 DOI: 10.35382/TVUJS.14.1.2024.3265

STUDY ON MODIFICATION OF RED PUMPKIN (*Cucurbita moschata*) STARCH TO CREATE RESISTANT STARCH USING ENZYME

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Abstract – *This study aims to determine the parameters of the enzymatic hydrolysis process of red pumpkin starch to increase the resistant starch content in the material. The liquefaction stage was designed with two factors: α-amylase concentration and different times. The results of the α-amylase liquefaction process showed that the highest reducing sugar content was 14.92% at an α-amylase concentration of 11 U/g and time of 50 minutes, with a fixed starch: water ratio of 1:4. The saccharification experiment was carried out based on a multivariate model using the Central Composite Design method and yielded expected results, with variance analysis in the regression model showing significance ($p < 0.05$). The reliability of the model $R^2 = 0.997$ showed that the regression model was suitable for the dataset with 99.7% accuracy. CV = 2.08% indicated that the accuracy and reliability of the experiments were good. At a glucoamylase concentration of 112.424 U/ml, a temperature of 48.590^oC, and a time of 179.732 minutes, the highest resistant starch content reached 35.968%. Solubility and swelling increased with temperature: at 90^oC, the solubility of native starch and modified starch were 12.12% and 68.85%, respectively, while swelling of native starch was 6.77%, and modified starch achieved 3.39%. The transparency of native starch was 5.37% T, while modified starch achieved 2.17% T.*

Keywords: α-amylase, Cucurbita moschata, glucoamylase, pumpkin starch, resistant starch, starch modifications.

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Received date: 18^h December 2023; Revised date: 07^h March 2024; Accepted date: 28^h March 2024

I. INTRODUCTION

The prevalence of obesity poses serious health consequences. In developing countries, including Vietnam, this trend has been rapidly increasing in recent years. Notably, the obesity rate among women is four times higher than among men [1]. The fundamental cause of obesity lies in the energy imbalance between calorie intake and expenditure. Our diets often contain an excessive proportion of rapidly digestible starch, which contributes to this health issue.

To mitigate this problem, many people are interested in products containing '*slowly digestible carbohydrates*' – specifically, those rich in resistant starch (RS). According to nutritional purposes, starch in food can be categorized into three types: RDS (rapidly digestible starch), SDS (slowly digestible starch), and RS (resistant starch) [2]. Among these, RS stands out as a type of starch that resists digestion by pancreatic amylase enzymes. Instead, it undergoes fermentation by gut microbiota, producing short-chain fatty acids beneficial for intestinal health. RS plays a crucial role in preventing fat accumulation, reducing the risk of diabetes, and cardiovascular diseases [3], and providing positive effects on the digestive system, particularly the colon [4, 5]. Additionally, RS has been associated with lowered blood cholesterol levels and reduced postmeal glucose. Previous studies even suggest that RS supplementation increases satiety, leading to reduced caloric intake and potentially aiding in weight management [6–8].

Recent studies have explored the production of RS from various sources such as cereals, fruits, and legumes [9, 10]. Red pumpkin (*Cucurbita moschata*) is a promising source of starch, containing over 60% starch. However, there is limited research on modifying red pumpkin starch to

produce RS. Currently, there are many methods to modify starch to create RS, such as physical methods (heat-moisture, moisture-heat, recrystallization, polymer coating, extrusion), enzymatic methods (pullulanase, isoamylase, α–amylase, glucoamylase or transglucosidase), and chemical methods (acid method, cross-linking, oxidation, substitution method, esterification and etherification). However, each method has its advantages and disadvantages. In this study, the enzymatic treatment method was chosen because it is an environmentally friendly biological method, that does not affect human health but has high efficiency. The activity of α-amylase on starch molecules is to randomly cut the α-1,4 glycosidic bonds of the starch chain. Glucoamylase cuts effectively on the α-(1,6)-glycosidic bonds found at the branching points on the amylopectin molecule. The combination of amylase and glucoamylase enzymes aims to cut long-chain amylopectin and amylose molecules into a large amount of short-chain amylose, increasing the amount of resistant starch. The purpose of the research is to investigate the conditions for hydrolysis of starch using α-amylase and glucoamylase to increase the resistant starch content in the material source. This contributes to applications in the field of food processing, resulting in products beneficial for human health.

