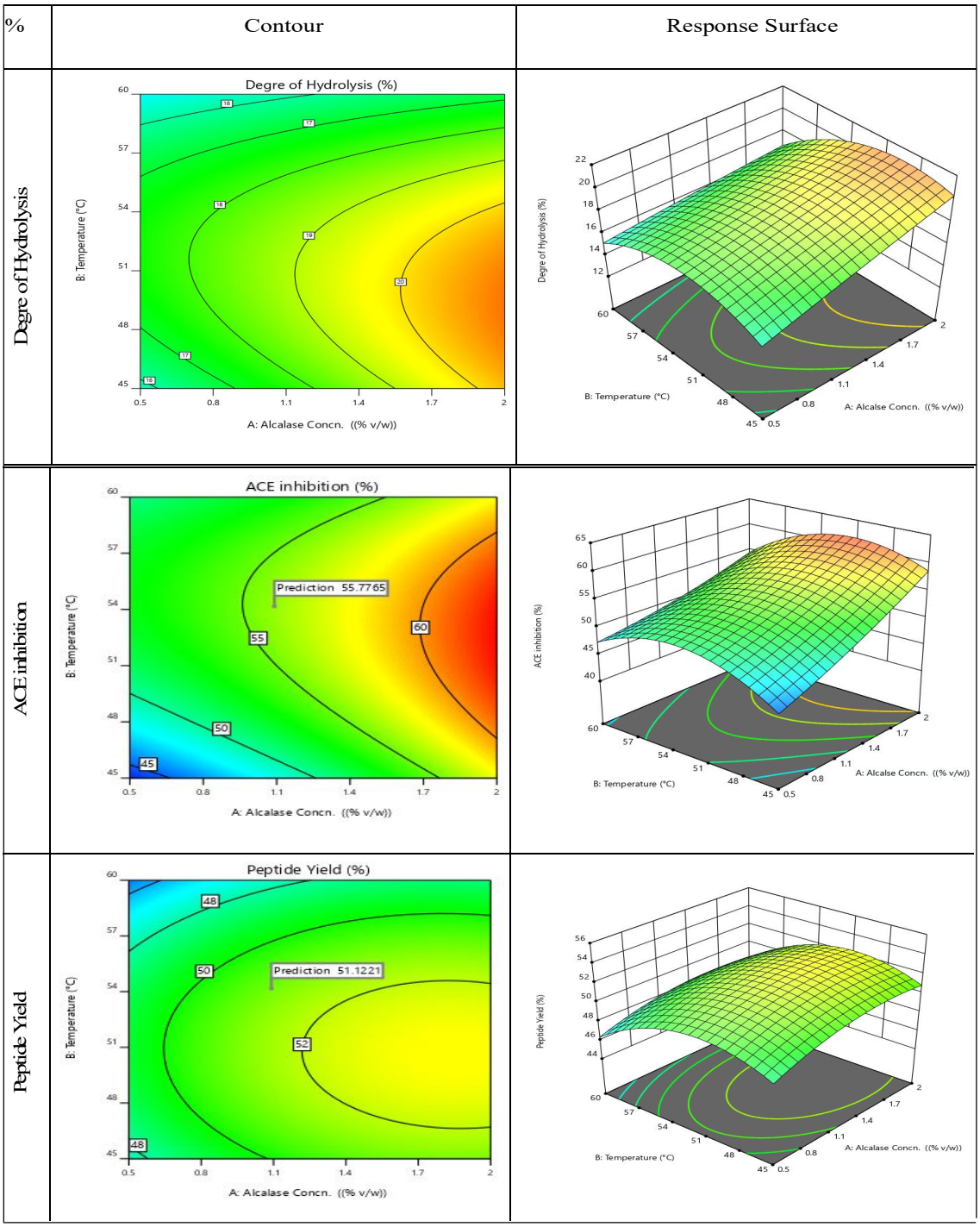


Fig 2: The 2D contour plot and 3D response surface plots of the relative effects of temperature (°C) and alcalase concentration (%, v/w) on A) Degree of hydrolysis, B) ACE inhibition and C) Peptide yield of ACE-Inhibitory peptide extracted from RFW.

