



Cellulase-Assisted Enhancement of Phenolics in Black Glutinous Rice Tape: An RSM Optimization Study

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Abstract

Black glutinous rice (BGR) *tape* is a traditional fermented product rich in phenolic compounds, which may provide health benefits. However, the dense aleurone layer and limited microbial activity during fermentation may leave a portion of bound phenolics. This study aimed to maximize the total phenolic content (TPC) in BGR *tape* by combining cellulase treatment with statistical optimization. Using response surface methodology (RSM), a two-factor central composite design (CCD) was employed to evaluate the effects of incubation time and enzyme concentration on TPC response (Folin-Ciocalteu, mg GAE/g sample). A quadratic model was developed and assessed through analysis of variance (ANOVA) and diagnostics. The ANOVA results indicated the model was significant, and the lack-of-fit was not significant. The findings showed that the most influential variables were the linear term of incubation time ($p = 0.0001$), followed by the quadratic term of incubation time ($p = 0.01667$), and then the linear term of enzyme concentration. The quadratic term of enzyme concentration and the interaction between factors had no significant effect. Response surface and contour plots revealed that longer incubation times increased TPC, though extended incubation eventually led to a decrease. RSM analysis identified an optimal fermentation time of 71.16 h and an enzyme concentration of 21.13 U/g of enzyme, predicting a TPC of 0.414 mg GAE/g with a desirability of 1. These results suggest that the optimal condition for optimizing TPC in BGR *tape* involves extended incubation periods and low to moderate enzyme concentrations. The model provides a quantitative basis for process optimization within the studied ranges. Overall, RSM proves to be an effective method for optimizing phenolic enhancement in BGR *tape* fermentation.

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Introduction

Research on natural ingredients high in bioactive substances such as phenolics and anthocyanins has increased due to growing interest in functional foods. The demand from consumers worldwide for meals that promote health has grown considerably in recent decades, encouraging the food industry to produce foods enhanced with naturally occurring bioactive substances. Phenolic compounds are one of the main bioactive compounds that are contained in functional foods for their health benefits (Bortolini et al., 2022; Foss et al., 2022). These compounds are widely studied for its antioxidant, anti-inflammatory, antidiabetic, and cardioprotective properties (Khoo et al., 2017; Sahu et al., 2023). In this regard, investigating phenolic-rich indigenous crops presents encouraging prospects for creating functional foods that are culturally appropriate.

Black glutinous rice (BGR, *Oryza sativa* var. *glutinosa*) is one such ingredient widely cultivated and

consumed in Asia that is recognized for its high anthocyanin and phenolic content. These bioactive compounds contribute to the dark-purple color in food matrices and possess a strong antioxidant capacity (Khoo et al., 2017). In Indonesia and Southeast Asian countries, BGR is commonly fermented into *tape*, a traditional sweet fermented food characterized by its soft texture, aromatic flavor, and mild alcoholic note (Kanino, 2019). The fermentation process, carried out by a mixed-culture starter (*ragi tape*) containing molds such as *Amylomyces rouxii*, *Mucor* spp., and *Rhizopus* spp., converts starch into sugars and generates secondary metabolites that improve nutritional and functional attributes (Gunam et al., 2021; Hermanto et al., 2021). As a cultural and traditional delicacy, BGR *tape* can be developed further as a functional food. During *tape* fermentation in BGR, an increase in free and free-conjugated phenolic compounds and alterations of phenolic composition, such as protocatechuic acid,

vanillic acid, vanillin, ferulic acid, caffeic acid, and isoferulic acid, were reported (Azkia et al., 2024). However, the extraction of the active components may be hindered by the dense bran layer. (Yang et al., 2022). Furthermore, the enhancement is limited since endogenous microbial enzymes might not completely break down the cell wall matrix, resulting in some bound phenolics remaining unreleased (Azkia et al., 2023).

