Indonesian Food Science and Technology Journal
IFSTJ : Vol 7 No 1; December 2023 ; (PP : 1-8)
ISSN : 2615-367X

INDONESIAN FOOD SCIENCE AND TECHNOLOGY JOURNAL (IFSTJ)

Journal homepage : online-journal.unja.ac.id/ifstj/issue/archive

Optimization Process of the Pepsin-Solubilized Collagen from Lizardfish (*Saurida tumbil* Bloch, 1795) Skins by-Product

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***Abstract*— By-products from the marine fish processing are rich in organic compounds that can be converted into value-added products like collagen, and it is thought as an ideal candidate polymer for such research and medical applications. The lizardfish (*Saurida tumbil* Bloch, 1795) skin collagen had been investigated by our previous work, but an effective extraction method is needed to increase the yield of collagen. The purpose of this study was to optimize the method used to extract pepsin-solubilized collagen (PSC) from lizardfish skin. We employed an approach of one factor at a time (OFAT), along with response surface methodology (RSM) utilizing a central composite design (CCD), to attain the highest possible yield of the extracted collagen. Additionally, its properties were also assessed comparatively. The suggested optimal conditions for extraction were a pepsin concentration of 1.87%, a liquid-solid ratio of 24.90 mL/g, and a hydrolysis period of 38.09 h. Using these conditions resulted in a PSC yield of 21.82 g/100g, which closely matched the predicted collagen value.**

***Keywords*— Marine source; pepsin-aided extraction; optimization process; collagen yield**

Manuscript received August 29, 2023; revised Nov 21, 2023; accepted December 28, 2023. Available online December 31. 2023

I. INTRODUCTION

Fish collagen has drawn considerable attention over the last decades. Fish source, particularly its by-products (skin, bones, scale, and swim bladder), is potentially used as a promising raw material for collagen production due to its abundance, safer from infectious diseases, and good characteristics [1]. Some fish collagens derived from different species and fish parts have been studied and characterized, including the bone, scale and skin of bigeye tuna (*Thunnus obesus*) [2], grass carp (*Ctenopharyngodon idellus*) [3], golden pompano (*Trachinotus blochii*) [4], tilapia (*Oreochromis niloticus*) [5], barracuda (*Sphyraena* sp.) [6], purple-spotted bigeye snapper (*Priacanthus tayenus*) [7], red-bellied pacu (*Piaractus brachypomus*) [8], tiger grouper (*Epinephelus fuscoguttatus*) [9], striped marlin (*Kajikia audax*) [10], needlefish (*Tylosurus acus melanotus*) [11], sturgeon fish (*Huso huso*) [12], ray fish (*Zearaja chilensis*) [13], parrotfish (*Scarus sordidus*) [14], and seabass (*Lates calcarifer*) [15]. Among them, tilapia skin collagen has been proved as a wound healing acceleration agent [16] and grass carp scale collagen could be used for bone tissue regeneration [17].

Lizardfish (*S. tumbil*) holds economic significance as a vital tropical marine fish species in Malaysia. Over the period from 2018 to 2021, its average production reached 84,364.33 tons [18], and this fish is recognized for its important role as a primary source for surimi production, attributed to its good gel strength and whiter flesh portion [19,20]. After surimi processing, as aforementioned, a high quantity of byproducts was collected. Converting lizardfish byproducts into collagen have been documented in our previous works, and the results reported that the collagen extracted from the lizardfish skin shows higher yield (15.23%) compared to other portions of the scales (0.78%) and bones (4.99%) [21–24]. From these data, the skin portion is more suitable for further collagen production; however, an effective approach in optimizing extraction process variables is required to enhance the yield of fish collagen. The concentration of pepsin, ratio of liquid to solid components, and time of hydrolysis are important parameters affecting the extractability of lizardfish skin collagen. Among optimization techniques, response surface methodology (RSM) is widely favored for refining these processes that explores the relationship between several variables and generates a mathematical model to predict the values of the response variables [25]. To date, a number of existing studies on the fish collagen extraction with response surface methodology have been demonstrated [26,27], but limited information relating to the tropical marine fish skin collagen. Thus, the objective of our present work was to analyze the effects of independent factors on the yield of pepsin-solubilized collagen derived from lizardfish (*S. tumbil*) skin using RSM with a central composite design (CCD).

