Journal of Biotechnology **14**(4): 735-742, 2016

OPTIMIZATION FOR CONTINUOUS OVERFLOW PROTEOLYTIC HYDROLYSIS OF SPENT BREWER'S YEAST BY USING PROTEASES

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Received: 9.11.2016
Accepted: 20.12.2016

SUMMARY

A large amount of spent yeast as by-product is annually generated from brewing industry and it contains about 50-55% protein with good balance of amino acids. The hydrolysate produced from spent brewer's yeast may be used in food application. The yield of proteolylic hydrolysis for spent brewer's yeast and amino acid contents of hydrolysates depend on factors such as temperature, pH value, type of used enzyme and ratio enzyme/substrate, time. Besides, applied hydrolysing methods (batch-, or continuous method) has effected on degree of hydrolysis. With the purpose of how proteolytic hydrolysis having effects on the spent brewer's yeast for food application in industrial scale, continuous overflow method was used in this study. Bitterness of hydrolysate and the yield of continuous overflow proteolytic hydrolysis process are the two interested factors for protein hydrolysis. In this report, it is dealt with determination for optimal conditions to obtain the highest yield of hydrolysis process and the lowest bitterness of hydrolysate. Response surface methodology (RSM) was used to determine optimal condition for continuous overflow proteolytic hydrolysis of spent brewer's yeast. The optimal conditions for obtaining high degree of hydrolysis and low bitterness are determined as followings: ratio of enzyme mixture (alcalase 7.5 U/g and flavourzyme 10 U/g), pH at 7.5, hydrolysis temperature at 51^{o}C and hydrolysis time of 9 hours. Under the optimal conditions, the yield of hydrolysis was 59.62% ± 0.027 and the bitterness equivalently with concentration of quinine was 7.86 ± 0.033 µmol /ml.

Keywords: *Continuous overflow hydrolysis, degree of hydrolysis, enzymatic hydrolysis, optimization, spent brewer's yeast.*

INTRODUCTION

Spent brewer's yeast, the byproduct from the brewing industry, is being produced in large amount annually from main beer manufacturers due to increased volume of beer production. It is generally used primarily as inexpensive animal feed after inactivation by heat and much of this byproduct is considered industrial organic waste that causes a great deal of concerns. Such wastes are generally incinerated or put into landfill, in which case, remaining proteins and amino acids, and other useful substances were not recovered. In addition, incineration of organic wastes often gives toxic emission whose distribution degree is even higher than that of organic solid wastes. Attempts have been made to recover higher value protein and amino acid products from spent Brewer's yeast (Zhang *et al.,* 2008) by employing various processes such as autolysis, plasmolysis (Vukasinovic-Milic *et al.,* 2006), acid or alkali catalyzed hydrolysis, or enzymatic hydrolysis (Bayarjargal *et al.,* 2011; Tavano, 2013) by batch, overflow or continuous methods.

Review of published researches to date (Cheryan, Deeslie, 1983) indicated there were several problems in this area. One was the high cost of using large quantities of enzymes in batch – type operations (Cheftel *et al.,* 1971). Batch processes were energy and labor intensive and the equipment might require considerable floor space. In addition, batch processes were slow and rarely went to completion, thus resulting in low yields and/or poor productivity (Cheryan, Deeslie, 1983). Hence the extent of reaction must be carefully controlled. However, the product from batch hydrolysis is generally non uniform in composition and may

contain several fractions of varying molecular weights. In addition, uncontrolled or excessive hydrolysis could lead to production of off-flavors and bitterness is pronounced when low molecular weight peptides produced (Adler-Nissen, 1976). The major advantage of ultrafiltration (UF) reactors were that they allow the use of soluble enzymes in contact with the substrate, thus avoiding problems of steric hindrance, loss of activity, and diffusional resistance problems typical of other conventional immobilization methods (Cheryan, Deeslie, 1983). But the cost of UF reactors was big when it was applied on an industrial scale. Moreover, it was clear that this system was needed to have crude filter and crystal filter before UF reactors. This leads to the great investment cost. The scientific aims of this study is to describe the design and performance of a continuous overflow using seamlessly connected tanks or the continuous hydrolysis of yeast's protein.

