OPTIMIZATION OF CONDITIONS FOR LIMITED PROTEOLYTIC HYDROLYSIS OF SPENT BREWER'S YEAST TO PRODUCE LOW-WEIGHT MOLECULAR PEPTIDE WITH SOME BIOACTIVITIES

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ABSTRACT

Spent brewer's yeast (*Saccharomyces cerevisiae*) is a rich source of protein, vitamins and widely used as a raw material for producing bioactive compounds and food ingredients. In the present study, the Box-Behnken design was used to determine the optimal conditions for hydrolysing spent brewer's yeast using protease (Neutrase) to produce low- weight molecular peptide. The spent brewer's yeast concentration, concentration of protease, and hydrolysis time are the most important factors used to optimize the protein hydrolysis. For achieving high yields of peptide, the hydrolysis conditions were determined: yeast concentration of 22.72 % (w/w), protease concentration of 4.07 % and hydrolysis time of 16.73 hours. The maximum yield of low-MW peptides was achieved 223.18 mg/g. The obtained bioactive peptide showed capability of antibacterial against *Salmonella typhi*, *Listeria monocytogenes*, *Staphylococcus aureus* and high antioxidant activity at peptide concentration of 100 (µg/ml).

Keywords: spent brewer's yeast, protease, low weight molecular peptide, antibacterial activity, antioxidant activity.

1. INTRODUCTION

Low molecular weight peptides are biologically active peptides (Bio- peptides). In addition to nutritional value, bio-peptides are also capable of affecting the physiological functions of the body, helping to improve the health of humans as antibacterial, antioxidant capacities, angiotensin-converting enzyme (ACE) inhibition, immune-modulatory [1]. Low molecular weight peptide (MW) having biological activity can be extracted from animal, plant, or microbial fermentation product, limited protein hydrolysate obtained from different sources by using protease [2]. Peptides fraction with molecular mass smaller than 3 kDa obtained from trypsin hydrolysis of *S. cerevisiae* showed the most antioxidant activity against DPPH and ABTS radicals [3]. The fraction protein hydrolysate (MW = 5 - 10 kD) obtained after autolysis

treatment of *Kluyveromyces marxianus* showed antibacterial activity against *St. aureus* and *Lis. monocytogenes* [4]

In recent years, biological peptide having been studied in Vietnam mainly obtained from protein of fermented milk and black bean, soybean. Yeast protein would be a good material for the production of bioactive peptides with economic advantages because it is a source of by-product in brewery industry.

The aim of this research is to optimize the spent brewer's yeast hydrolysis process to obtain the high content of low MW peptide with antioxidant and antibacterial activities by proteolytic hydrolysis of spent brewer's yeast.

2. MATERIALS AND METHODS

2.1. Materials

Spent brewer's yeast (*Saccharomyces cerevisiae*) was obtained from Sabeco- Hanoi Beer Company. Ortho-phthalaldehyde (OPA); furanacrylolyl; 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, were obtained from Sigma-Aldrich (Munich, Germany). Ultrafiltration membranes with 10 kDa cut-off were purchased from Millipore (United States). Neutrase 1.5 MG was trade mark by Novozyme, Denmark.

2.2. Enzymatic hydrolysis and purification method

The spent brewer's yeast after pretreatment with 3 times wash with water which were centrifuged at 8000 rpm in 10 minutes. The slurry was suspended in distilled water to achieve a concentration of 15 - 25 % (w/v). The suspensions were placed in 100 ml glass vessels immersed in a temperature-controlled water bath. After the heat treatment, it was followed by cooling and 3 – 5 % of Neutrase were added. The initial pH was adjusted to 6.5 with NaOH (1 N) solution for hydrolysis using Neutrase. The hydrolysis was performed at 50 ^{o}C, the hydrolysis process was allowed for 14 - 18 hours. To determine low molecular weight peptides contents of the hydrolysate, 5 ml aliquots of reaction mixture were taken, heated at 95 °C for 5 min to inactivate enzyme, and centrifuged (12000 rpm; 4 °C) for 20 min. To purify antioxidant and antibiotic peptides, the hydrolysates derived enzymatic treatments were passed through ultra-filtration membranes with a cut-off of 10 kDa. The filtrates were used for determine low molecular weight peptides contents, analysis of antioxidant and antibiotic activity [5].

