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Research paper

Optimisation of Enzymatic Hydrolysis Condition of Soybean (Glycine Max (L.) Merr.) Tempeh Protein Hydrolysate Using Response Surface Methodology (RSM)

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Abstract

The beneficial properties of overripe tempeh as a functional ingredient protein isolate are overlooked by most food manufacturers. The present study aims to optimise the enzymatic hydrolysis conditions to obtain tempeh protein hydrolysate (PH) that can be used as potential functional foods. The enzymatic hydrolysis (using Flavourzyme) conditions, namely, temperature (°C), enzyme to substrate concentration (%) and hydrolysis time (min) on both total flavonoid content (TFC) and glutamic acid content (GAC), as responses, were optimised using response surface methodology (RSM) by employing three factors, 3-level, and central composite rotatable design (CCRD). Enzyme inactivation was successfully performed by keeping the hydrolysate at 85°C in a water bath for 10 min. Based on the results, the optimum conditions for the hydrolysis of 6.0 g of soy protein isolate (SPI) from soybean tempeh were at temperature 55°C with 2.6% enzyme to substrate concentration heated for 128 min which resulted in 8.93 g QE/100 g DEW of TFC and 12.96 g/100 g DEW of GAC. The results also showed that TFC and GAC were significantly influenced by all the factors studied. Therefore, the results suggested that soybean by-product such as overripe tempeh can be converted into hydrolysate which is a good source of protein fortification of various food products as well as a potential functional food ingredient.

Keywords: *Enzymatic Hydrolysis, Glutamic Acid Content, RSM, Soybean Tempeh, Total Flavonoid Content*

1. Introduction

Tempeh (also spelt as tempe), an indigenous fermented food from Indonesia, was known since several centuries ago. Soy tempeh is a fantastic source of protein as compared to meat and animal milk, with no cholesterol and none of the saturated fat associated with health problems. Known as a good source of various nutrients, tempeh is an excellent probiotic food that supplies good amounts of magnesium, manganese, calcium and iron. The beneficial organisms present in tempeh are responsible for its numerous health benefits, notably in terms of digestion. Freshly made raw tempeh remains edible for a few days at room temperature [1] and can be extended for another few days by quick freezing (-15°C) and lyophilisation [2].

It is well known that proteins are important in food processing and food product development, as they are responsible for various functional properties that influence consumers' acceptability. Both animal and plant proteins are commercially used as functional ingredients. However, plant proteins are most abundantly found in the world since they have been tested as functional and nutritional ingredients in various food products [3]. Soy protein hydrolysates (PH) are known to possess various physiological activities, including hypolipidemic and hypocholesterolemic properties [4], antioxidant activity [5], reduction of blood pressure [6], improvement in both arterial compliance and endothelial function [7], insulin resistance and weight loss in obesity [8].

Previous studies have demonstrated that enzymatic hydrolysis is commonly used to produce soybean hydrolysates with biological activity [9]. Enzymatic proteolysis is helpful to release bioactive peptides encrypted in food proteins. According to Pacheco-Aguilar et al. [10], a series of smaller polypeptides produced by the controlled enzymatic hydrolysis of protein are able to improvise the functional characteristics of the hydrolysates for different applications. Enzymatic hydrolysis is influenced by several factors such as pH, temperature, time and enzyme concentration that cooperatively influence the enzyme activity thereby making the process more controllable [11].

Flavourzyme is a fungal protease/peptidase complex produced by submerged fermentation of a selected strain of *Aspergillus oryzae* which has not been genetically modified and suitable for hydrolysis of proteins under neutral or slightly acidic conditions. Flavourzyme has been used to produce a protein hydrolysate with acceptable functional properties [11]. Generally, there are several controlled variables during the hydrolysis process namely temperature, time, pH level and enzyme concentration [12]. In order to obtain the optimum hydrolysis, conditions with the targeted responses should be conducted. Hence, the objective of the study was to optimise the enzymatic hydrolysis condition of protein hydrolysate derived from soy protein isolate (SPI) of tempeh by using Flavourzyme.

