International Journal of Food Science and Agriculture, 2023, 7(4), 511-517
https://www. hillpublisher. com/journals/jsfa/
ISSN Online: 2578-3475
ISSN Print: 2578-3467

Optimization of By-product Enzymatic Hydrolysis Process of Spanish Mackerel Based on Orthogonal Test

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How to cite this paper: Chuanqi Chen, Xudong Wu, Qingxiang Zhang, Shaoxuan Yu, Haifang Xiao, Zhengpeng Wei, Lanlan Zhu. (2023). Optimization of By-product Enzymatic Hydrolysis Process of Spanish Mackerel Based on Orthogonal Test. *International Journal of Food Science and Agriculture*, 7(4), 511-517.
DOI: 10.26855/ijfsa.2023.12.015

Received: November 28, 2023
Accepted: December 25, 2023
Published: January 22, 2024

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Abstract

Objective: In order to obtain the enzymatic hydrolysis solution of Spanish mackerel by-products with good taste and high peptide content. **Methods:** In this study, the influence parameters of the enzymatic hydrolysis process were optimized based on the orthogonal test. The effects of enzymolysis time, enzymolysis temperature, pH value, and enzyme dosage on peptide content and flavor dilution value (TD value) of enzymolysis solution were investigated. **Results:** The optimum enzymolysis conditions were as follows: enzymolysis time was 4 h, enzymolysis temperature was 35 ℃, enzymolysis pH was 8.0, and enzyme addition amount was 16000 U/g. At this time, the peptide content was 107.84 mg/mL and the TD value was 28.53. **Conclusion:** The peptide content and TD value in the verification test were higher than those in other experimental groups, indicating that the optimized enzymatic hydrolysis conditions were accurate. This study provided a theoretical study for the utilization of high value of Spanish by-products.

Keywords

Spanish mackerel, Enzymatic hydrolysis, Orthogonal test

1. Introduction

Spanish mackerel (*Scomberomorus niphonius*), the scientific name of Spanish mackerel, belongs to the class bony fish, percoform, Spanish mackerel family, mainly distributed in China's Bohai Sea, Yellow Sea, East China Sea, South China Sea waters, is one of the important Marine economic fish [1]. Studies have shown that Spanish mackerel is rich in protein, fat, as well as some polyunsaturated fatty acids, vitamins, and minerals, and is a high-quality raw material for food processing [2, 3]. At present, a large number of Spanish mackerel heads, Spanish mackerel skin, Spanish mackerel bone, and other processing by-products are produced in the processing of Spanish mackerel products, accounting for 45%~50% of the raw materials of Spanish mackerel [4]. Due to the lack of effective processing means, most of them are sold as low-value feed bases, resulting in a great waste of protein resources. Therefore, it is very important to improve the effective utilization rate of the by-products by enriching the processing means of the by-products of Spanish mackerel. At the same time, the in-depth development of the by-products of Spanish mackerel and the extension of the related industry chain has become a research hotspot at present.

Enzymatic hydrolysis is an important technical link for the efficient utilization of by-product protein resources of Spanish mackerel. Under the action of enzymes, macromolecular proteins, and fats are decomposed into peptides, amino acids, fatty acids, etc. On the one hand, it has a positive contribution to the nutrition and flavor characteristics of the enzymatic hydrolysis products [5, 6]. On the other hand, enzymatic hydrolysis products can also be used as important base materials for high-value products such as condiments, functional peptides, and nutritional fortifiers [7]. In conclusion, optimizing the enzymatic hydrolysis process of Spanish mackerel by-products to prepare umamiceptide can provide technical support

for the high-value utilization of Spanish mackerel by-product protein resources.

In this paper, umami peptides were prepared by hydrolysis of Spanish mackerel byproducts by protease. The effects of different enzymatic parameters on the content of peptides and the dilution value of taste were studied. Based on the single factor test, the orthogonal test was used to optimize the optimum enzymatic hydrolysis conditions of Spanish mackerel by-products, which provided a theoretical study for the comprehensive utilization of low-value aquatic protein resources.

2. Materials and Methods

2.1 Materials and Reagents

The Spanish mackerel by-product was provided by Shandong Rongcheng Taixiang Food Co., LTD., and was frozen at -20℃ before the test.

Trypsin, Papain Shanghai Maclin Biochemical Technology Co., LTD.; Alkaline protease fertilizer Qiansheng Biotechnology Co., LTD.; Neutral protease Shanghai Xianding Biotechnology Co., LTD.; Hydrochloric acid, trichloroacetic acid, sodium hydroxide, etc. are all domestic analysis pure Sinopharm Group chemical reagent Co., LTD.