The hydrolysate has bitter taste. The bitter taste of protein hydrolysate is formed due to the presence of hydrophobic amino acids or peptides contain hydrophobic amino acid residues (Shaa, Hayashi, 2001; Sujit, Hymavathi, 2011; Teruyoshi, Tadao, 2011; Dougherty, 2007). Bitterness increased as hydrophobic amino acid content increased (Haefeli, Glaser, 1990; Fitzgerald, Ocuinn, 2006; Raksakulthai, Haard, 2003). In order to increase sensory and nutritional value of products, yeast hydrolysate is added in food (such as: salad dressings, spreads, ice cream, coffee whitener, cracker, and meat products like sausages). With the aim to obtain the maximum degree of hydrolysate (DH) and minimum bitterness of hydrolysate, response surface methodology (RSM) was used to determine optimum condition for continuous overflow proteolytic hydrolysis of spent brewer's yeast by using combination of alcalase and flavourzyme.

MATERIALS AND METHODS

Materials

The spent brewer's yeast *Saccharomyces* used as a substrate was donated by brewer's Sai Gon Ha Noi. Flavourzyme and alcalase were obtained from Novozymes, Denmark. Proteolytic activity is 289 U/g and 328 U/g. Flavourzyme is a food grade exoprotease from *Aspergillus oryzae,* its main enzyme component is EC 3.4.11.1. Alcalase is a food grade endoprotease from *Bacillus licheniformis*, its main enzyme component is the serine protease subtilsin A (EC 3.4.21.62).

Methods

Technological methods

Washing process: Spent brewery's yeast was washed once with NaOH 0.1N for removing polyphenols and 3 times with cold water for the removing remained solids, and then centrifuged at 4000 rpm at 4 ^{o}C for 15 min to recover solids, which were materials for further studies.

Pretreament yeast cell: Sludge of treated yeast was adjusted to pH 5.5 (using HI 2211 pH/ORP meter) by NaOH 0.2N. The ratio of yeast: water was 1:1.5 (w/w), and autolysis was carried out in 24 hours.

Hydrolysis process: After autolysis process, autolysate was adjusted to pH 7.5 and added with combination two enzymes (alcalase and flavourzyme) and then continuous hydrolysis process was performed on continuous overflow system (include one tank of 50 liters and 5 tanks of 5 liters) (Fig. 1) using the agitators with agitation speed (M) of 100 rpm under different conditions. Autolysate was continuous overflow from big tank (Fig. 1/ 1) to small tanks (Fig. 1/ 2–6). After hydrolysis, the sludge yeast in tanks was heated by heating bars. Hydrolysate was circulated by pump (P) though if necessary. The sample was inactivated by 0.5M TCA and the sludge was removed by using centrifuge (6000 rpm, at 4^{o}C for 10 min). The obtained hydrolysate was recovered in order to determine amino acid content (by HPLC) and bitterness of hydrolysate (by sensory method).

Analysis methods

Sensory method: Sensory evaluation for bitter taste of the yeast protein hydrolysate was conducted by a panel consisting of 7 females and 5 males between the age of 22 and 40 years old. The panel members were trained for a period of one month, four times per week, with using quinine as standard (S6672804614, Merck, Germany). Quinine threshold was determined at 8 µmol/l, calibration curve equation of quinine was determined: $y = 0,8557\ln(x) + 4,3554$, với $R^2 = 0,9829$, where: y - Quinine concentration (µmol/l); x - Bitter taste point (0 -10).

Determination of degree of hydrolysis: In protein hydrolysis, the key parameter for monitoring the reaction is the degree of hydrolysis (DH), which is determined as the percentage of amino acids before and after hydrolysis process for spent brewery's yeast. The following formula was used for

calculation according to Vukasinovic-Milic *et al.* (2006): DH = $N_s/N_t \times$ 100%; - where N_s is amino acid content in hydrolysate, it was determined by Ninhydrin method using glutamic standard (Merck KGaA, Germany). N_t is the total nitrogen content in yeast dry before hydrolysis, it was measured by the Kjeldahl method.