II. MATERIAL AND METHODS

A. Chemicals and sample preparation

Pepsin (1:10,000) from bovine origin was purchased from HiMedia (Maharashtra, India). A total of one kilogram of lizardfish (S. tumbil) skin obtained after separation under a deboner machine was used for the study of collagen extraction. Prior to extraction, fish skins were trimmed into small pieces (approximately 1.5 × 1.5 cm^2) with a stainless-steel scissor. Subsequently, these trimmed skins were washed with distilled water (dH2O) and packed into a polyethylene container. The prepped lizardfish skin samples were then placed in a freezer for further experimentation.

B. Extraction of pepsin-solubilized collagen (PSC)

PSC extraction was referred to our previous study with minor amendment [24]. The frozen fish skins were thawed overnight before used. Ten kilograms of samples were immersed with ten volumes of NaOH (0.1 M) for 6 h with replacing every 3 h. Subsequently, the alkaline-treated samples were rinsed with cooled dH2O until a pH of 7.0 was reached. The samples with adjusted pH were defatted by dissolving ten volumes of butyl alcohol (10%) for 24 h. The defatting solution was refreshed every 12 h. Further, the defatted fish skins were rinsed with cooled dH2O for approximately 15 min. After pre-treatment processes, the pre-treated fish skins were extracted in acidic condition by dissolving 15 volumes of AcOH (0.5 M) and 1.5% (w/w) pepsin enzyme for 48 h. The extracted fish skins were passed through two layers of cheesecloth for filtration. Following that, the resulting supernatant was subjected to precipitation by the addition of sodium chloride (NaCl) (2.5 M) along with Tris-HCl (0.05 M) at a neutral pH of 7.0. The precipitate was then centrifuged at 15,000× g for 30 min using a centrifuge device. After this step, the resulting pellets were resuspended in a solution of acetic acid (0.5 M). These resuspended samples were then dialyzed, utilizing a dialysate procured from Sigma-Aldrich, USA. Then, the treated samples were lyophilized using a freeze-dryer apparatus. All stages of collagen production were carried out in a cool environment at 4°C. Finally, the freeze-dried PSC was stored in a freezer until further evaluation. In the context of yield calculation, we adopted the method described from previous work, as follows:

$$Yield\ (\%) = \frac{Weight\ of\ dried\ collagen}{Initial\ weight\ \ of\ lizardfish\ skin} \times 100 \quad (1)$$

C. Experimental study

One factor at a time (OFAT) method

OFAT study on the extraction yield from the lizardfish (S. tumbil) skin collagen was determined. Three independent variables were selected, composing of pepsin concentration (0.1−2.0%), liquid-solid ratio (10−30 mL/g) and hydrolysis time (24−72 h). In terms of OFAT analysis, one factor was altered independently while keeping the other two constants. Initially, when different pepsin concentrations (0.1, 0.5, 1.0, 1.5 and 2.0%) were tested, the liquid-solid ratio was fixed at 15 mL/g and the hydrolysis time at 48 hours. Subsequently, various liquid-solid ratios (10, 15, 20, 25, and 30 mL/g) were examined while maintaining the initially determined optimal pepsin concentration, and the hydrolysis time was unchanged. Furthermore, the optimal pepsin concentration and liquid-solid ratio were chosen based on the previous experiment, and various hydrolysis times (24, 36, 48, 60, and 72 hours) were employed to attain the highest achievable yield of collagen. Finally, the selected extraction parameters were further optimized using RSM [26,28].