II. LITERATURE REVIEW

Recent studies have explored the production of RS from various sources such as cereals, fruits, rice, and legumes. Doan Thu Thuong et al. [11] have validated the method of determining resistant starch content according to AOAC 2002.02 method. The method has been applied to analyze 36 real samples, and the results show that the content of resistant starch varies, ranging from 1–42% depending on the origin, structural characteristics, and the way starch is modified. Roman et al. [12] studied to improve the properties of bread by changing the RS starch composition. Bread is categorized as having a high amount of rapidly digested starch that may result in a rapid increase in postprandial blood glucose and, therefore, poor health outcomes. This is mostly the result of the complete gelatinization that starch undergoes during baking. The inclusion of RS ingredients in bread formulas is gaining prominence, yet many RS ingredients contain RS structures that do not resist baking and, therefore, are not suitable to result in a meaningful RS increase in the final product. In 2022, Shen et al. [13] studied RS in rice because RS starch is receiving more and more attention. This review focuses mainly on the genetic regulation of RS content and discusses the emerging genetic and molecular mechanisms of RS formation in rice. The formation of RS is influenced by the intrinsic properties of starch and non-starch components, as well as storage and processing conditions.

Nguyen Thi Mai Huong et al. [9] studied the enrichment of RS from mung bean starch using pullulanase. The results showed that the optimal sample was obtained at the condition of 17.5 hours hydrolysis time, 45 U/g pullulanase concentration, and 1:20 starch: water ratio, which resulted in the highest level of RS with 60.98%. In the study by Zhang et al. [10], the pullulanase enzyme was used to hydrolyze corn starch to produce RS starch. The results showed that at 32 hours, pH 5.0, temperature of 46^{o}C, and pullulanase concentration of 12 ASPU/g, the highest RS starch content was 44.7%. Pongjanta et al. [14] obtained similar results by using pullulanase to hydrolyze rice starch to produce RS type III. The highest RS starch content was achieved at a pullulanase concentration of 12 U/g, with a value of 19.81%.

In several studies, different concentrations of α-amylase and glucoamylase enzymes were used to hydrolyze various sources of starch. For instance, when rice bran starch was hydrolyzed with 1.17% α-amylase concentration and a hydrolysis time of 13.36 minutes, the lowest viscosity of 14.82 cP and the highest total soluble solids of 13.37^{o}Brix were achieved. Similarly, when glucoamylase concentration was 1%, the temperature was 73.85^{o}C, and the hydrolysis time was 137.52 minutes, the highest reducing sugar content of 9.52% was obtained [15]. On the

other hand, sweet potato starch with α-amylase concentration of 0.03% and hydrolysis time of 2 hours resulted in the highest reducing sugar content of 27.8 mg/mL, while glucoamylase concentration of 0.075% and hydrolysis time of 80 hours produced the highest reducing sugar content of 61.02 mg/mL [16].

III. RESEARCH METHODS

A. Materials

Red pumpkin (*Cucurbita moschata*): Selected yellow-orange-skinned pumpkins with a diameter of 10 – 15cm, flesh thickness of approximately 3.5 cm, and a weight of 2.2 – 2.5 kg were purchased from Ba Thu agent at Tra Vinh market. α-amylase (thermostable α-amylase) from Novozyme: Publicized activity of 120 KNU-S/g (KNU is the amount of enzyme which breaks down 5.26 g starch), liquid form, viscosity of 1-25(cPs), recommended pH 5.5 – 6.5, t_{opt} 85 – 90^{o}C, source from *Bacillus licheniformis*. Glucoamylase, Oxford lab - India (buy at Phuong-Tram Agricultural Product & Chemical Trading Co. Ltd, Vietnam), liquid, brown, pH 3.0 – 5.5, recommended temperature 40–70^{o}C (t_{opt} 60–65^{o}C), publicized activity of 300U/ml, source from *Aspergillus sp.* 3,5-dinitrosalicylic acid (DNS), KOH, CH_3COOH, CH_3COONa, NaOH, D-glucose, $KNaC_4H_4O_6.4H_2O$ from China.

B. Methods

The process of creating starch from red pumpkin involves three steps. The first step is to determine the composition of pumpkin starch. Red pumpkin material was extracted by peeling, washing, grinding with water at a ratio of 1:2, filtering (a mixture mixed with water at a ratio of 1:4 to reduce the viscosity of the filtrate), drying at room temperature for 12 hours, then dring at 50^{o}C until the moisture reached 10 – 12%, ground finely (sieve size $\geq$ 35 mesh) to obtain pumpkin starch. The second step refers to the starch liquefaction stage. The red pumpkin starch was mixed with water in a 1:4 ratio and gelatinised at a temperature of 85^{o}C for 15 minutes, according to research by Zhang et al. [17]. The liquefied mixture was treated with α-amylase concentrations ranging from 7 to 13 U/g for varying durations (30 – 70 minutes). The mixture was then cooled down to continue the saccharification. The third step mentions the saccharification process. A multivariate model is employed in this process.