Experimental design method and optimization: Experimental design: The response surface method with CCOD (central composite orthogonal design) were used to study the effects of independent factors: Enzyme: substrate ratio (E/S) ratio of flavourzyme (E/S ratio of alcalase was not changed), temperature, pH, time of hydrolysis. Desirable responses are the followings: Degree of hydrolysis (Y_1, %) and bitterness of hydrolysate (Y_2, %) (Table 1). This design has 27 trials including 16 trials for factorial design, 8 trials for axial points and 3 trials for central points with α = 1.41421 (Table 2).

Optimization: For prediction of the optimal point, second-order polynomial models were fitted to correlate relationship between independent variables and response. CCOD was performed to evaluate the optimal operating conditions to obtain maximum degree of hydrolysis and minimum bitterness (BT) of hydrolysate.

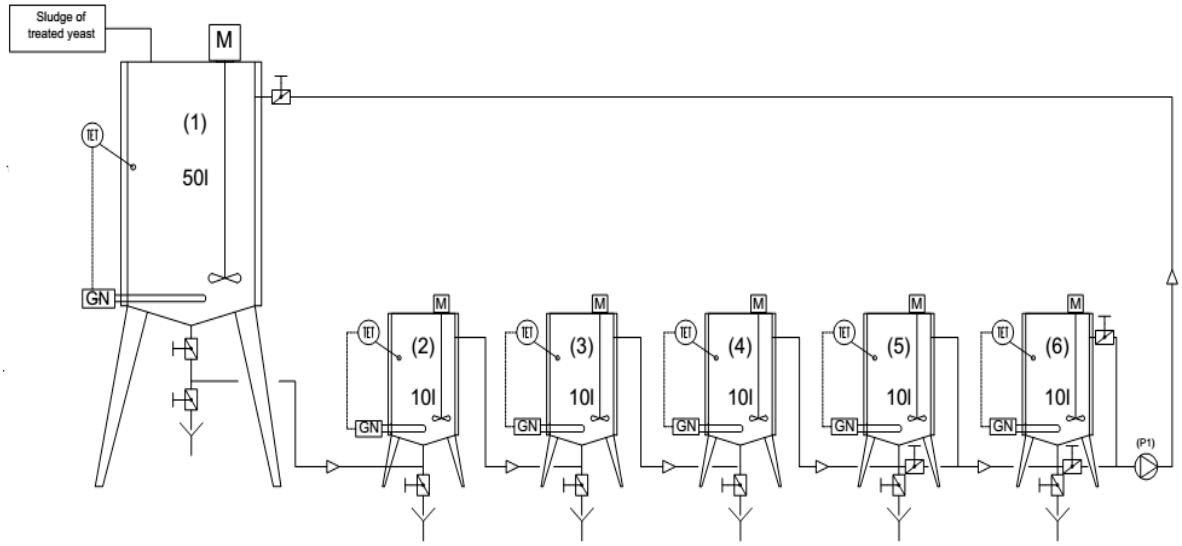

Figure 1. Diagram of continuous overflow hydrolysis (1. Big tank; 2-6: small tanks; M: Motor for paddle; GN: Heating bar; TET: Thermal sensor; P1: Pump).

Table 1. The variables of hydrolyses and their levels.

Variables	Symbols	units	Levels				
			- α	- 1	0	+ 1	+ α
Hydrolysis temperature	A	^{0}C	36	40	50	60	64
pH of hydrolysis	B		5.4	6	7.5	9	9.6
Ratio of E/S	C	U/g	4	5	7.5	10	11
Hydrolysis time	D	Hour	5.4	6	7.5	9	9.6

Statistical analysis

Design Expert software version 10.0 (Stat-Ease, Minneapolis) was used for the regression analysis of experimental data, to plot response surface and to optimize by desirability methodology. ANOVA was used to estimate the statistical parameters.