2.3. Analysis methods

2.3.1. Determine low molecular weight peptides contents by OPA method

The extent of hydrolysis was studied using o-phthaldialdehyde (OPA) spectrophotometric assay, which has been previously described by Frank C. C et al. [6]. A fresh OPA solution was prepared daily as follows: 25 ml of 100 mM sodium tetrahydroborate, 2.5 ml of 20 % SDS (w/v), 40 mg of OPA (dissolved in 1 ml methanol) and 100 µl of β- mercaptoethanol were adjusted to a final volume of 50 ml with distilled water. To assay proteolysis with yeast protein as substrate an aliquot 50 µl sample was added directly to 200 µl of OPA reagent. The solution was mixed briefly and incubated for 2 min at room temperature. Subsequently the absorbance

was read at 340 nm using a UV-visible spectrophotometer. Free amino groups were calculated from a standard curve constructed by using peptone (Himedia, India) 0–10 mg/ml [6].

2.3.2. Method for antioxidant activity determination using the DPPH free radical scavenging assay.

The samples were pre-diluted at a factor of 5 and DPPH stock solution (0.1 mM) in ethanol was prepared. A 190 µl DPPH stock solution was added to 10 µl sample with different peptide concentrations and incubated at 37 ^{o}C for 30 min in the dark. After incubation, the absorbance of the sample was then measured at 517 nm using a spectrophotometer. A control sample is acid ascorbic range of 0–60 µg/ml was prepared as mentioned above. The antioxidant activity was expressed as percentage of DPPH free radical scavenging activity (%DPPH) and calculated using formula [7, 8]:

$$\%DPPH = (A_{control} - A_{sample}) / A_{control} \times 100\%$$

where $A_{control}$: the absorbance of control sample; A_{sample}: the absorbance of sample.

2.3.3. Determination of antibacterial activity by colony counting method

Three strains of bacteria were conducted as *Salmonella Typhi*, *Listeria monocytogenes*, *Staphylococcus aureus*. After the proliferation into 10 ml of LB media, then incubating at 37 °C in 24 h, the cell density of cultures was achieved 2,2.108; 1,6.108; 3,2.108 CFU/ml. Each of 0.1 ml bacterial culture suspension with density of 105 CFU was supplemented in 2 ml LB liquid with translated peptide at different concentrations 0-10-20-30 µg/ml (control sample - without peptide) and incubated at temperature 37 °C for 24 h. The bacterial cultures were diluted at concentrations ranged from 10^1 - 10^6. Amount of bacteria in the incubated media was determined by implanting on Petri dishes filled with LB agar, incubating at 37 °C for 24 h. Antibacterial activity of peptide was evaluated by comparison of bacterial amount between the samples cultured in medium supplemented with translated peptide and control sample [9].

2.3.4. Optimization of Spent brewer's yeast hydrolysis process under experimental planning methods quadratic Box – behnken using Design Expert 7.1.5 software *(State-Ease, Inc.,)*

Three factors are selected to determine the impaction to the spent brewer's yeast hydrolysis process to optimize the yield of produced bioactive peptides (Y): X1 - spent brewer's yeast concentrations, X2 - enzyme concentration, X3 - hydrolysis time. Experimental matrix is set with 17 experiments combined with the level (high, low, medium) of the surveyed factors, combined 3 separate experimental factor (experiments in mind) is replicated 5 times. The quadratic model used by software to describe the tested experimental conditions is a second degree polynomial equation as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_1X_3 + b_6X_2X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2 . \quad (1)$$

3. RESULTS AND DISCUSSION

3.1. Optimization of conditions for spent brewer's yeast hydrolysis by protease to low molecular weight peptides

The influence of three independent variables (including concentration of spent brewer's yeast, concentration of protease and hydrolysis time) on the yield of produced peptides released from yeast's protein was investigated. The matrix of variation of independent variables is presented in Table 1. The experimental design is shown in Table 2.