Response surface methodology (RSM)

RSM, a statistical technique, is employed to construct an empirical model and identify the specific independent variables that yield the desired response [26]. To assess the interactions between different factors and their effects on the response, a central composite design (CCD) featuring three independent variables at five levels (-1.682, -1, 0, +1, and +1.682) was utilized in this study. A total of 20 experimental runs were conducted, comprising 8 fractional factorial points, 6 axial points and 6 central points, as outlined in Table 1. These levels were determined and encoded using the following formula:

$$Z = \frac{Z_i - Z_0}{\Delta Z} \quad (2)$$

where Z and Z_1 denote the coded and actual levels of the independent factors, respectively. ΔZ signifies the step change value, and Z_0 represents the true value at the central point.

TABLE 1:
INDEPENDENT VARIABLE AND THEIR LEVELS IN RSM STUDY

Independent variables	Symbol	Level of variable used				
		−α*	−1	0	1	+α
Pepsin concentration (%)	X_1	0.7	1	1.5	2	2.3
Liquid-solid ratio (mL/g)	X_2	16.6	20	25	30	33.4
Hydrolysis time (h)	X_3	15.8	24	36	48	56.2

* α: axial point of CCD

The data from the CCD were explained by fitting through a second-order polynomial response surface model, as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j>1} \beta_{ij} X_i X_j + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \varepsilon \quad (3)$$

where Y represents the dependent variable (PSC yield, g/100g), while β0, βi, βii, and βij, denote a constant value, linear, quadratic, and interaction coefficients, respectively. Xi and Xj represent the coded values of the independent variables, and ε is the random error component measured by fitting the model to the data. The efficiency of the model and the statistical significance were examined using the F and R tests. Analysis of variance (ANOVA) was used to examine the effect of the individual variables, using a Design-Expert 13, USA.

III. RESULT AND DISCUSSION

In this work, we highlighted the optimization process of the PSC yield from lizardfish (*S. tumbil*) skin. As aforementioned, this research was based on our previous report on the collagens extracted from the bone, scale and skin of same fish species [21–24]. We used a OFAT method to find the central point of each selected extraction condition that was a common approach prior to employing the RSM study, as carried out by other researchers [26].

A. Single-factor data

Effect of pepsin concentration on yielding collagen

The effect of varying pepsin concentrations on the obtained yield of PSC derived from lizardfish (*S.tumbil*) skin was examined, and the data illustrated in Fig. 1A. The extraction process was carried out using different pepsin concentrations: 0.1%, 0.5%, 1.0%, 1.5%, and 2.0%. The remaining two extraction parameters (liquid-solid ratio: 15 mL/g, hydrolysis time: 48 h) were held constant. When the concentration of enzyme was increased from 0.1% to 1.5%, the yield of PSC increased significantly from 16.09% to 21.36% (based on wet basis of lizardfish skin), and then slightly increased when pepsin concentration exceeded 1.5%. The extraction yield exhibited an upward trend as the pepsin concentration was raised within the range of 0.1% to 2.0%. However, there were no significant differences ($p > 0.05$) observed between the concentrations of 1.5% and 2.0%. Besides that, considering the higher expense industrial viewpoint [29], the addition of 1.5% pepsin enzyme during collagen extraction was chosen for the next study.