RESULTS AND DISSCUSSION

Model building and statistical significance test

Table 2 shows the process variables and experimental data of 27 runs. The experimental results were fitted with a second-order polynomial equation by a multiple regression analysis.

Table 2. Experimental design and results.

Exp No	A (°C)	B	C (U/g)	D (hour)	Y_1 (%)	Y_2 (µmol/l)	Exp No	A (°C)	B	C (U/g)	D (hour)	Y_1 (%)	Y_2 (µmol/l)
1	40	6	5	6	38.03	22.53	15	40	9	10	9	43.66	14.85
2	60	6	5	6	42.83	14.95	16	60	9	10	9	48.31	13.88
3	40	9	5	6	38.26	21.62	17	36	7.5	7.5	7.5	36.12	21.33
4	60	9	5	6	42.45	15.14	18	64	7.5	7.5	7.5	42.60	15.28
5	40	6	10	6	40.92	18.43	19	50	5.4	7.5	7.5	45.64	14.56
6	60	6	10	6	46.10	14.17	20	50	9.6	7.5	7.5	45.04	14.75
7	40	9	10	6	41.23	17.23	21	50	7.5	4	7.5	56.99	11.18
8	60	9	10	6	46.03	14.12	22	50	7.5	11	7.5	61.72	8.13
9	40	6	5	9	40.85	18.17	23	50	7.5	7.5	5.4	56.00	11.18
10	60	6	5	9	45.49	13.13	24	50	7.5	7.5	9.6	59.58	8.23
11	40	9	5	9	40.16	18.57	25	50	7.5	7.5	7.5	57.59	10.13
12	60	9	5	9	44.20	14.66	26	50	7.5	7.5	7.5	57.53	10.02
13	40	6	10	9	44.27	14.75	27	50	7.5	7.5	7.5	57.45	10.24
14	60	6	10	9	49.30	12.63							

Table 3. Regression analysis of overall degree of hydrolysis (DH) Y_1 and minimum bitterness (BT) Y_2.

Source	Overall DH Y_1			Overall BT Y_2		
	Mean Square	**F value**	**p-value (Prob > F)**	**Mean Square**	**F value**	**p-value (Prob > F)**
Model	101.329	24750.9	< 0.0001	27.2378	2956.66	< 0.0001
A	108.0031	26381.1	< 0.0001	88.2895	9583.81	< 0.0001
B	0.95278	232.729	< 0.0001	0.12465	13.5311	0.0032
C	58.6554	14327.3	< 0.0001	26.4864	2875.09	< 0.0001
D	32.4470	7925.58	< 0.0001	23.5831	2559.94	< 0.0001
AB	0.24509	59.8652	< 0.0001	1.29581	140.660	< 0.0001
AC	0.24509	59.8652	< 0.0001	9.88676	1073.206	< 0.0001
AD	0.02320	5.66771	0.0347	5.48463	595.355	< 0.0001
BC	0.03626	8.85580	0.0116	0.07459	8.09627	0.0147
BD	0.83532	204.038	< 0.0001	1.72226	186.951	< 0.0001
CD	0.28424	69.4294	< 0.0001	0.21758	23.6180	0.0004
A^2	726.516	177461	< 0.0001	149.385	16215.7	< 0.0001
B^2	325.297	79457.8	< 0.0001	45.9525	4988.13	< 0.0001
C^2	8.22148	2008.20	< 0.0001	0.46710	50.7039	< 0.0001
D^2	0.28988	70.8067	< 0.0001	0.37375	40.5703	< 0.0001
Lack of Fit	0.00396	0.82741	0.6612	0.00864	0.7154	0.7084