2.2 Instruments and equipment

UV-2600A Spectrophotometer (Shimadzu International Trading Co., LTD.), 85-2 Constant temperature magnetic Agitator (Henan Gongyi Yuhua Instrument Co., LTD.), FE28-Micro pH meter (Mettler Toledo Technology Co., LTD.), JYC-C020 Tissue Crusher (Shandong Jiuyang Co., LTD.), D1524 high-speed refrigerated centrifuge (Beijing Dalong Xingchuang Test Instrument Co., LTD).

2.3 Methodologies

2.3.1 Enzymatic hydrolysis of by-products of Spanish mackerel

Take mackerel head, mackerel skin, and mackerel bone as raw materials, ground them, and add a certain proportion of deionized water homogenate, adjust the PH of the homogenate, and keep them warm for 30 minutes. Then add the protease and put it into the water bath for enzymatic hydrolysis, after a certain time, remove the enzyme in the boiling water bath for 15min, remove and cool to room temperature for filtration, and centrifuge the filtered supernatant at 4000 r/min for 15min to obtain the enzymatic hydrolysis solution of Spanish wael by-product, refrigerate for use.

2.3.2 Screening of proteases

Selecting proteolytic enzymes from different sources for enzymolysis, namely papain in plant protease, trypsin in animal protease, alkaline protease, and neutral protease in microbial protease. According to the optimal pH value and enzymolysis temperature, the enzymolysis was carried out at the dosage of 12000U/g for 4h. The best protease was selected based on peptide content and taste dilution factor (TD value).

2.3.3 Experimental design of enzymatic hydrolysis process optimization

Single factor test: trypsin was selected as the enzymolysis protease for the by-product of Spanish mackerel after protease screening. Single factor test was conducted with peptide content and taste dilution factor as screening indexes, and enzymolysis times of 2h, 3h, 4h, and 5h were selected as test levels, respectively. pH value 6.0, 7.0, 8.0, 9.0; Enzymatic hydrolysis temperature 35℃, 40℃, 45℃, 50 ℃; Enzyme dosage 4000U/g, 8000U/g, 12000U/g, 16000U/g. The enzymolysis conditions were preliminarily optimized, and the experimental parameters of the best factors were determined. Each treatment was repeated 3 times, and the results were averaged.

Based on the results of the single-factor experiment, four parameters of enzymatic hydrolysis pH, enzymatic hydrolysis time, enzymatic addition amount, and enzymatic hydrolysis temperature were selected as the main influencing factors of the enzymatic hydrolysis process. With the aid of orthogonal experimental design ideas, a table of 4 factors and 3 levels was set, as shown in Table 1. Each experimental group was parallel 3 times.

Table 1. Orthogonal experiment design coding level table

Factor	Coding level		
	-1	0	1
A: Enzymatic hydrolysis time/h	3	4	5
B: Enzymatic hydrolysis temperature/℃	35	40	45
C: pH value	7.0	8.0	9.0
D: Enzyme additions/ ($U.g^{-1}$)	8000	12000	16000

2.3.4 Determination of peptide content

The content of the peptide was determined by the biuret method [8]. The OD value of peptide in the clear solution was determined, bovine serum albumin was used as the standard material (Y=0.1311X+0.0038, R^2=0.9993), the concentration of peptide was calculated, and then multiplied by the dilution of the sample, that is, the peptide content P in the enzymatic hydrolysis solution of Spanish mackerel by-product.

$$P(mg.mL^{-1})=(A-0.0038)*N/0.1311 \quad (1)$$

Where: A-OD value; N - sample dilution ratio; P - Peptide content in the supernatant, ($mg.mL^{-1}$).

2.3.5 Taste dilution analysis (TD value)

Referring to Brock and Seo et al., the dilution factor of protease hydrolysis products was determined by comparative taste dilution analysis, and the taste characteristics of the products could be initially judged [9, 10]. The higher the value, the more obvious the taste characteristics. At room temperature, 1g freeze-dried enzymolysis solution sample was weighed, dissolved in 100ml pure water, and gradually diluted according to the volume ratio of 1:1. The sample solution of each component was gradually diluted 5mL each time, and each sample was presented to the sensory evaluator in the order of increasing concentration. The enzymolysis solution at each dilution level was evaluated by three-point determination. When the difference in taste between the diluent of a dilution level and the two blank groups is just enough to be identified, the dilution multiple or dilution level is called the dilution value (TD), and the TD value of each sample is the average of the results assessed by the sensory evaluators, and the difference between the results of the sensory evaluators should be less than or equal to a dilution level. In addition, in sensory evaluation, each evaluator describes the taste characteristics of each sample presented.