To overcome this limitation, enzymatic treatment using cellulase has been proposed as a selective and environmentally friendly method to enhance phenolic recovery. Cellulase addition allows hydrolysis of β -1,4-glycosidic linkages in cellulose, degrading the cell wall and releasing the bound phenolics (Sapwarobol et al., 2021). Previous studies showed a significant increase in TPC and antioxidant activity on rice bran, fruit peels, and seed matrices with cellulase-assisted treatments (Prabhu and Jayadeep, 2015; Peixoto Araujo et al., 2019; Jiang et al., 2024). Similarly, Yang et al. (2022) reported that pre-gelatinization combined with cellulase treatment enhanced cellulase and glucoamylase activities in black rice wine fermentation, resulting in higher phenolic release. The addition of cellulase in conventional BGR *tape* fermentation has not yet been investigated, despite these findings.

In a previous study, the extraction of phenolics from rice bran by cellulase addition was conducted by the one-factor-at-a-time method, which varied the enzyme type, temperature, time, pH, and enzyme concentration (Martillanes et al., 2021). Despite its simple method and informative result, this method may overlook interactions or curvature between factors; it also requires numerous runs and may fail to achieve optimal conditions. In contrast, Response Surface Methodology (RSM) models main effects, interaction effects, and quadratic effects to predict responses across the design space, enabling efficient optimization. Within RSM, the Central Composite Design (CCD) is widely used due to its efficiency and supports rigorous model checks and response prediction (Myers et al., 2016; Fahimitabar et al., 2021).

This study examines and optimizes incubation time and cellulase concentration to enhance the phenolic recovery of BGR *tape* fermentation, using statistical RSM based on CCD.

Materials and Methods

Materials

The main ingredients utilized in this study were black glutinous rice (SariPedaas Ketan Hitam, THK Internasional, Indonesia), *ragi tape* acquired from Na Kok Liong (NKL, Surakarta, Indonesia), and distilled water. The *ragi tape* typically contains a consortium of microorganisms, including *Amylomyces* sp., *Mucor* sp.,

Saccharomyces sp., and *Candida* sp (Ninsix, 2013). The enzyme used in this study, cellulase sourced from *Aspergillus* sp. (C2605, 1000 units/g), was obtained from Sigma-Aldrich, USA. The chemicals used in this research, including methanol, analytical standards gallic acid, sodium carbonate, and Folin-Ciocalteu reagent, were sourced from Merck, Germany.

Sample Preparation

The preparation of BGR *tape* was conducted using a modified method of Azkia et al. (2023). BGR was weighed 100 g, washed, and soaked overnight (12 hours) in distilled water at a 2:3 (w/v) ratio. The rice was drained and steamed for 20 minutes, then mixed with 60 mL of boiling distilled water and steamed again for an additional 20 minutes. This process was repeated twice. The sample was aseptically transferred to a container, cooled, and inoculated with 0.3% (w/w) *ragi tape*. The experimental conditions, including cellulase addition and fermentation time, are outlined in Table 1 of the Central Composite Design. Finally, the fermented samples were collected and stored in a sealed, dark container at 4 °C for further analysis.

Free Phenolic Extraction

The extraction of free phenolics refers to the method of Azkia et al. (2023) with modifications. A total of a two-gram sample, containing both solid and liquid components from the *tape*, was weighed, crushed, and dissolved in 20 mL of a solvent (80:20 methanol-water). The mixture was subjected to ultrasound treatment (Pulse 150™ Ultrasonic Homogenizer, Benchmark Scientific, USA) at 30% power for 15 minutes, maintaining a temperature below 35°C. After the extraction, the supernatant was collected from the solid material using a centrifuge (Frontier™ 5000 Series Multi Ohaus FC5706, Shanghai) at 5500 rpm for 5 minutes, with the centrifuge operating at room temperature. The extract was concentrated in an oven (Memmert UN55, Germany) at 50°C. Subsequently, the extract volume was adjusted to 5 mL using fresh solvent and filtered through a 0.22 μ m nylon filter for analysis.