2. Materials and Methods

2.1. Materials

Soybean (*Glycine max (L.) Merr*.) was purchased from Country Organic Farm in Selangor, Malaysia. Tempeh inoculum in powder form (*Rhizopus oligosporus*) was bought from Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. Flavourzyme from *Aspergillus oryzae*, a food grade enzyme used in this study was purchased from Novo Nordisk's Enzyme Business in Wuxi, China with an activity of 500 LAPU/g. Analytical grade solvents/chemicals used were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China) and Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Production of tempeh

The tempeh was produced in a clean and controlled environment. Soybean was weighed and pre-washed before being soaked in tap water for 18 hours (1:3 v/v). After soaking and manual dehulling, the legume was boiled for 30 min, followed by draining and cooling to room temperature. The legumes were then inoculated with tempeh starter mould (*Rhizopus oligosporus*) (2 g/kg dry legume). Next, the inoculated legumes were packed in perforated polyethene bags of 18.45 X 26.40 cm and incubated at 30^{o}C in an incubator chamber (Model KBF 115, Binder GmbH, Tuttlingen, Germany).

2.3. Sample preparation

Tempeh stored at day 3 (72 hours) was submerged into liquid nitrogen before grinding. For tempeh flour, tempeh was ground using a blender, sifted using 100 mm mesh size and kept frozen at -20^{o}C in polythene bags until further analysis. Defatted flour was prepared by immersing in n-hexane with continuous stirring at 300 rpm for 30 min. The filtered defatted sample was air dried overnight in a cabinet dryer. Next, the SPI from defatted tempeh flour was prepared according to the method described by Chang-Qing and Hai-We [13].

2.4. Optimisation of soybean tempeh protein hydrolysate (PH) production

Enzymatic hydrolysis was carried out based on XiangZhen et al. method [14] with some modifications.

Experimental design: Table 1 shows the symbol and coded factor levels for the independent variables and Table 2 shows the nine experimental combinations which were generated from Design Expert software Version 8.0.1.0 (Stat-Ease Inc., Minneapolis, USA). Factors considered in this study and their levels were based on information from literature and preliminary laboratory investigations of numerous protein hydrolysates research. Experimental data were fitted with statistical models to produce the response surface model. These models were deemed suitable when it is significantly based on ANOVA, insignificant lack of fit and R^2 of more than 0.75. The chosen models were subsequently optimised based on the optimisation criteria of optimum temperature, E/S ratio and hydrolysis time while TFC and GAC were set for maximum.

Glutamic acid analysis (GAC): The GAC was determined by using L-Glutamic Acid assay kit (K-GLUT) from Megazyme (Megazyme International Ireland, 2015).

Total flavonoid content (TFC): The TFC was determined using the method by Kim et al. [15].Distilled water (4 ml) was added to one ml of PH extract. Then, 5% sodium nitrite solution(0.3 ml) was added, follow by 10% aluminum chloride solution (0.3 ml). Test tubes were incubated at ambient temperature for 5 minutes, and then 2 ml of 1M sodium hydroxide was added to the mixture and then the volume of reaction mixture was made up to 10 ml with distilled water. The mixture was thoroughly vortexed and the absorbance of the pink colour developed was determined at 510 nm. A calibration curve was prepared with catechin and the results was expressed as mg quercetin equivalents (QE)/100 g.

Table 1: Coded and uncoded levels of independent variables for thermal treatment experimental design

Independent variables		Coded and uncoded levels				
Symbols	Factors	-α -1.682	-1	0	+1	+α +1.682
X_1	Temperature (°C)	30	40	55	70	80
X_2	Reaction Time (min)	20	60	120	180	220
X_3	Enzyme/Substrate Ratio (%)	0	1	2	3	4

Table 2: Actual and coded levels of temperature (X_1), hydrolysis time (X_2) and E/S ratio (X_3) used for optimisation of SPI from tempeh