2.4 Data processing

Origin 2017 and Microsoft Excel 2019 software were used for experimental design and data analysis.

3. Results and analyses

3.1 Screening of proteases

Choosing the right protease is important because it determines the sequence that will eventually be hydrolyzed to amino acids [11]. Proteases have certain specific effects on substrates, mainly manifested in different peptide bonds [12]. The peptide content and flavor intensity of the by-products of Spanish mackerel under the action of different proteases are shown in Figure 1 and Table 2. As can be seen from Figure 1, after the byproducts of Spanish mackerel were enzymolized by various proteases, the highest peptide content in the enzymolysis solution was trypsin, up to 78.50mg/mL, which was significantly different from other proteases. The next was papain with 66.63mg/mL peptide content. The peptide contents of neutral protease and alkaline protease were 62.12mg/mL and 60.30mg/mL, respectively, and there was no significant difference between the two enzymes.

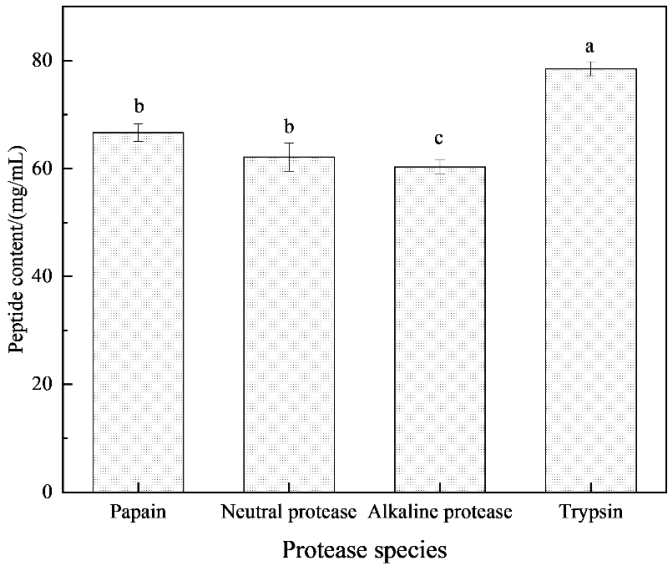

Figure 1. Polypeptide content of byproducts of Spanish mackerel hydrolyzed by various proteases.

As can be seen from Table 2, neutral protease has the highest taste intensity but has a larger bitter taste, papain and alkaline protease have a slightly fishy bitter taste. The product obtained by trypsin enzymolysis is rich in strong Spanish mackerel taste and has no obvious bitter taste, showing a high taste intensity. Therefore, trypsin was selected as the most suitable enzyme for the preparation of umami peptide by-product of Spanish mackerel.

Table 2. Sensory evaluation and TD values of by-products from enzymatic hydrolysis of Spanish carp by each protease

Enzyme species	Sensory description	TD value
Papain	Light yellow, caused by a slightly bitter, slightly beany odor	24.2
Neutral protease	Light yellow, with a greater bitter taste, and without the characteristic umami taste of Spanish fish	31.6
Alkaline proteases	Light yellow, slightly turbid, slightly bitter, slightly beany	26.2
Trypsin	Bright yellow with characteristic umami taste and no off flavor from tangy Spanish fish	27.6

3.2 Single-factor test for enzymatic hydrolysis of Spanish mackerel byproducts by trypsin

3.2.1 Effect of temperature on the peptide content and TD value in the by-product enzymatic hydrolysis solution of Spanish mackerel

Figure 2(a) shows the influence of different temperatures on the peptide content of the Spanish mackerel by-product enzymatic hydrolysis solution. With the increase in temperature, the peptide content and TD value of the Spanish mackerel by-product enzymatic hydrolysis solution also increase. When the temperature was 40℃, the peptide content and TD value reached the maximum. When the reaction temperature is 50℃, the peptide content is significantly decreased compared with the reaction temperature of 45℃ ($p < 0.05$), mainly because the increase of temperature in a certain range makes the enzyme activity of trypsin gradually enhanced, the reaction speed gradually accelerated, the reaction efficiency constantly improved and tended to be stable, so the peptide content in the by-product enzymatic hydrolysis solution of Spanish mackerel is relatively high. When the temperature is increased to the optimum temperature of trypsin, the activity of trypsin is the highest and the efficiency of enzymatic hydrolysis is the best. However, too high a temperature may lead to the inactivation of trypsin or denaturation of substrate protein [13], thus affecting the peptide content in the by-product enzymolysis solution of Spanish mackerel. Therefore, 40℃ was selected for the follow-up test.