Analysis of Total Phenolic Content

The total phenolic content (TPC) was determined using a modified method of Putra et al. (2022). A total of 50 μ L sample extract was placed into a cuvette, followed by the addition of 500 μ L of Folin-Ciocalteu reagent diluted with water in a 1:2 ratio. The mixture was left to stand for 5 minutes. Subsequently, 1 mL of 7.5% sodium carbonate was added, and the solution was homogenized. It was then stored in the dark at room temperature for 45 minutes. The TPC was measured at a wavelength of 760 nm using a UV-Vis spectrophotometer (BioDrop Duo+ Microvolume UV/Vis

Table 1. Experimental variables and their coded levels for the central composite design

Independent variable	Unit	Symbol code	Levels of coded variables				
			$-\alpha$	-1	0	+1	$+\alpha$
Incubation Time	h	A	14.06	24	48	72	81.94
Enzyme Concentration	U/g	B	7.57	20	50	80	92.43

Table 2. Experimental factors in coded and actual units and experimental responses

No.	Coded Value		Uncoded Value		TPC (mg GAE/g wet sample)
	Enzyme Concentration (A)	Incubation Time (B)	Enzyme Concentration (U/g)	Incubation Time (h)	
1	-1	-1	20	24	0.353
2	-1	+1	20	72	0.410
3	+1	-1	80	24	0.341
4	+1	+1	80	72	0.406
5	- α	0	50	14.06	0.314
6	+ α	0	50	81.94	0.405
7	0	- α	7.57	48	0.413
8	0	+ α	92.43	48	0.376
9	0	0	50	48	0.382
10	0	0	50	48	0.377
11	0	0	50	48	0.370
12	0	0	50	48	0.378
13	0	0	50	48	0.396

Spectrophotometer, Biochrom, United Kingdom) and was expressed as milligrams of gallic acid equivalent (GAE) per gram of wet sample. The calibration curve for gallic acid spanned a concentration range of 10 to 200 mg L⁻¹.

Experimental design and optimization

The response surface methodology was employed to optimize the TPC of *tape* fermented BGR using the CCD technique. For this purpose, two controllable factors were defined: the effect of enzyme concentrations (A, U/g) and fermentation time (B, h). Each factor was analyzed at five levels, encoded with symbols and numbers as shown in Table 1. A rotatable CCD employed with a total of 13 experimental units that consist of five central point treatments (0,0), four axial points of lowest (-1) and highest (+1) level, and four expanded points of axial point treatments to expand the design at minimum (- α) and maximum (+ α). Each treatment unit used 2 grams of *tape* and was conducted in triplicate. The statistical software Design-Expert version 13 (Stat Ease, Inc., USA) was used to generate the optimization treatments from a CCD. The results are shown in Table 2.

Results and Discussion

Model fitting and statistical analysis

The influence of incubation duration and enzyme concentration on the total phenolic compounds in BGR *tape* was examined using Central Composite Design (CCD). This study employed a rotatable CCD design of five center runs and an α value of 1.4142 to provide reasonable stability, enhance prediction capabilities across the design space (Myers et al., 2016; Kasina et al., 2020). The findings regarding total phenolic compounds are detailed in Table 2.

Furthermore, the analysis of variance (ANOVA)

result is presented in Table 3. Based on the ANOVA, the relative influence on TPC followed the order $A > AA > B$ (incubation time) > AB (enzyme concentration), with F-values of 101.41, 9.76, and 7.53, respectively. The AB and BB terms were not significant. A significant model term presented by a p-value of 0.0002 ($p < 0.05$) and an F-value of 25.08. This result indicates the adequacy of the model form. The fit statistics further supported the model performance ($R^2 = 0.9471$, adjusted $R^2 = 0.9094$, predicted $R^2 = 0.8249$), suggesting a strong fit and good predictability with minimal overfitting across the studied ranges of incubation time and cellulase concentration, as it explains the data variations.