Table 1. Levels and code of variables chosen for Box-Behnken design.

Symbol	Variables	Units	Coded levels	
			-1	+1
X1	Brewer's yeast concentration	%	15	25
X2	Protease concentration	%	3	5
X3	Hydrolysis time	h	14	18

Table 2. The experimental design for the enzymatic hydrolysis of spent brewer's yeast.

Run	Brewer's yeast concentration (%)	Protease concentration (%)	Hydrolysis time (h)	Yield of produced Peptide (mg/g)
1	**15**	**3**	**16**	**120.87**
2	25	3	16	200.87
3	15	5	16	171.5
4	25	5	16	209.6
5	15	4	14	138.65
6	25	4	14	196.5
7	15	4	18	150.25
8	25	4	18	212.1
9	20	3	14	154.3
10	20	5	14	210.55
11	20	3	18	172.12
12	20	5	18	219
13	20	4	16	213.68
14	20	4	16	218.12
15	**20**	**4**	**16**	**222.27**
16	20	4	16	205.10
17	20	4	16	204.94

As it can be seen in Table 2, the maximum yield of peptide (222.27 mg/g) released from substrate was produced when the concentration of enzyme was 4 %, the amount of brewer's yeast was 20 % (w/v), the hydrolysis time was 16 hours. The minimum peptide amount of 120.87 mg/g was obtained at an protease concentration of 3 %, the hydrolysis time of 16 hour and 15 % (w/w) substrate. The amount of obtained peptide was smaller due to the reduce of protease concentration and hydrolysis time. The statistical significance of model equation was checked using F-test analysis of variance (ANOVA) (Table 3).

The model F-value of 25.28 implies that the model was significant. A p-value 0.0002 (< 0.05) indicates that the model terms are also significant. In this case, the correlative effects of the brewer's yeast concentration, protease concentration, the hydrolysis time were significant model terms. Also, p-values ≥ 0.05 indicates that the model terms were not significant. The lack of fit F-value of 1.33 implies that the lack of fit is not significant relative to the pure error. There is a 38.09 % chance that a lack of fit F-value this large could occur due to the noise.

Table 3. Analysis of variance (ANOVA) for enzymatic hydrolysis evaluation.

Source	SS	Df	MS	F-ratios	p- value
Model	15587.49	9	1731.94	25.28	0.0002
Brewer's yeast concentration(X_1)	7068.61	1	7068.61	103.16	< 0.0001
Protease concentration (X_2)	3299.77	1	3299.77	48.16	0.0002
Hydrolysis time (X_3)	357.45	1	357.45	5.22	0.0563
X_1^2	438.90	1	438.90	6.41	0.0392
X_2^2	4.00	1	4.00	0.06	0.8160
X_3^2	21.97	1	21.97	0.32	0.5889
$X_{1\times} X_2$	2817.03	1	2817.03	41.11	0.0004
$X_{1\times} X_3$	532.62	1	532.62	7.77	0.0270
$X_{2\times} X_3$	666.83	1	666.83	9.73	0.0169
Residual	479.64	7	68.52		
Lack of Fit	239.96	3	79.99	1.33	0.3809
pure error	239.68	4	59.92		
SS total	16067.13	16	1731.94		

According to the data presented in Table 3, the quadratic model fitted well for variable optimization as the p-value was lower than 0.05. For peptide release after hydrolysis by the commercial protease, the summary showed that the model was globally significant, so it can be interpreted.