Run	Temperature (°C) X_1	Reaction Time (min) X_2	Enzyme/Substrate Ratio (%) X_3
1	40.00 (-1.000)	60.00 (-1.000)	1.00 (-1.000)
2	70.00 (1.000)	60.00 (-1.000)	1.00 (-1.000)
3	40.00 (-1.000)	180.00 (1.000)	1.00 (-1.000)
4	70.00 (1.000)	180.00 (1.000)	1.00 (-1.000)
5	40.00 (-1.000)	60.00 (-1.000)	3.00 (1.000)
6	70.00 (1.000)	60.00 (-1.000)	3.00 (1.000)
7	40.00 (-1.000)	180.00 (1.000)	3.00 (1.000)
8	70.00 (1.000)	180.00 (1.000)	3.00 (1.000)
9	30.00 (-1.682)	120.00 (0.000)	2.00 (0.000)
10	80.00 (1.682)	120.00 (0.000)	2.00 (0.000)
11	55.00 (0.000)	20.00 (-1.682)	2.00 (0.000)
12	55.00 (0.000)	220.00 (1.682)	2.00 (0.000)
13	55.00 (0.000)	120.00 (0.000)	0.31 (-1.682)
14	55.00 (0.000)	120.00 (0.000)	3.68 (1.682)
15	55.00 (0.000)	120.00 (0.000)	2.00(0.000)
16	55.00 (0.000)	120.00 (0.000)	2.00 (0.000)
17	55.00 (0.000)	120.00 (0.000)	2.00 (0.000)
18	55.00 (0.000)	120.00 (0.000)	2.00 (0.000)
19	55.00 (0.000)	120.00 (0.000)	2.00 (0.000)
20	55.00 (0.000)	120.00 (0.000)	2.00 (0.000)

*Data Analysis: D*ata were tabulated and analysed using Statistical Analytical System (SAS) Version 6.12 for ANOVA test and DUNCAN. All experiments were done in triplicates. The optimisation was carried out using Design Expert Software Version 6.0.10 (StatEase 2003). Validation was performed using Root Mean Squared Deviation (RMSD).

2.5. Statistical Analysis

The data were tabulated and analysed using SAS software (Version 9). All data were presented as a mean value with their standard deviation indicated (mean ± SD). Analysis of variance (ANOVA) was performed with a confidence interval of 95% ($p<0.05$).

3. Results and Discussions

The TFC and GAC results for enzymatic hydrolysis of the SPI from tempeh using central composite rotatable design (CCRD) are shown in Table 3. The TFC and GAC experimental data were fitted using linear, quadratic and cubic models. Statistical results suggested that the quadratic model was the best fitting model for both TFC and GAC.

Table 3: Actual levels of independent variables along with the observed values for TFC and GAC

Run	Temperature (°C) X_1	Reaction Time (min), X_2	Enzyme/Substrate Ratio (%), X_3	TFC (g QE/100g)	GAC (g/100 g)
1	40.00	60.00	1.00	7.14	11.21
2	70.00	60.00	1.00	5.31	10.97
3	40.00	180.00	1.00	7.37	11.41
4	70.00	180.00	1.00	4.97	1097
5	40.00	60.00	3.00	7.15	11.35
6	70.00	60.00	3.00	5.82	11.04
7	40.00	180.00	3.00	7.56	11.74
8	70.00	180.00	3.00	5.26	10.97
9	30.00	120.00	2.00	6.52	11.26
10	80.00	120.00	2.00	4.61	10.95
11	55.00	20.00	2.00	5.21	10.97
12	55.00	220.00	2.00	7.92	12.10
13	55.00	120.00	0.31	7.08	10.27
14	55.00	120.00	3.68	8.66	12.44
15*	55.00	120.00	2.00	8.93	13.41
16*	55.00	120.00	2.00	8.85	13.39
17*	55.00	120.00	2.00	8.84	13.56
18*	55.00	120.00	2.00	8.77	13.48
19*	55.00	120.00	2.00	8.60	13.45
20*	55.00	120.00	2.00	8.79	13.60

*replication of the centre point

The response surface equation for the fitting of TFC and GAC data based on the quadratic models are shown in Table 4. According to the ANOVA, both models were significant. The R^2 values for both models were higher than 0.75, indicating a good fit. The R^2 values for TFC and GAC were 0.9946 and 0.9987, respectively. The lack-of-fit test was not significant for both TFC and GAC which also showed a good fit between the experimental data and the model.

Table 4: Model equations fitted for TFC and GAC experimental data for hydrolysis of SPI with Flavourzyme