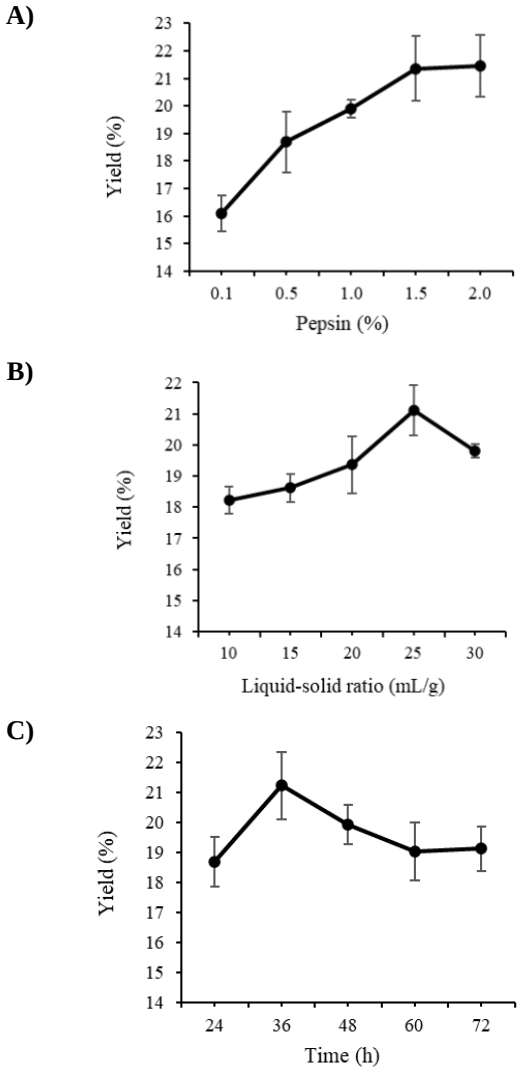

Fig. 1. Effect of various conditions on the yield of PSC derived from lizardfish skin. A) Pepsin concentration (%); B) liquid-solid ratio (mL/g); C) hydrolysis time (h).

Effect of liquid-solid ratio on yielding collagen

The liquid-solid ratio used in this preliminary work of PSC extraction ranged between 10 mL/g to 30 mL/g of lizardfish skin, and the other two extraction factors were designed by keeping enzyme concentration (1.5%) and hydrolysis time (48 h). The obtained results revealed that as the liquid-solid ratio increased within the range of 10 mL/g to 25 mL/g, there was a notable rise in the PSC extraction yield, ranging from 18.22% to 21.12% (Fig. 1B). Thus, a liquid-solid ratio of 25 mL/g for fish skin was selected for further extraction process.

Effect of hydrolysis time on yielding collagen

Another important factor used during yield extraction of PSC from the lizardfish (*S. tumbil*) skin is hydrolysis time. Different time of hydrolysis was set, initiating from 24 h to 72 h, and other two extraction parameters were set by the selection of previous optimization (pepsin concentration: 1.5% and liquid-solid ratio: 25 mL/g). As presented in Fig. 1C, the highest yield of extracted collagen (21.23%) was observed at 36 h, and after 36 h of hydrolysis, the yields decreased gradually. This finding was similar to another researcher [26]. From the single-factor study, enzyme concentration (0.1–2.0%), liquid-solid ratio (10–30 mL/g) and hydrolysis period (24–72 h) were applied for response surface methodology (RSM) study.

B. RSM

Model fitting process

RSM used in the present study was CCD with three independent variables, i.e., X_1: pepsin concentration (1.5%), X_2: liquid-solid ratio (25 mL/g) and X_3: hydrolysis period (36 h), resulting in 20 runs of selected treatments, and their responses are highlighted in Table 2. The obtained results showed the extraction yields of PSC from lizardfish (*S. tumbil*) skin were between 18.67% and 21.69%. The highest collagen yield was observed in the experimental conditions of enzyme concentration, liquid-solid ratio, and extraction time were 2.34%, 25 mL/g, and 36 h, respectively. Furthermore, the quadratic model was selected due to the highest F value (40.33) compared to other models (*viz*. linear: 14.37, 2Fl: 0.57 and cubic: 0.04), and this model had a p value of <0.0001. In addition to this, the selected model showed insignificant lack-of-fit, while other models were significant.