Analysis of variance for two models is shown in Table 2. F-value of two models are 24750.9 (Y_1), 2956.66 (Y_2), respectively. It is indicated that all the regression models were highly significant at the confidence level of 99.99% ($p<0.0001$). The signifcance of each regresssion coeffecient measured by p-value and F-value is listed in Table 3. The p-value less than 0.05 indicates that the coefficient is significant. As shown, all regression coeffecients of Y_1 model and Y_2 model are significant at 99.99% confidence level with $p < 0.0001$ (except for a cross coeffecient of AD and BC in Y_1; B, BC, and CD in Y_2 model is less significant). F-value for lack of fit of Y_1 model is 0.82741 ($p = 0.6612$) and Y_2 model is 0.7154 ($p = 0.7084$), respectively. This means that the two models were fit with experiment. Moreover, the coeffecients of determination (R^2) of the two models were 0.9999 (Y_1), 0.9996 (Y_2), indicating that 99.99% and 99.96% of variability in the response could be predicted by the models. The models for the response variables could be expressed by the following second – degree model in terms of coded factors:

$$Y_1 = 57.47 + 2.33A – 0.22B + 1.72C + 1.28D – 0.12AB + 0.12AC - 0.038AD + 0.048BC – 0.23BD + 0.13CD – 9.22A^2 – 6.17B^2 + 0.98C^2 + 0.18D^2$$

$$Y_2 = 10.12 – 2.11A + 0.079B – 1.15C – 1.09D + 0.28AB + 0.79AC + 0.59AD – 0.068BC + 0.33BD + 0.12CD + 4.18A^2 + 2.32B^2 – 0.23C^2 - 0.21D^2$$

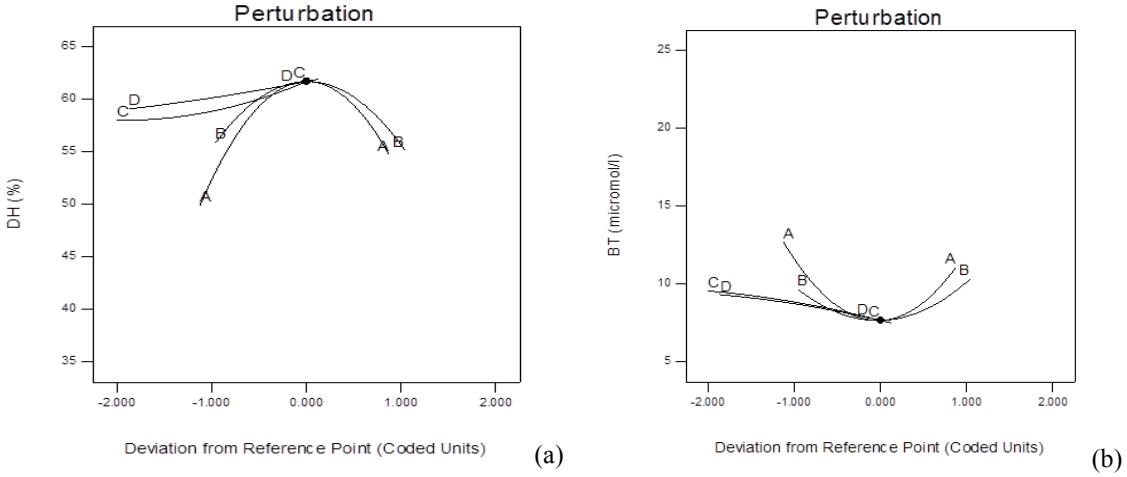

Figure 2. Influence of factors on degree of hydrolysis (DH) and minimum bitterness (BT) of hydrolysate.