Studying the parameters from the ANOVA statistical analysis, it can be easily observed that the concentration of protease had the greatest influence on the yield of enzymatic hydrolysis. Also, was an important factor that increased the yield of substrate hydrolysis. The hydrolysis time effect on brewer's yeast hydrolysis was insignificant for the studied range, as the p-value was higher than 0.05. After the analysis of variance, which gives the level of peptide amount as a function of the investigated factors affecting the hydrolysis process, the regression equation (1) becomes:

$$Y = +212.83 + 29.73.X_1 + 20.31.X_2 + 6.68.X_3 - 10.48.X_1X_2 + 1.00.X_1X_3 - 2.34X_2X_3 - 25.87.X_1^2 - 11.25.X_2^2 - 12.58.X_3^2$$

The terms that do not contribute significantly to the prediction of Y were omitted from the full model and a reduced second-order polynomial equation was obtained. This implies that the effects of enzyme concentration and hydrolysis time were significant as it is obvious from their high coefficients. The results of multiple linear regression analysis (reduced model) reveal that, by increasing the time of hydrolysis and the enzyme concentration, the yield of peptides (expressed by Y) will increase, as the b_1 and b_3 coefficients are positive.

The suggested hydrolysis conditions for obtaining low molecular weight peptides were as follows: the spent brewer's yeast concentration of 22.72 %, protease concentration of 4.07 % and hydrolysis time 16.73 h. The following results showed that yield of producing peptide was

estimated 223.18 mg/g. Thout it had a little compatibility with last experiment (222.27 mg/g), but it convinced that the simultaneous effect of three independent variables was significant. The desirable response for yield of producing peptides and optimal conditions for hydrolysing spent brewer's yeast to bioactive peptide is presented in Fig. 1.

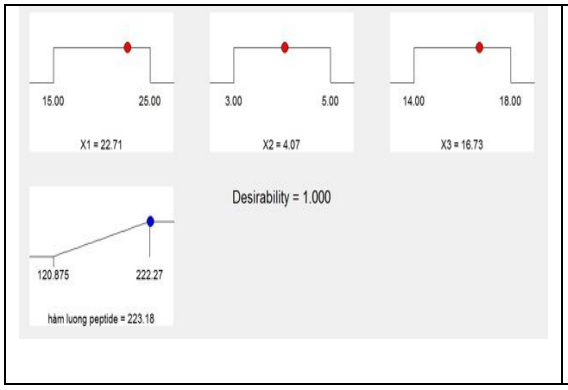

Figure 1. The desirable response for yield of producing peptides and optimal conditions for hydrolysing spent brewer's yeast to bioactive peptides.

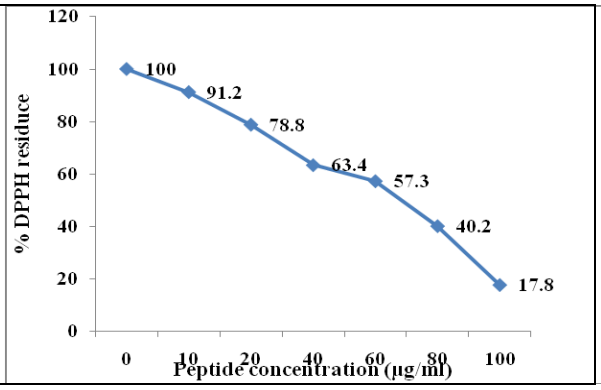

Figure 2. DPPH residue after reaction with biopeptides.

3.2. Determination of some bioactives

3.2.1. Antibacterial activity

The inhibition of malignant bacteria was progressed with three strains: *Salmonella typhi, Listeria monocytogenes, Staphylococcus aureus.* The results are shown in Table 4.

Table 4. The results of colony counting in media containing bioactive peptide.