The experimental design follows a central composite design with three factors and five levels for each factor. The experiments include repeated units at factorial points and center points, resulting in 16 factorial points and 12 center points (with $\alpha = \pm 1.5$). The values '0', '+1' and '-1' represent the center value, upper boundary value, and lower boundary value, respectively. The values '+α' and '-α' correspond to extreme values above and below the center, known as 'star points' for the considered variables (Table 1). Data is encoded for three independent factors: glucoamylase enzyme concentration (X_1), hydrolysis temperature (X_2), and hydrolysis time (X_3), with the maximum RS content (Y_{max}) as the dependent response.

The proposed polynomial regression equation is applied to model the process (1):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{n} \beta_{ij} X_i X_j + e \quad (1)$$

Where: Y is the dependent variable, β_o is the intercept coefficient, β_i is the coefficient of the quadratic equation, β_{ii} is the coefficient of the quadratic equation of the variable X_i, and β_{ij} is the interaction coefficient and e is the random error.

Table 1: Data coding for saccharification experiments according to CCD

Coded symbols	Independent variable	Units	Levels				
			-1.5	-1	0	1	+1.5
X_1	Enzyme	U/ml	45	60	90	120	135
X_2	Temperature	°C	35	40	50	60	65
X_3	Time	minute	30	60	120	180	210

The mathematical model calculates the optimal parameters for the modification conditions. The optimal starch sample will then be evaluated for RS content and compared with the actual sample

under the same conditions. The RS content in these research samples is determined using the Englyst et al. [2], modified by Juansang et al. [18]. The formula for determining SDS content is as follows:

$$RSD = (G_{20}*100)/TG$$

$$SDS = (G_{120} - G_{20})*100/TG$$

$$RS = 100 - (RSD + SDS)$$

In which: G_{20} is the glucose content after hydrolysis 20 minutes, G_{120} is the glucose content after hydrolysis 120 minutes, and TG is the total glucose content.

The solubility of starch was calculated according to Schoch et al. [19]:

$$\text{Solubility (\%)} = \frac{m_2 - m_1}{m} * 100$$

In which, m: mass of starch analyzed, m1: mass of container before drying, m2: mass of container after drying.

The swelling degree of starch was calculated according to Hung et al. [20]:

$$\text{Swelling (g/g)} = \frac{m_2 - m_0}{m_1}$$

In which, m_o: mass of centrifuge tube, m_1: mass of starch for analysis, m_2: mass of sample after treatment.

Determination of the clarity (%T) was carried out according to the method of Reddy et al. [21]: 0.05 g of modified starch dissolved in 5 ml of distilled water in a test tube. The mixture is heated at 100^{o}C for 0.5 to 1 hour, stirring the mixture every 5 minutes with a Vortex machine. The clarity of the mixture was determined through the transmittance (%T) measured using a spectrophotometer (Genesys 20, Thermo Scientific–USA) absorbing at a wavelength of 650 nm, along with a control sample of distilled water.

C. Statistical analysis

The results were processed using statistical software such as SPSS Statistics 20 IBM and Excel 2017. The optimization experiments were processed using Design-Expert 11.0.4.0 from Stat-Ease.

IV. RESULTS AND DISCUSSION

A. Composition of red pumpkin starch

The starch sample was dried to a moisture content of approximately 10 – 12%, ground finely (sieve size $\geq$ 35 mesh), and analyzed for chemical composition (Table 2). The moisture content of 11.26% is considered low and suitable for storage standards (< 13%). According to Zhu et al. [22], the ash content of the raw material is approximately 0.2%, which is considered sufficient for extraction purposes.

Table 2: Composition of pumpkin starch

Humidity (%)	Ash (%)	Lipid (%)	Protein (%)
11.26 ±0.007	0.21 ±0.011	3.32±0.068	10.65±0.10

The chemical composition of red pumpkin starch contains a relatively high proportion of lipids and proteins. This indicates that the starch content of the sample is 85.8%, which is lower than that obtained using the potato extraction method by Pezez et al. [23]. The high lipid and protein content also affects the RS content due to the amylo-lipid complex containing hydrophobic fatty acid tails located inside the amylose helix. The formation of the amylo-lipid complex impedes the swelling of the starch granules and creates bonds between starch polymers [24]. Protein interferes with the combination of starch molecules, and according to Carvalho et al. [25], protein can cause the degradation of amylose-rich starch.