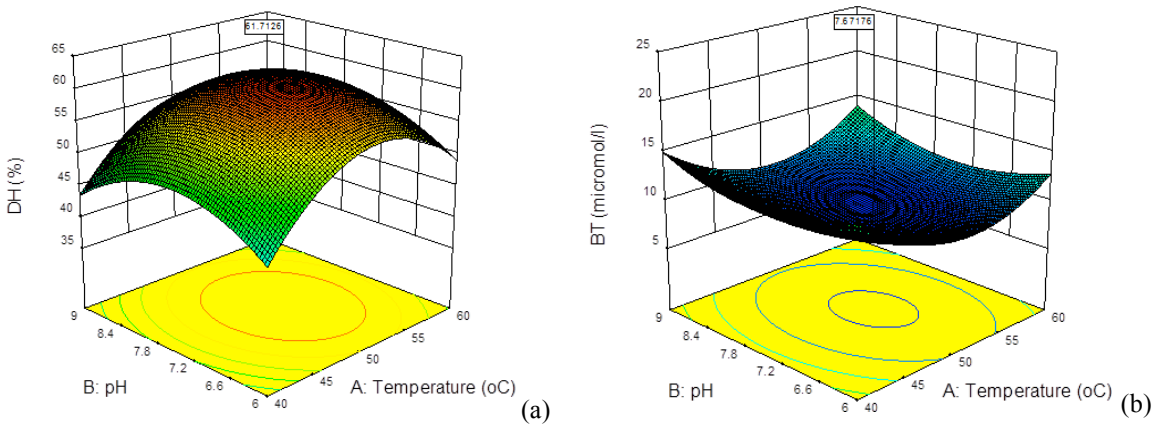

Figure 3. Response surface plot of protein hydrolysis process for degree of hydrolysis (DH) and minimum bitterness (BT) of hydrolysate.

Considering in turn the effect of each factor (when others are fixe at zero level) on the DH (Fig. 2a) and BT (Fig. 2b), it showed that hydrolysis temperature (A) and pH (B) significantly affect the overall DH (Y_1) and BT (Y_2); besides, E/S ratio and hydrolysis time are the less significant factors. The effects of these factors showed more details in the response surface of Y_1 and and Y_2 function (Fig.3). Meanwhile, temperature and pH decided to catalytic activity of the enzyme so their effect on DH and BT are clearly, this study result is the same to study of Tavano (2013).

Optimization and verification of the models

The algorism of fastened targets according to desirability methodology invented by Derringer, Suich (1980) was applied. Employed to optimize the process parameters of DH and BT of protein hydrolysis from spent brewer's yeast as follows: E/S ratio (alcalase of 7.5U/g and 9.99U/g), pH at 7.44, hydrolysis temperature at 51.29^{o}C, hydrolysis time of 8.82 hours. Under the optimal conditions, the corresponding response value predicted for the final DH of 61.71% and BT of 7.67 µmol quinie/l. The final DH, BT and combination have achieved of 99.81%, 100% and 99.90% desirability of proposed objectives, respectively (Fig. 4).

In order to confirm the predicted results, we selected hydrolysisconditions (ratio E/S of flavourzyme 10U/g, pH 7.5, 51^{o}C, 9 hours). Under these conditions, we carried out experiments (five times). The mean value of the maximum DH has reached 59.62% ± 0.027, the average value of BT reached 7.86 ± 0.033 µmol quinine/l (Table 4). There was a good coordination between the observed and the predicted values in models. DH in this study is higher than that in the study of Chae, Joo (2001), ie. DH of 48.3% obtained when the yeast cells were treated using mixture of 0.6% protamex and 0.6% flavourzyme.

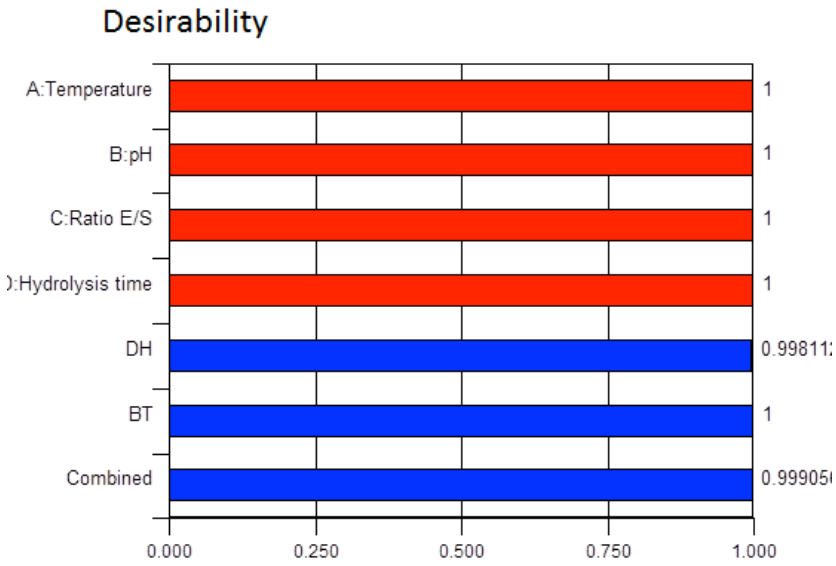

Figure 4. Responsible desirability level.