The estimated regression coefficients of the TPC response were fitted by a second-order regression as follows:

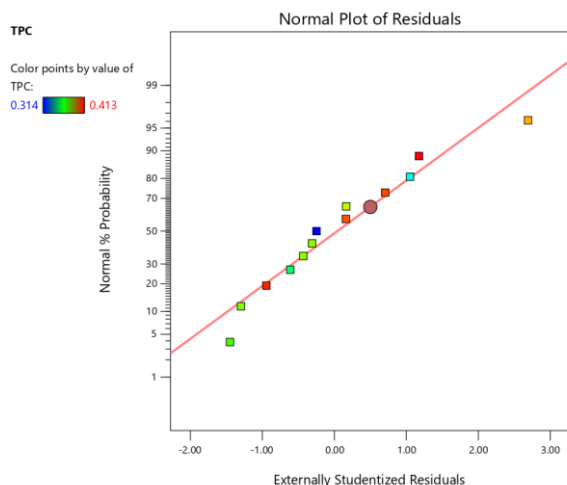
$$TPC \text{ (mg GAE/g sample)} = 0.31678 + 0.002904 A - 0.001204 B - 0.000018 A^2 + 0.00000786 B^2 + 0.0000003 AB$$

where A (incubation time) and B (cellulase concentration) are coded variables. A positive coefficient suggests that the variable can enhance the response, while a negative coefficient indicates that an increase in the variable results in a lower response (Freire Balseca et al., 2024). This regression equation showed that increasing the incubation time results in an increase in TPC. However, the significant A^2 term indicates a quadratic relationship, suggesting an optimal point beyond which further extension of incubation time may result in a decrease in TPC. Furthermore, the regression analysis established an inverse correlation between TPC and cellulase concentration (B). Moreover, the positive coefficient of B^2 suggest that this effect diminishes at higher concentrations, producing a curvature in the response surface.

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model for TPC optimization in BGR *tape* fermentation.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0097	5	0.0019	25.08	0.0002	significant
A-Incubation Time	0.0079	1	0.0079	101.41	< 0.0001	
B-Enzyme Concentration	0.0006	1	0.0006	7.53	0.0287	
AB	0.0000	1	0.0000	0.2065	0.6632	
A ²	0.0008	1	0.0008	9.76	0.0167	
B ²	0.0003	1	0.0003	4.50	0.0717	
Residual	0.0005	7	0.0001			
Lack of Fit	0.0002	3	0.0001	0.6144	0.6409	not significant
Pure Error	0.0004	4	0.0001			
Cor Total	0.0103	12				

Additionally, the normal probability plots of the residuals were evaluated to determine whether a dataset follows a normal distribution. It is utilized to identify significant deviations, outliers, and kurtosis (Freire Balseca et al., 2024). Figure 1 displays the normal probability plots of the residuals for the designated model. It was observed that there are no major outliers, as the residuals are evenly distributed around the straight line, typically ranging from -1.6 to ± 3.0 , and no point exceeds 3. The color-coded TPC values are scattered along the line without a clear pattern, indicating that the residual distribution is independent of the response magnitude. This result suggests that the errors are approximately normal and supports the adequacy of the fitted quadratic model.

Figure 1. Normal probability plots for residuals of the TPC in BGR *tape* fermentation.

Effects of incubation time and enzyme concentration on TPC

According to the 3D response surface and contour plots (Figure 2), TPC increases substantially

with incubation time across the studied range (24-72 h), with a minor decline at longer incubation times. This result supports the significant quadratic effect of incubation time (AA), indicating that this factor exerts the strongest influence on the TPC response. In contrast, the cellulase concentration (B) exhibits a marginal, primarily adverse effect, characterized by a relatively weak curvature (B² borderline, $p = 0.0717$). These trends are further illustrated by the Pareto chart (Figure 3), which clearly ranks incubation time as the most influential variable in the model which determines TPC level. Overall, the surfaces suggest an operational range at extended incubation times with low to moderate enzyme concentrations within the studied region.