3.2.2 Effect of pH value on peptide content and TD value of Spanish mackerel by-product enzymatic hydrolysis solution

The dissociation state of the enzyme active group is closely related to the pH value of the system, thus affecting the enzyme activity [14]. In the range of pH 6.0~9.0, the changes in peptide content and TD value of enzymatic hydrolysis products were analyzed. As can be seen from Figure 2(b), the TD value of peptide content showed a trend of first increasing and then decreasing with the increase of pH value. The optimal pH value is conducive to the binding of enzyme and substrate, and the enzymatic hydrolysis efficiency can also reach the highest value [15]. The TD value reaches its maximum at pH 7.0, and the peptide content reaches its maximum at pH 8.0. When the pH value is 9.0, the molecular structure of the enzyme begins to change and the catalytic activity decreases, resulting in a decrease in peptide content and TD value. After comprehensive consideration, pH 8.0 was selected as the optimal pH for enzymatic hydrolysis.

3.2.3 Effect of enzyme addition level on peptide content and TD value in the enzymatic hydrolysis solution of Spanish mackerel by-product

As shown in Figure 2(c), the peptide content and TD value of the enzymolysis solution increased with the increase of trypsin addition in the range of 4000U/g-12000U/g. The main reason was that the increase in enzyme content accelerated the enzymolysis reaction rate and made the substrate protease hydrolysis more complete, increasing the number of target products. However, when the enzyme dosage was greater than 12000U/g, the peptide content and TD value of the enzymolysis product decreased, which may be due to the excessive enzyme further hydrolyzed the peptide segment, resulting in the reduction of the umami peptide segment [16]. Therefore, the optimal concentration of trypsin was 12000U/g.

3.2.4 Effect of enzymatic hydrolysis time on the peptide content and TD value in the by-product enzymatic hydrolysis solution of Spanish mackerel

As shown in Figure 2(d), with the increase of time, trypsin continued to cut the macromolecular protease cleavage site, resulting in a continuous increase in peptide content, and umami peptide segments were successively produced, reaching the maximum value at 4 h. After more than 4h, the peptide content and TD value of the by-product enzymatic hydrolysis solution of Spanish mackerel decreased with the increase of time. This may be because peptides are hydrolyzed to amino acids with the prolongation of enzymatic hydrolysis time, and some umami peptides are cut by trypsin, resulting in a decrease in peptide content and TD value. Therefore, the optimal enzymatic hydrolysis time was finally determined to be

4 h, which was verified in the studies of Chen [17].

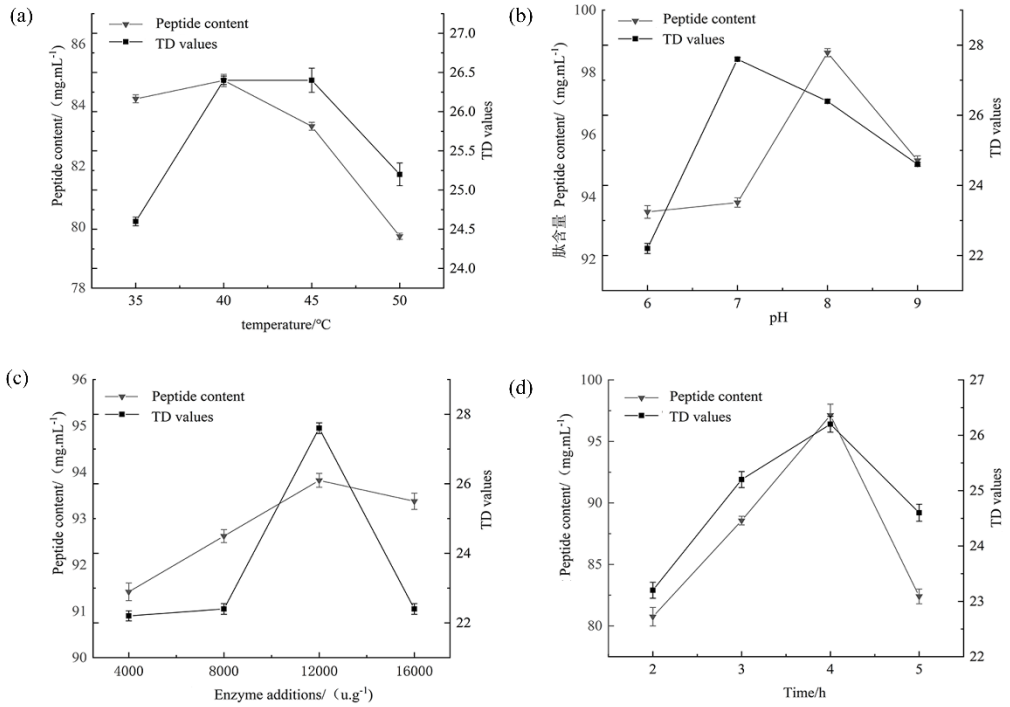

Figure 2. Effects of temperature (a), pH(b), enzyme addition amount (c), and enzymatic hydrolysis time (d) on the peptide content and TD value of the by-product enzymatic hydrolysis solution of Spanish mackerel.