B. Effect of α-amylase concentration and hydrolysis time on DE

The results show that there is an interaction between α-amylase concentration and hydrolysis time in reducing sugar content ($p < 0.05$). When the α-amylase concentration increased from 7 U/g to 13 U/g with a hydrolysis time of 30 and 50 minutes, the reducing sugar content increased gradually.

If the duration is extended to 60 and 70 minutes, the reducing sugar tends to decrease. At an α-amylase concentration of 11 U/g after 50 minutes, the highest reducing sugar content

Table 3: Effect of α-amylase concentration and hydrolysis time on DE (%)

α-amylase (U/g)	Time (minutes)				
	30	40	50	60	70
7	3.74^{aA}±0.07	4.40^{aB}±0.07	8.94^{aC}±0.12	9.14^{aD}±0.07	7.10^{aE}±0.07
9	4.40^{bA}±0.07	7.61^{bB}±0.07	12.38^{bC}±0.07	9.25^{aD}±0.07	8.51^{bE}±0.07
11	12.30^{cA}±0.18	12.62^{cB}±0.07	14.92^{cC}±0.23	13.30^{cD}±0.18	10.71^{cE}±0.18
13	12.46^{cA}±0.20	13.94^{dB}±0.18	14.67^{dC}±0.12	13.28^{cD}±0.20	10.11^{dE}±0.18

Note: Different letters in the same row and column represent statistically significant differences at the 0.05 level.

reached 14.92%. If the α-amylase concentration is increased, the reduced sugar content tends to decrease. This can be explained by the enzyme's kinetic equation: under suitable substrate concentration conditions, the reaction rate is linear with the enzyme concentration. However, when the enzyme concentration increases and the duration is prolonged to a limit, the reaction rate no longer increases because the substrate is gradually depleted during the reaction, leading to a decrease in sugar formation. These results are consistent with the enzyme kinetics theory of Nguyen Duc Luong et al. [26]

C. Effect of glucoamylase concentration, temperature, and hydrolysis time on RS production

The efficiency of starch hydrolysis by enzymes depends on several conditions, especially enzyme concentration, temperature, and hydrolysis time. The experimental matrix describing the process according to the Central Composite Design model yielded observed and predicted values as shown in Table 4 below.

The results show that the data is dispersed as shown in Figure 1, satisfying the conditions for running the optimization model.

The ANOVA shows that the model is significant with $p < 0.0001$ (Table 5). Lack of fit ($p > 0.05$) indicates that the model is a good fit for the data. The model's reliability, $R^2 = 0.9975$, and CV = 2.08%, indicate that the experiments were conducted with high accuracy and reliability.

Table 4: Experimental values and predicted values in modeling

No.	X_1	X_2	X_3	RS content (%)	
				Experimental	Predicted
2	60	40	60	18.71	18.60
11	120	40	180	34.13	34.83
7	120	60	60	15.92	16.32
9	60	40	180	12.05	12.08
5	60	60	60	21.94	21.64
16	120	60	180	35.74	35.79
4	120	40	60	16.71	16.64
1	60	40	60	18.71	18.60
3	120	40	60	16.08	16.64
8	120	60	60	16.89	16.32
15	120	60	180	35.74	35.79
13	60	60	180	16.72	16.41
14	60	60	180	16.72	16.41
6	60	60	60	21.94	21.64
12	120	40	180	35.05	34.83
10	60	40	180	12.05	12.08
19	135	50	120	21.41	21.08
20	135	50	120	21.41	21.08
27	90	50	210	32.53	32.81
24	90	65	120	28.94	29.34
18	45	50	120	7.59	8.02
21	90	35	120	26.62	26.33
17	45	50	120	7.59	8.02
22	90	35	120	26.62	26.33
25	90	50	30	22.33	23.09
28	90	50	210	33.16	32.81
23	90	65	120	28.94	29.34
26	90	50	30	23.56	23.09
32	90	50	120	29.74	29.17
30	90	50	120	29.74	29.17
29	90	50	120	28.94	29.17
31	90	50	120	28.40	29.17

The regression equation has the form:

$$Y = 29.1652 + 4.3554X_1 + 1.0023X_2 + 3.2406X_3 - 0.8412X_1X_2 + 6.1753X_1X_3 + 0.3218X_2X_3 - 6.4956X_1^2 - 0.5922X_2^2 - 0.5399X_3^2$$