Responses	Model equation	Model significance	Lack of fit	R^2
TFC	Actual equation: $+244.73039 - 7.04160X_1 - 2.08596X_2 - 29.20225X_3 + 0.084541X_{12} + 1.72384X_{13} - 0.0134X_{23} + 0.051830X_{11} + (1.93883 \times 10^{-3})X_{22} - 3.74044X_{33} - 2.45633X_{123} - (6.19028 \times 10^{-4})X_{112} - 0.015197X_{113} - (7.74450 \times 10^{-5})X_{122}$ Coded equation : $+88.08 - 8.11X_1 + 3.16X_2 + 2.67X_3 - 1.92X_{12} + 0.78X_{13} - 2.352X_{23} - 0.058X_{11} - 11.89X_{22} - 8.35X_{33} - 1.112X_{123} - 3.74X_{112} - 2.13X_{113} - 1.21X_{122}$	< 0.0001 (Significant)	0.0650 (Not significant)	0.9946
GAC	Actual equation: $-4.86676 + 0.41812X_1 + 0.053484X_2 + 3.47296X_3 - (9.26806 \times 10^{-5})X_{12} - (3.40417 \times 10^{-3})X_{13} + (2.70625 \times 10^{-4})X_{23} - (3.7394 \times 10^{-3})X_{11} - (1.91552 \times 10^{-4})X_{22} - 0.75293X_{33} - 0.1224X_{123} - 0.87862X_{112} - 0.2026X_{113} - 0.01621X_{122}$ Coded equation : $+13.49 - 0.091X_1 + 0.33X_2 + 0.65X_3 - 0.083X_{12} - 0.051X_{13} + 0.016X_{23} - 0.84X_{11} - 0.69X_{22} - 0.75X_{33} - 0.032X_{123} - 0.27X_{112} - 0.58X_{113} - 0.13X_{122}$	< 0.0001 (Significant)	0.8808 (Not significant)	0.9987

Note: X_1 = temperature (°C); X_2 = hydrolysis time (min); X_3 = enzyme/substrate ratio (%)

Analysis of coefficients for each model used to fit the data of TFC and GAC are shown in Table 5. The results showed that among the independent variables, temperature and hydrolysis time displayed a significant effect ($p<0.05$) for both TFC and GAC. However, the enzyme/substrate ratio did not show any significant effect on both TFC and GAC. For the interaction variables, a coefficient model for TFC showed significance ($p<0.05$) for X_{11}, X_{33}, X_{12}, X_{112} and X_{122} while the coefficient model for GAC showed significance for X_{12}, X_{13}, X_{23}, X_{113} and X_{122}.

In Table 5, the interaction between temperature (X_1) and hydrolysis time (X_2) was shown to be significant ($p<0.05$). Figure 1 shows the response surface for the interaction between temperature and time for TFC. It can be observed that TFC decreased when the temperature was increased at each hydrolysis time. The decreased in TFC might be due to the conversion or release of glucosides or bound phenolics into free phenolic derivatives during hydrolysis with the enzyme [19]. Kavita et al. [20] proved that heating had a positive effect on the flavonoids. However, exposure at high heat with longer time will reduce the flavonoids content. At a lower temperature, only a slight increase in TFC was produced by increasing the hydrolysis time. However, at a higher temperature, increase in hydrolysis time resulted in a decrease in TFC value. These results showed that temperature has more influence on TFC than GAC during hydrolysis. Increasing both temperature and hydrolysis time produced a decrease in TFC as suggested by the negative value of the coefficient (-1.920) (Table 5).

Table 5: Analysis of coefficients for models used to fit TFC and GAC experimental data for hydrolysis of SPI with Flavourzyme

Independent variables	TFC			GAC		
	Coefficient	F	Prob < F	Coefficient	F	Prob < F
Temperature (°C), X_1	-8.110	27.45	0.0008*	-0.091	8.66	0.0258*
Hydrolysis Time (min), X_2	+3.160	45.24	<0.0001*	+0.330	116.40	<0.0001*
Enzyme/Substrate Ratio (%), X_3	+2.670	5.49	0.0471	+0.650	432.21	<0.0001*
Interactions						
X_{11}	-0.058	18.61	<0.0001*	-0.840	1871.02	0.0187
X_{22}	-11.890	4.01	0.0058	-0.690	1256.81	0.0981
X_{33}	-8.350	60.53	<0.0001*	-0.750	1498.32	0.5565
X_{12}	-1.920	92.89	<0.0001*	-0.083	10.21	<0.0001*
X_{13}	+0.780	13.90	0.0058	-0.051	3.83	<0.00

X_{23}	- 2.350	5.68	0.0062	+0.016	0.39	01* <0.00
X_{112}	-3.740	78.66	<0.000 1*	-0.270	44.02	01* 0.264 0
X_{113}	-2.130	13.17	0.0067	-0.580	203.46	0.000 6*
X_{122}	-1.210	19.70	0.0022 *	-0.130	10.16	<0.00 01*
X_{123}	- 0.122	11.23	0.0052	-0.032	1.52	0.018 9