3.3 Orthogonal test results and analysis

According to the 2.3.3 Test design scheme, an orthogonal test design with four factors and three levels was carried out. The test scheme and results are shown in Table 3.

Table 3. Peptide contents and TD values of byproduct enzymatic lysates from Spanish carp under different parameters

Sample number	A: Time /h	B: Tempera-ture/℃	C: pH value	D: Enzyme addi-tions/(U.g^{-1})	Mean values of peptide con-tents/(mg.mL^{-1})	TD value	Composite score
1	3	35	7	8000	89.92	25.2	115.12
2	3	40	8	12000	89.62	23	112.62
3	3	45	9	16000	83.01	26.8	109.81
4	4	35	8	16000	106.15	27.4	133.55
5	4	40	9	8000	89.32	22.2	111.52
6	4	45	7	12000	89.92	26.4	116.32
7	5	35	9	12000	86.91	25	111.91
8	5	40	7	16000	104.94	28	132.94
9	5	45	8	8000	86.91	25.2	112.11
Mean 1	112.52	120.19	121.46	112.92			
Mean 2	120.46	119.03	119.43	113.62			
Mean 3	118.99	112.75	111.08	125.43			
R	7.95	7.45	10.38	12.52			

As can be seen from Table 3, the range of each factor is as follows: D factor >C factor >A factor >B factor. That is the main and secondary factors affecting the peptide content and TD value are as follows: the amount of protease has the

greatest effect on the enzymolysis solution of Spanish mackerel by-product, followed by pH value, enzymolysis time, and enzymolysis temperature. From the analysis in Table 3, it can be seen that the theoretical best enzymatic hydrolysis process of Spanish Spanish byproducts is $A_2B_1C_1D_3$. In the list of orthogonal tests, the comprehensive score of $A_2B_1C_2D_3$ is the highest 133.55, so it is necessary to add a group of verification experiments, and the verification results are shown in Table 4.

Table 4. Verification experiments

Assembly	Time /h	Temperature/℃	pH values	Enzyme additions/(U.g^{-1})	Mean values of peptide contents/(mg.mL^{-1})	TD values	Composite score
$A_2B_1C_1D_3$	4	35	7	16000	106.58	27.54	134.12
$A_2B_1C_2D_3$	4	35	8	16000	107.84	28.53	137.37

It was verified that the comprehensive score of the two groups was higher than that of the experimental group, but the alkaline pH value of A2B1C2D3 made the $A_2B_1C_2D_3$ score higher than that of the experimental group. It may be that the proteases used in this experiment react more completely under alkaline conditions than under neutral conditions. Table 4 shows that the $A_2B_1C_2D_3$ peptide content is 107.84mg/mL and the TD value is 28.53. Therefore, the optimal combination is: enzymolysis time is 4 h, enzymolysis temperature is 35 ℃, enzymolysis pH is 8.0, and enzyme addition amount is 16000 U/g.

4. Conclusion

Taking the by-product of Spanish mackerel as raw material, the most suitable protease for preparing umami peptide was trypsin through the determination of polypeptide content. Single-factor experiments were conducted with peptide content and TD value as indexes, enzymolysis temperature, pH value, time, and enzyme addition amount as influencing factors. The optimal reaction conditions of enzymolysis were as follows: reaction temperature 40℃, pH 8.0, time 4h, enzyme addition amount 12000U/g.

According to the results of the orthogonal test, the enzymatic hydrolysis conditions of Spanish mackerel byproducts are as follows: enzymatic hydrolysis time is 4 h, enzymatic hydrolysis temperature is 35 ℃, enzymatic hydrolysis pH is 8.0, the enzyme addition amount is 16000 U/g. Under these conditions, the peptide content was 107.84mg/mL and the TD value was 28.53.

Funding

Major Scientific and Technological Innovation Project of Shandong Province: Development of key technologies for targeted and efficient preparation of marine active peptides (2022CXGC020414).

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