Solid/liquid ratio

DH, ACE inhibition and PY increased significantly ($p<0.05$) with increase in S/L ratio (Fig 1D). Initially (at <0.4) there was a gradual increase followed by a sharp increase (from 0.4 to 0.8) and finally very minimal increase (beyond >0.8). The increase in solid (homogenized RFWP) per unit of liquid (reaction buffer) increases the substrate availability and thereby provide more active sites for the enzymatic activity. In alcalase hydrolysate of Basa fish skin highest ACE inhibition was reported at S/L ratio 1:8.05 g/mL (Zhang *et al*., 2016) while in the minced striped snakehead (62% ACE inhibition) at 1:15 S/L (Ma *et al*., 2021). Highest PY found in our study is 49.9%.

Optimization of enzymatic extraction process variables

RSM-BBD (32 runs) was performed to study the combined effect of 4 independent factors (X_1: Alcalase Concentration, X_2: Temperature, X_3: Time and X_4: S/L Ratio) on 3 dependent responses (DH, Y_1; ACE inhibition, Y_2 and PY, Y_3). A multiple regression analysis was performed using RSM to determine all the coefficients of linear (X_1, X_2, X_3, X_4), quadratic (X_1^2, X_2^2, X_3^2, X_4^2) and interaction (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4) terms to fit a full response surface model for the responses. In our study, a linear model term X_1 (alcalase concentration) found significant ($p<0.05$) for all the responses while X_2 (temperature) was significant ($p<0.05$) for PY only. X_3 and X_4 did not affect any response significantly ($p>0.05$). Additionally, a quadratic model term, X_2^2 showed significant ($p>0.05$) effect on all responses while other quadratic model term X_3^2 showed significant ($p>0.05$) effect on ACE inhibition and PY only.

Fig 2 illustrates contour (2D) and response surface (3D). Alcalase concentration significantly ($p<0.05$) influenced all the dependent variables. Temperature affected PY significantly ($p<0.05$) but not DH and ACE inhibition, however, temperature demonstrated significant effect on all the dependent variables of 2nd order reaction but with negative effect on DH and PY. Time and S/L ratio were insignificant ($p>0.05$) for all the dependent variables. A final response surface regression equation obtained by RSM is as below (Eq. 4).

$$Y= 127.59 + 7.81\ X_1 - 1.2\ X_2 - 28.26\ X_2^2 - 4.56\ X_3^2 \quad \text{.....(4)}$$

In our study the desirability profile indicated optimum DH (19.27%), ACE inhibition (54.98%) and PY (51.37%) with 1.08 % (v/w) alcalase concentration, 52.10°C temperature, 129.18 min hydrolysis time and 0.8:1 solid-liquid ratio. Taking the actual operations these may be adjusted to 1.1% (v/w), 52°C and 129 min. respectively. Similar results were reported in salmon skin hydrolysate (2.5% v/w alcalase, 55.3°C temperature resulted in 77% DH) (See *et al*., 2011) and minced striped snakehead (5% w/v alcalase, 55°C temperature, 3 h hydrolysis and 1:25 g/mL S/L ratio exhibited 54.35% ACE inhibition) (Ma *et al*., 2021).

CONCLUSION

We report generation of more waste in smaller rohu fish than medium and bigger fish; highest fat and ash in fish head, moisture in scales and protein in swim bladder and edible flesh (<60%). ACE inhibitory peptides at optimum conditions (1.1% v/w alcalase concentration, 52°C hydrolysis temperature, 129 min. hydrolysis time and 0.8:1 solid-liquid ratio) showed 19% DH, 55% ACE inhibition and 51% PY. Results indicated the potential application of rohu fish waste-based ACE inhibitory peptide in nutritional supplements, pharmaceutical products and functional foods as an alternative source of hypertension treatment following future research.

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Disclosure statement

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