Bacteria	**Media**	**Colony count result (× 10 CFU/ml)**		**Reduction rate of control comparison (%)**
		0 h	After 24 h	
Salmonella typhi	LB (Control)	22	8300	-
	LB + 10 µg/ml	22	4400	46.99
	LB + 20 µg/ml	22	2300	72.29
	LB + 30 µg/ml	**22**	**84**	**98.90**
Listeria monocytogenes	LB (Control)	16	8700	-
	LB + 10 µg/ml	16	5300	39.08
	LB + 20 µg/ml	16	4700	45.98
	LB + 30 µg/ml	**16**	**1200**	86.21
Staphylococcus aureus	LB (Control)	32	10400	
	LB + 10 µg/ml	32	6600	36.54
	LB + 20 µg/ml	32	4300	58.65
	LB + 30 µg/ml	32	**2500**	75.96

As it can be seen, three strains of bacteria were cultured with LB media supplement low weight molecular peptide with different concentration of peptides (10-20-30 µg/ml). After 24 h incubating, the amount of colony was decreased. In particular, at concentration of 30 µg/ml, *Salmonella typhi* reduced to 98.9 %; *Listeria monocytogen* reduced to 86.21 %.

3.2.2. Antioxidant activity

The results of the test for antioxidant activity (Fig. 2) showed that DPPH concentration had been changed when DPPH reacted with low weight molecular peptide. With concentration of peptide ranged from 10-100 (µg/ml), the percentage of DPPH free radical scavenging activity had reduced from 91.2 % to 17.8 %. So that, produced peptide by proteolytic hydrolysis has high antioxidant activity at peptide concentration of 100 (µg/ml).

4. CONCLUSIONS

The hydrolysis conditions for producing low weight molecular peptide using commercial protease (Neutrase) were optimized by applying method Box-behnken. Hydrolysis time and the concentration of enzyme have significant influence on the yield of hydrolysis. By using spent brewer's yeast concentration of 22.72 %, with protease concentration of 4.07 % and hydrolysis times 16.73 h, a high hydrolysis yield was achieved. Under optimal conditions for hydrolysis, the maximum peptide yield was reached the value of 223.18 mg/g.

The obtained low weight molecular peptide showed antibacterial activity against *Salmonella typhi, Listeria monocytogenes, Staphylococcus aureus* at concentration of 10 - 30 µg/ml, the strongest inhibitory activity was achieved at 30 µg/ml and had high antioxidant activity with peptide concentration 100 µg/ml.

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TÓM TẮT

NGHIÊN CỨU TỐI ƯU CÁC ĐIỀU KIỆN THỦY PHÂN BÃ NẤM MEN BIA TẠO PEPTIDE PHÂN TỬ LƯỢNG THẤP VÀ MỘT SỐ HOẠT TÍNH SINH HỌC

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Bã nấm men bia là một nguồn giàu protein, vitamin và được sử dụng rộng rãi như là một loại nguyên liệu để sản xuất các hợp chất peptid có hoạt tính sinh học và thành phần bổ sung vào thực phẩm. Trong nghiên cứu này, phương pháp quy hoạch thực nghiệm Box-Behnken được sử dụng để tối ưu hóa quá trình thủy phân bã nấm men bia nhờ chế phẩm protease (Neutrase) nhằm thu peptid phân tử lượng thấp. Nồng độ bã nấm men bia, nồng độ enzym và thời gian thuỷ phân là những yếu tố quan trọng nhất ảnh hưởng tới sự tối ưu hóa quá trình thủy phân. Để có hiệu suất tạo peptid cao, điều kiện thủy phân đã được xác định là: nồng độ nấm men 22,72 % (w/w), nồng độ protease 4,07 % và thời gian thuỷ phân 16,73 giờ. Hàm lượng các peptid phân tử lượng thấp thu được lớn nhất là 223,18 mg/g. Các peptid sinh học thu được có khả năng kháng khuẩn đối với *Salmonella typhi, Listeria monocytogenes, Staphylococcus aureus* và khả năng chống oxi hoá cao ở nồng độ peptid là 100 µg/ml.

Từ khóa: bã nấm men bia, protease, peptid có phân tử lượng thấp, hoạt tính kháng khuẩn, hoạt tính chống oxi hoá.