The increase in phenolics along with the incubation time of fermentation aligns with prior observations in BGR systems. The enhancement of free phenolic compounds over a 24-72 h incubation period was reported during the fermentation of BGR using *ragi tape* (Azkia et al., 2023). Mu et al. (2019) also reported a similar result of TPC increase during BGR rice wine fermentation up to 60 h. A mixed community of microorganisms, such as molds (*Amylomyces rouxii*, *Mucor* spp., and *Rhizopus* spp.), yeast (*Saccharomyces* spp., *Candida utilis*), and some lactic acid bacteria (*Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *L. fermentum*), may produce hydrolytic enzymes during fermentation, such as protease, β -glucosidase, xylanase, cellulase, and esterase, disrupt cell-wall polysaccharides and cleave glycosidic/ester linkages, thereby releasing phenolics bound to the structural proteins, cellulose, and arabinoxylan, which elevates measured TPC (Gunam et al., 2021; Azkia et al., 2024). Time-dependent shifts in phenolic profiles (e.g., protocatechuic acid, ferulic acid, and vanillic acid) further contribute to the observed increase in TPC (Shin et al., 2019; Azkia et al., 2023).

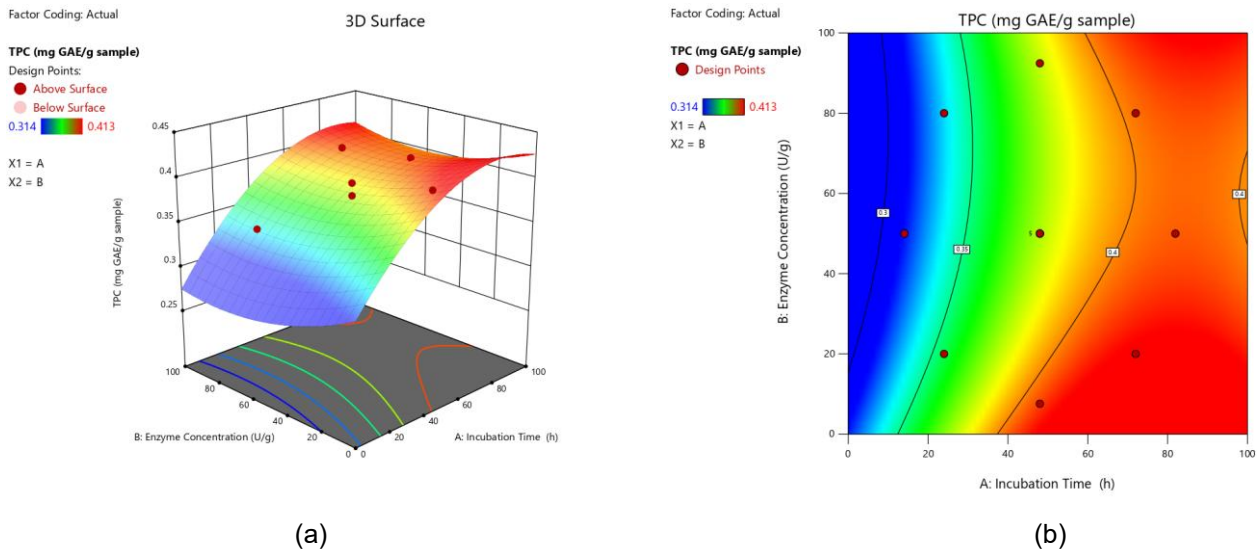


Figure 2. (a) Response surface plot of TPC in BGR *tape* fermentation considering the factors of incubation time and enzyme concentration; (b) contour plot of TPC in BGR *tape* fermentation considering the factors of incubation time and enzyme concentration

However, at extended incubation time, the model and surface indicate a downturn in TPC, confirming the existence of an optimal point. This decline is plausibly due to oxidative and/or enzymatic degradation of phenolics, polymerization, and complexation with proteins or cell-wall fragments, which reduce extractable phenolics (Wang et al., 2018; Mu et al., 2019). This outcome is consistent with a prior study on the pre-fermentation of BGR wine that found that TPC increased up to 60 hours of incubation time before declining further. Moreover, the phenolic compounds may be utilized by the microorganisms involved in fermentation, contributing to the loss in TPC (Mu et al., 2019). Additionally, a longer incubation period for BGR rice wine was found to enhance protease activity. Proteins released by this protease activity may bind with phenolics and form a complex, lowering the TPC value over an extended incubation period (Mu et al., 2019; Martillanes et al., 2021).