TABLE 2:
CCD AND RESPONSE FOR THE YIELD OF PSC

Pepsin concentration (%) X_1	Liquid-solid ratio (mL/g) X_2	Hydrolysis time (h) X_3	Yield of PSC (%)	
			Actual value	Predicted value
−1	+1	+1	19.90	20.14
+1	+1	−1	21.56	21.62
0	+1.682	0	21.04	20.87
−1	−1	−1	18.67	18.61
0	0	0	21.47	21.34
0	0	0	21.23	21.34
0	0	0	21.36	21.34
+1	+1	+1	21.46	21.5
0	0	−1.682	20.71	20.89
−1.682	0	0	18.69	18.66
0	0	0	21.51	21.34
0	0	0	21.27	21.34
−1	+1	−1	20.21	20.15
−1	−1	+1	19.36	19.28
+1	−1	+1	21.46	21.5
+1	−1	−1	21.19	20.93
+1.682	0	0	21.69	21.75
0	−1.682	0	19.37	19.57
0	0	+1.682	21.50	21.35
0	0	0	21.23	21.34

Upon selecting the appropriate model, an ANOVA test was conducted on the response surface quadratic model, and the significant effect of each factor used in this work is outlined in Table 3. The results revealed a model F value of 59.82, signifying the model's significance with a mere 0.01% likelihood that such a large F value could result from random variation. Within this study, the terms X_1, X_2, X_3, X_1X_2, X_2X_3, X_{12} and X_{22} were recognized as significant model components. The lack of fit F value recorded was 3.61 with an p value was around 0.093, indicating that the lack of fit was not statistically significant. The chances of a lack of fit F value of this magnitude occurring due to random noise were approximately 9.25%. Additionally, the coefficient of determination (R^2 = 0.9818) yielded by ANOVA for this model, along with the adjusted coefficient of determination (Adj R^2 = 0.9654), further underscored the model's significance. The equation involving coded factors could be employed to predict the response for given levels of each factor, as demonstrated below:

The equation involving coded factors could be employed to predict the response for given levels of each factor, as demonstrated below:

$$Y = 21.34 + 0.92X_1 + 0.39X_2 + 0.14X_3 - 0.21X_1X_2 - 0.02X_1X_3 - 0.17X_2X_3 - 0.40X_1^2 - 0.40X_2^2 - 0.08X_3^2 \quad (4)$$

where Y represented the yield of pepsin-solubilized collagen, and X_1, X_2, X_3 described the enzyme concentration, liquid-solid ratio, and hydrolysis time, respectively

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TABLE 3:
ANALYSIS OF VARIANCE FOR QUADRATIC MODEL

Source	Sum of squares	Df	Mean square	*F* Value	*p* Value	Remark on significance
Model	18.66	9	2.07	59.82	< 0.0001	significant
X_1	11.58	1	11.58	334.07	< 0.0001	
X_2	2.02	1	2.02	58.42	< 0.0001	
X_3	0.26	1	0.26	7.46	0.0212	
X_1X_2	0.37	1	0.37	10.54	0.0088	
X_1X_3	0.01	1	0.01	0.16	0.6984	
X_2X_3	0.23	1	0.23	6.77	0.0264	
X_1^2	2.34	1	2.34	67.37	< 0.0001	
X_2^2	2.27	1	2.27	65.61	< 0.0001	
X_3^2	0.09	1	0.09	2.60	0.1381	
Residual	0.35	10	0.03			
Lack of Fit	0.27	5	0.05	3.61	0.0925	not significant
Pure Error	0.08	5	0.02			
Cor total	19.01	19				
R^2					0.9818	
Adj R^2					0.9654	