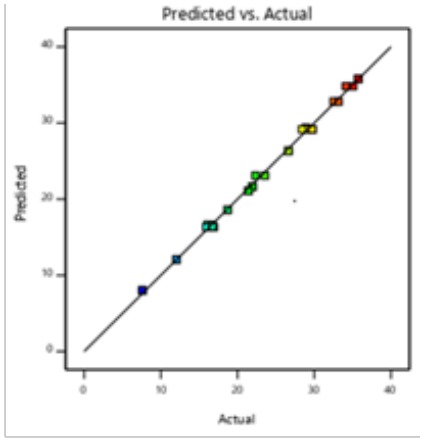

Fig. 1: Distribution of actual and predicted RS content from the model

Table 5: ANOVA analysis for RS content results

Source	SS[a]	df[b]	MS[c]	F-value	p-value	
Model	2046.95	9	227.44	979.92	< 0.0001	Significant
X1	474.23	1	474.23	2043.23	< 0.0001	
X2	25.11	1	25.11	108.21	< 0.0001	
X3	262.53	1	262.53	1131.10	< 0.0001	
X1 X2	11.32	1	11.32	48.78	< 0.0001	
X1 X3	610.14	1	610.14	2628.80	< 0.0001	
X2 X3	1.66	1	1.66	7.14	0.0139	
X_1^2	625.61	1	625.61	2695.45	< 0.0001	
X_2^2	5.20	1	5.20	22.40	0.0001	
X_3^2	4.32	1	4.32	18.62	0.0003	
Residual	5.11	22	0.2321			
Lack of Fit	1.77	5	0.3537	1.80	0.1662	Not significant
Pure Error	3.34	17	0.1963			
R^2	0.9975					
Adjusted R^2	0.9965					
Predicted R^2	0.9949					
Adequate Precision	103.1299					

Note: [a]sum of squares, [b]degree of freedom, [c]mean of squares

The results of the regression equation found by solving the equation in the model are only coded variables that receive values when $p < 0.05$, so it is necessary to convert them to real variables.

The regression equation on real variables has the form:

$$RS = -43.1659 + 1.1728A1 + 0.8804B2 - 0.2456C3 - 0.0028A1B2 + 0.0034A1C3 + 0.0005B2C3 - 0.0072A_1^2 - 0.0059B_2^2 - 0.00015C_3^2$$

Where:

A_1(U/g): Real variable of enzyme value

B_2(^oC): Real variable of temperature value

C_3(minute): Real variable of time value.

From the mathematical equation, we optimize the hydrolysis process with the desire to achieve the highest content of RS formed.

Table 6: Optimal condition results for three factors

Optimal conditions	
Temperture (^oC)	48.590
Time (minute)	179.732
Glucoamylase (U/ml)	112.424
RS (%)	35.968

By using the central composite design and response surface methodology, we have accurately determined the glucoamylase concentration, temperature, and processing time at which the RS content can be guaranteed to be the highest level (Figure 2).

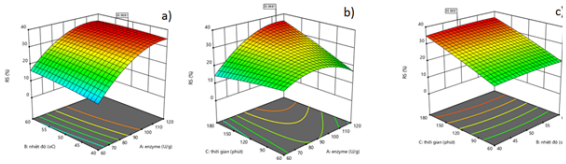

Fig. 2: Response surface plot
a) Impact of temperature and enzyme on RS content; b) Impact of time and enzymes on RS content; c) Impact of temperature and temperature on RS content

When sufficient substrate is available, the speed of the enzymatic reaction is proportional to the enzyme concentration. The greater the concentration of enzyme, the bigger the amount of substrate that is converted. There are also cases that when the enzyme concentration is too large, the reaction speed slows down according to Michaelis et al. [27]. This is because each enzyme only works best in a certain state, and this state depends on temperature, concentration, time, pH, and enzyme concentration [23]. Short hydrolysis time and low enzyme concentration do not allow enough contact between the substrate and enzyme, causing a low amount of reducing sugar content to be produced. If the time and enzyme concentration are increased, there is not enough substrate for the reaction. Therefore, when increasing the glucoamylase concentration to 112.424 U/ml, time of 179,732 minutes, and temperature of 48.590^{o}C, the RS content will reach the highest number with 35.968%.