*Significant p<0.05

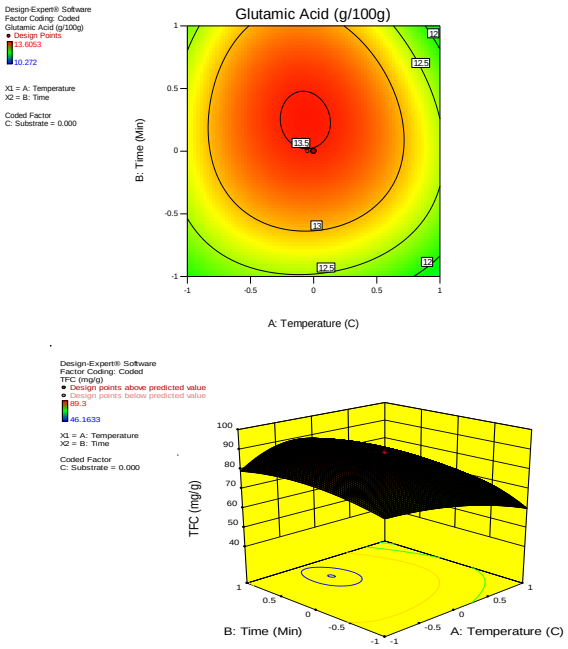

Fig. 1: Response surface for TFC as a function of time (min) and temperature (°C) during hydrolysis of SPI from tempeh using Flavourzyme

The effect of reaction time and temperature of reaction on TFC can be clearly seen in Figure 2 based on the 2D contour plots. The increment towards the red colour represents the high value of TFC, while the lower temperature with increase in the hydrolysis time leads to an increase of TFC. The predicted values calculated from actual or coded equation of TFC were in a strong agreement with the experimental values as displayed in Figure 3 where all the points are located close to the regression line. Hence, this quadratic model is well suited for this experimental setup.

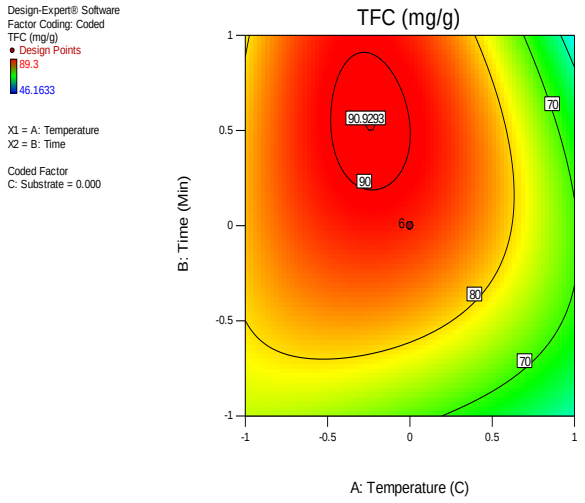

Fig. 2: 2D contour plots represent the effect of reaction time and temperature on TFC

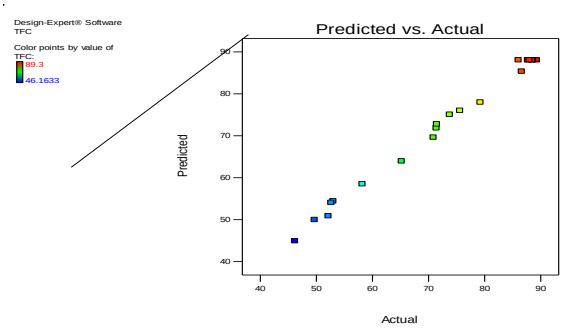

Fig. 3: Experimental TFC vs. the predicted TFC under optimum extraction

Figure 4 shows the GAC as a function of hydrolysis time and temperature for the hydrolysis of SPI using Flavourzyme. At a lower hydrolysis time, GAC value did not change drastically when the temperature was increased. However, higher hydrolysis time with increasing temperature resulted in a decrease of GAC. Similar to TFC, the decrease in GAC may be due to the loss of protein caused by the deamination process [21]. GAC value did not show any appreciable change when hydrolysis time was increased at a higher temperature. But, at a lower temperature, an increase in GAC can be observed with an increase in hydrolysis time. This happened because higher hydrolysis time allows extensive hydrolysis to occur resulting in a higher GAC. Increasing both hydrolysis time and temperature produced a decreasing GAC as projected by the negative value of the coefficient (-0.083) (Table 5.0).