6.33% higher than in their control counterparts. Martillanes et al. (2021) also reported an increase in *trans*-ferulic acid and *p*-coumaric acid in rice bran due to cellulase treatment with enzyme concentration of 0.5 to 2.0%. This increase may be caused by the activity of cellulase, which releases bound phenolics from cell-wall matrices, thus liberating free phenolics (Martillanes et al., 2021; Yang et al., 2022; Azkia et al., 2024).

In the present study, cellulase concentration (*B*) had a significant but small linear effect ($p = 0.0287$), the quadratic term was borderline (B^2 , $p = 0.0717$), and the interaction of both factor terms (AB) was not significant. Thus, at a constant incubation time, increasing enzyme concentration produced a slightly adverse marginal change in TPC. These findings align with a study of cellulase treatment in rice straw that resulted in an increase in TPC after 24 hours, followed by a slight decrease at longer incubation times (Xue et al., 2017). Moreover, Ferraioli et al. (2025) reported an optimal bioactivity under mild enzymatic conditions, reinforcing the significance of controlled hydrolysis. Additionally, cellulase hydrolysis also releases proteins that can complex phenolic compounds, thus reducing the response of TPC. Ferraioli et al. (2025) proposed another possible mechanism: during hydrolysis, the released protein may competitively inhibit the enzymes, thereby reducing their activity.

In summary, it is advisable to operate at longer incubation times with low to moderate levels of cellulase, as increasing enzyme concentration yields diminishing returns on TPC. To optimize the TPC enhancement in BGR *tape* fermentation, the optimal condition was calculated to yield the maximum TPC response.

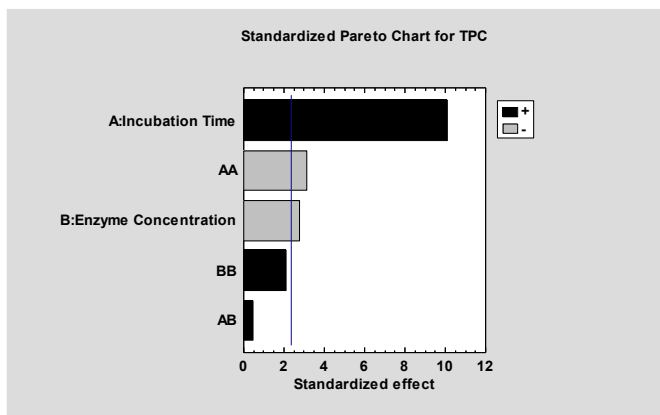


Figure 3. Standardized Pareto chart for TPC of BGR *tape* fermentation

Cellulase treatment has been reported to enhance the phenolic compounds in related matrices in previous studies. Yang et al. (2022) reported that the addition of cellulase in black rice wine increased the TPC level by

Optimization of TPC in BGR *tape* fermentation using RSM-CCD

This study aimed to identify the best incubation time and cellulase concentration parameters to enhance the TPC response during BGR *tape* fermentation. The optimization was conducted using Design-Expert version 13 software, which determined the maximum response

of TPC. The optimal conditions for incubation time and enzyme concentration were 71.16 h and 21.13 U/g, respectively. These optimal conditions predict a yield of 0.414 mg GAE/g sample for TPC with a desirability value of 1.00. These findings concur with the response surface plot (Figure 2) that suggests a recommended optimal point within a range of the variables, instead of a sharp point (Myers et al., 2016).

Conclusion

This current study showed that RSM using CCD can be applied to evaluate BGR *tape* fermentation variables, optimizing TPC response, and identifying its optimal process conditions. The CCD-RSM results were satisfactory for optimizing the independent variable and analyzing its effects on the response variable. The reduced quadratic polynomial models demonstrated an adequate level of prediction accuracy. The optimal condition to yield the maximum TPC in BGR *tape* fermentation was: incubation time of 71.16 hours and an enzyme concentration of 21.13 U/g. The predicted maximum TPC was 0.414 mg GAE/g sample.

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