Table 4. The verifying results for compatibility of the model with experimental values

Number	Temperature (^{o}C)	pH	E/S ratio (U/g)	Time (hour)	DH (%)	BT (µmol/l)
According to equations	51	7.5	10.0	9	61.91	7.52
According to actual	51	7.5	10.0	9	59.62 ± 0.027	7.86 ± 0.033

CONCLUSION

The statistical experimental design using the response suface and desirability methodology to optimize the process paremeters of the continuous overflow proteolytic hydrolysis of spent brewer's yeast by using proteases. The optimal conditions determined were as follows: The E/S ratio (alcalase: 7.5 U/g and flavourzyme 10 U/g), pH 7.5, temperature 51^{o}C and hydrolysis time 9 hours. Under these optimized conditions, the experimental value of the final DH reached 59.62% ± 0.027 and biterness was 7.86±0.033 µmol quinine/L, which are closely corresponding with the predicted values. It indicated that the models are satisfactory and accurate.

Acknowledgement: *We thank to School of Biotechnology and Food Technology, Hanoi University of Science and Technology and office's staffs of Polyco Ltd.Company.*

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TỐI ƯU HÓA QUÁ TRÌNH THỦY PHÂN BÃ NẤM MEN BIA BẰNG PHƯƠNG PHÁP LIÊN TỤC CHẢY TRÀN SỬ DỤNG PROTEASE

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TÓM TẮT

Bã nấm men là sản phẩm phụ được thu hồi từ ngành công nghiệp sản xuất bia thường được thải ra với khối lượng lớn và có hàm lượng protein cao (chiếm 50-55% lượng chất khô), tỷ lệ axit amin cân đối, cho nên việc tận dụng nguồn bã nấm men để sản xuất dịch thủy phân ứng dụng trong công nghệ thực phẩm là cần thiết và rất có ý nghĩa. Hiệu suất thủy phân bã men bia và hàm lượng amino acid trong dịch thủy phân phụ thuộc vào

nhiều yếu tố như nhiệt độ, pH, loại enzyme sử dụng và tỉ lệ enzyme/cơ chất, thời gian. Bên cạnh đó là phương pháp thủy phân (gián đoạn hay liên tục). Trong nghiên cứu này, với mục đích thủy phân bã men bia dùng làm thực phẩm ở quy mô công nghiệp, đã sử dụng phương pháp thủy phân liên tục chảy tràn. Vị đắng của dịch thủy phân và hiệu suất của quá trình là 2 yếu tố được quan tâm. Trong bào báo này, chúng tôi đề cập đến việc xác định các điều kiện tối ưu cho quá trình thủy phân protein đạt hiệu suất thủy phân lớn nhất và vị đắng dịch thủy phân nhỏ nhất .Đã sử dụng phương pháp bề mặt đáp ứng để xác định điều kiện tối ưu cho quá trình thủy phân gián đoạn nấm men ứng dụng trong công nghệ thực phẩm. Đã xác định được các yếu tố tối ưu như sau: tỷ lệ phối trộn hai enzyme (alcalase 7,5 U/g và flavourzyme 10 U/g), pH 7,5, nhiệt độ thủy phân 51^{o}C, thời gian thủy phân 9 giờ. Với điều kiện tối ưu đó, hiệu suất thủy phân và độ đắng của dịch thủy phân (được biểu diễn theo nồng độ quinine) lần lượt là: 59,62% ± 0,027 và 7,86 ± 0,033 µmol quinine/ml.

Từ khóa: *amino acid, bã nấm men bia, gián đoạn, hiệu suất thủy phân, thủy phân bằng enzyme, tối ưu hóa.,*