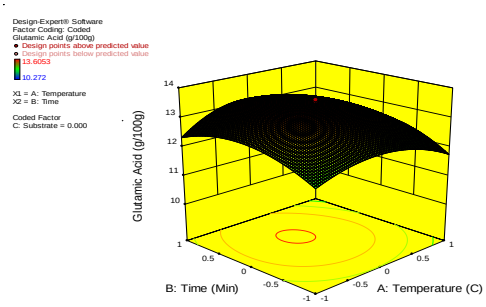

Fig. 4: Response surface for GAC as a function of time (min) and temperature (°C) during hydrolysis of SPI from tempeh using Flavourzyme

As seen in Figure 5, a 2D contour plot suggests the hydrolysis time and the temperature should not be too high or too low in order to maximize the GAC value. Figure 6 shows that the predicted values were very close to the actual values. As a proof, the high value of R^2 (0.9987) confirmed the true behaviour of the system which was clearly defined by the regression model. In addition, the suitability of the rendered model was supported by the closeness of adjusted-R^2 (0.9958) to presenting a high degree of correlation between the experimental and predicted values. The small E value (0.62%) suggested that the obtained model was acceptable.

The optimum point was determined based on the highest desirability to the responses. The analysis indicated that maximum TFC and GAC for hydrolysis of SPI by enzymatic hydrolysis can be achieved by using a 2.6% enzyme/substrate ratio at a temperature of 55°C and hydrolysis time of 128 min. From the optimisation study, TFC and GAC predicted were 90.633 mg QE/g and 13.64 g/100 g, respectively, with a desirability value of 0.991. Experimental runs in triplicate of the optimum point were carried out and the TFC and GAC values were compared with the predicted values for validation. Experimental results showed values of TFC at 8.93 ± 0.35 g QE/100 g and GAC at 12.96 ± 0.74 g/100 g. The results showed only a small RMSD value detected between the experimental and predicted TFC (1.313) and GAC (0.980) indicating the validity of the model. The low values of RMSD showed that the model satisfactorily predicted the response.

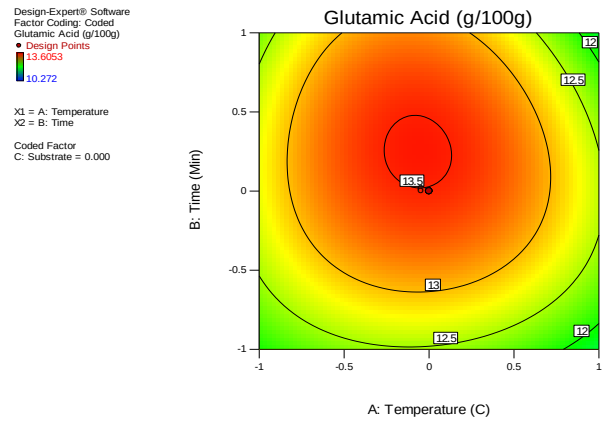

Fig. 5: 2D contour plots represent the effect of reaction time and temperature on GAC

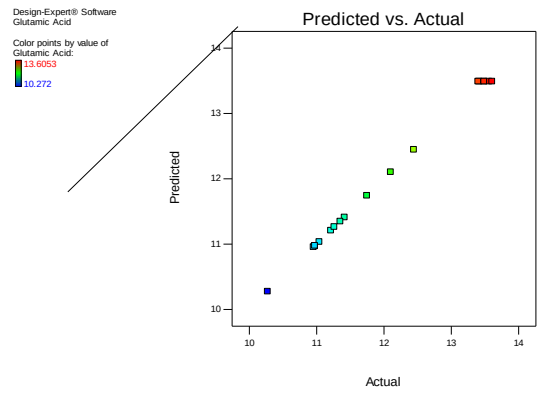

Fig. 6: Experimental GAC vs. the predicted GAC under optimum extraction conditions

4. Conclusion

Based on the results, enzymatic hydrolysis of SPI by using Flavourzyme was carried out at the optimum conditions suggested by the RSM. Therefore, the optimum hydrolysis conditions for 6.0 g of SPI from tempeh was at the temperature of 55°C and the hydrolysis time of 128 min by using 2.6% enzyme/substrate ratio. The TFC and GAC were significantly influenced by these two variables; the hydrolysis time and temperature of the process condition.

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