Interaction of each variable

Interaction between parameters used in this work (*i.e.*, X_1 and X_2, X_1 and X_3 and X_2 and X_3) was evaluated using three-dimensional (3D) response surfaces. Fig. 2A. shows the effect of pepsin concentration (X_1) and liquid-solid ratio (X_2) on the extraction yield of PSC, with the hydrolysis time was kept constant. The results exhibited that the PSC yield increased with an increase in the ratio of liquid-solid (15−25 mL/g). In terms of varying pepsin treatments, when the concentration of enzyme increased up to 2%, the solubilized collagen yields also increased. As demonstrated, the effect of protease enzyme concentration and liquid-solid ratio on the collagen yield was significant and it was in accordance with the data presented in Table 2. For the relationship between pepsin concentration (X_1) and hydrolysis time (X_2) during collagen production, with fixing another factor (liquid-solid ratio, X_3), as illustrated in Fig. 2B. A higher amount of yield was obtained at the concentration of enzyme up to 2% and it was also recorded at the hydrolysis time from 36 h to 48 h. However, their interaction was insignificant with a p value > 0.05, as shown in Table 2. Next, the effect of liquid-solid ratio (X_2) and hydrolysis time (X_3) on the collagen yield was also evaluated, and their 3D response surface plot is depicted in Fig. 2C. The extraction yield of PSC increased ready with increasing hydrolysis time up to 48 h, while the ratio of liquid-solid was observed ranging from 25 mL/g to 30 mL/g. Beside that range, the extraction yield of collagen decreased. This might be due to the increase in the reaction area of protease enzyme used although the obtained yield did not increase with a further increase in the liquid-solid ratio (particularly at above 25 mL/g). It was probably due to the complete reaction of lizardfish skin with liquid [30]. Both the ratio of liquid-solid and the hydrolysis time observed in the present study were significant. Liquid-solid ratio used in this preliminary work of PSC extraction ranged between 10 mL/g to 30 mL/g of lizardfish skin, and the other two extraction factors were designed by keeping enzyme concentration (1.5%) and hydrolysis time (48 h). The obtained results revealed that as the liquid-solid ratio increased within the range of 10 mL/g to 25 mL/g, there was a notable rise in the PSC extraction yield, ranging from 18.22% to 21.12% (Fig. 2B). Thus, a liquid-solid ratio of 25 mL/g for fish skin was selected for further extraction process.

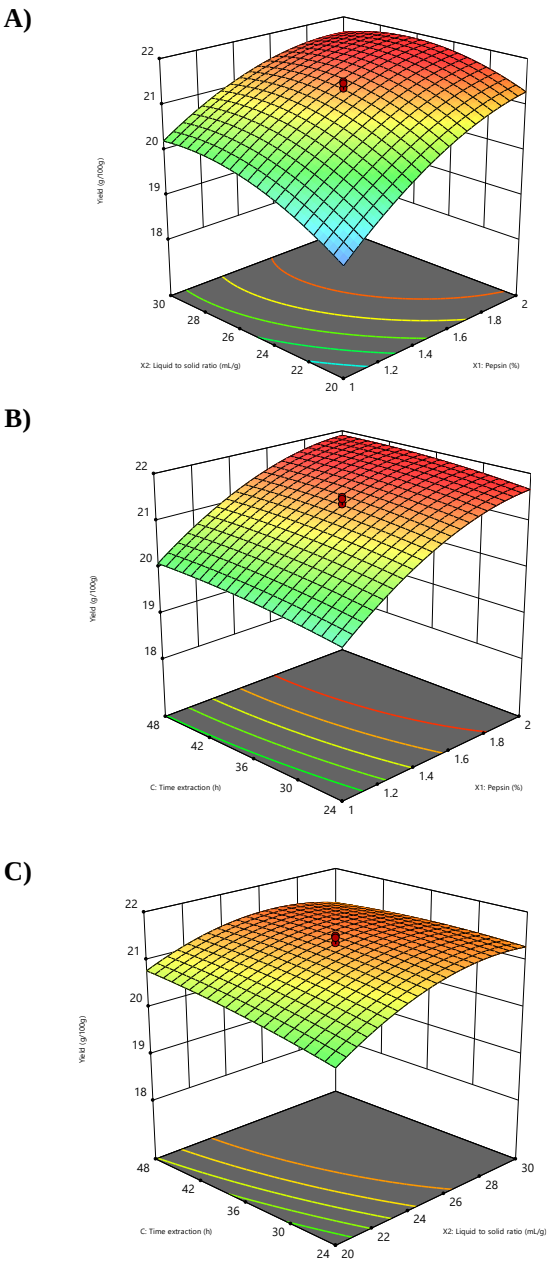

Fig. 2. The 3D response surface graph depicted the effect of the pepsin concentration (%, X1), liquid-solid (mL/g, X2) and hydrolysis time (g/100g, X3) on the yield of lizardfish skin collagen.