Experimental verification

To verify the accuracy of the value obtained from the regression equation, three independent experiments were carried out under the conditions of glucoamylase concentration = 112 U/ml, temperature of 50^{o}C, and hydrolysis time of 180 minutes. The result shows that the average value of RS content in the verification experiment is 35.904%, while the theoretical value is 35.968%.

Table 7: Results RS content from regression equations and experiments

		Theoretical	Actual	Control
Variable	Temperature (°C)	48.590	50	
	Time (minute)	179.732	180	
	Glucoamylase (U/ml)	112.424	112	
Response	RS content, %	35.968^a	35.904^a ±0.002	19.130^b ±0.001

Note: ^{ab} with the same letter, there is no statistically significant difference at the 0.05 level.

However, no significant difference between these two samples was found ($p > 0.05$). The RS content of the natural sample was relatively low compared to the theoretical sample.

D. Effect of temperature on the solubility of starch and modified starch

The results show that the solubility of modified starch is significantly higher than that of native starch, and the solubility increases with temperature. At 90^{o}C, the solubility of modified starch is 68.85%, while the solubility of starch is 12.12%.

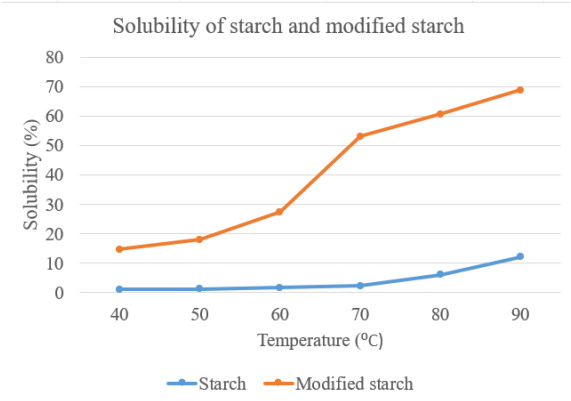

Fig. 3: Solubility of starch and modified starch

The increased solubility of modified starch is due to the production of short amylose chains from the hydrolysis process, which are easily dispersed and swollen out of the granules during gelatinization [28]. This result is similar to the study by Pham Van Hung et al. [1].

E. Effect of temperature on the gelatinization of starch and modified starch

In contrast to solubility, the gelatinization of modified starch is lower than that of starch. The amylose chains produced after branching by glucoamylase make a part of the structure of amylose and amylopectin in both crystalline and amorphous regions changed. The short-chain molecules in the starch structure that come out from a soluble phase reduce the proportion of the hydrated structure of the starch chain, thereby decreasing the degree of hydration and the ability of starch to swell [28].

Table 8: Effect of temperature on swelling of starch and modified starch

	Temperature (°C)				
	50	60	70	80	90
Starch	2.55[a]	2.83[b]	3.12[c]	4.15[d]	6.77[e]
	±0.03	±0.02	±0.02	±0.03	±0.01
Modified starch	2.24[a]	2.36[b]	2.59[c]	3.02[d]	3.39[e]
	±0.03	±0.04	±0.02	±0.02	±0.02

Note: Different letters in the same row represent significant differences at the 5% level.

F. Clarity of starch and modified starch

The results of Table 9 show that modified starch has lower transparency than starch. The hydrolysis process cuts long amylose and amylopectin chains into short chains, and the branched structures are cut into straight chains. Many straight chains create high cross-linking, which increases the turbidity of modified starch. Therefore, it reduces the light transmittance [29].

Table 9: Clarity of starch and modified starch

	Clarity (%T)
Starch	5.37±0.06
Modified starch	2.17±0.06

V. CONCLUSION

The process liquefaction with α-amylase concentration of 11 U/g and liquefaction time of 50 minutes gave the highest reducing sugar content of 14.92%. The saccharification stage was designed according to the Central Composite Design model to optimize the conditions for modifying butternut squash starch to achieve the expected results. The results were obtained at a glucoamylase concentration of 112.424 U/ml, a temperature of 48.590^{o}C, and a hydrolysis time of 179.732 minutes for the highest RS content of 35.968%. As the temperature increased, the solubility and swelling degree also increased. The solubility of modified starch is higher than that of native starch, and the swelling degree of modified starch is lower than that of native starch. According to the temperature survey, the solubility of modified starch increased from 14.62 to 68.85%, and that of native starch increased from 1.15 to 12.16%. The swelling degree of modified starch increased from 2.24 to 3.39%, and that of native starch increased from 2.55 to 6.77%. The transparency of modified starch is 2.17%, which is lower than that of native starch 5.37%T.

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