Validation of the optimal conditions

To predict the PSC yield of lizardfish skin through RSM study, the optimal conditions used in this study, such as pepsin concentration (X_1 = 1.87%), liquid-solid ratio (X_2 = 24.90 mL/g) and hydrolysis time (X_3 = 38.09 h) were selected. Using these proposed optimal conditions, the model predicted collagen yield is 21.82 g/100g. Our experimental results obtained the extraction yield of 21.83 ± 0.16 g/100g (based on wet basis of lizardfish skin), and this result was close to the predictive value of PSC yield, indicating that the selected model is accurate [29].

C. Yield of PSC

The yield of PSC from lizardfish (*S. tumbil*) skin obtained from the optimal extraction conditions was comparable to the PSC derived from various fish species, as presented in Table 4. Our findings showed a higher collagen yield when compared to the PSC isolated from different fish sources, such as the scales of sardinella (*Sardinella fimbriata*) (Y = 0.94 g/100g) [31], the bones of lizardfish (*S. tumbil*) (Y = 3.26 g/100g) [23], the cartilages of Siberian sturgeon (*Acipenser baerii*) (Y = 14.69 g/100g) [32], the skins of bigeye tuna (*T. obesus*) (Y = 16.70 g/100g) [2], and tilapia (*O. niloticus*) (Y = 19.61 g/100g) [33]. Although there was similar to the PSC yield found in the golden pompano (*T. blochii*) skin (Y = 21.81 g/100g) [4] with a lower concentration of pepsin. However, the extraction time and solvent used in the *T. blochii* skin-isolated collagen were greater than those of *S. tumbil* skin-extracted collagen. It can be assumed that the optimized extraction processes of PSC applied in this study were more efficient in producing lizardfish collagen.

TABLE 4:
THE PSC YIELD ISOLATED FROM DIFFERENT FISH SPECIES

Fish species	Byproduct	Pepsin concentration (%)	Liquid-solid ratio (mL/g)	Hydrolysis time (h)	Yield (g/100g)	References
S. tumbil	Skin	1.87	24.9	38.09	21.82	This study
P. tayenus	Skin	1	15	48	12.44	[7]
T. blochii	Skin	1	40	48	21.81	[4]
O. niloticus	Skin	0.1	50	24	19.61	[33]
E. macrura	Skin	10	5	48	7.1	[25]
T. obesus	Skin	0.5	40	48	16.7	[2]
I. platypterus	Skin	0.1	15	72	7.87	[34]
A. baerii	Cartilages	1	15	48	14.69	[23,32]
S. tumbil	Bone	1.5	15	48	3.26	[23]

Fish species	Byproduct	Pepsin concentration (%)	Liquid-solid ratio (mL/g)	Hydrolysis time (h)	Yield (g/100g)	References
P. heptacanthus	Scale	1	10	48	1.66	[35]
P. jullieni	Scale	1	10	24	1.16	[36]
S. fimbriata	Scale	1.5	10	30	0.94	[31]

IV. CONCLUSION

Our findings showed that the optimal extraction process (viz. pepsin concentration of 1.87%, liquid-solid ratio of 24.90 mL/g, and hydrolysis time of 38.09 h) developed by RSM with a central composite design was effective in obtaining maximum yield (21.82 g/100g) of the pepsin-solubilized collagen (PSC) derived from lizardfish *(S. tumbil*) skin. Using the optimized process, our next research will be focused on collagen hydrolysate with highlighting in its bioactive properties.

ACKNOWLEDGMENT

The authors would like to thank to the Ministry of Higher Education Malaysia for the funds provided through the Fundamental Research Grant Scheme (FRGS) with a grant number FRGS/1/2019/STG03/UMS/02/5., and we are also grateful to the Universiti Malaysia Sabah (UMS) for the support to accomplish this research paper.

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