

# Utilizing Black Turtle Beans (*Phaseolus vulgaris*) for Enhanced Natto Production: A Promising Alternative to Soybeans

Huong Huynh-Lien Ly<sup>1ab</sup>, Thanh Van Nguyen<sup>2a\*</sup>

**Abstract:** This study aims to replace soybeans, which pose allergy risks, with black turtle beans as a fermentation substrate for natto production. *Bacillus subtilis* MS05, known for its production of the fibrinolytic enzyme nattokinase, was employed to determine the optimal fermentation conditions. This was achieved through a multifaceted approach, incorporating one-factor-at-a-time experiments, a Plackett-Burman experimental design, as well as a response surface methodology with central composite design. The study reported the highest fibrinolytic activity at 418.32 FU/mL, which is equivalent to the nattokinase enzyme activity found in 5g of natto. This is sufficient to meet the daily nattokinase requirement of an adult consuming 25g of natto. Key factors that significantly influenced the fermentation process were identified as a peptone concentration of 2.8%, a bacterial density of 10<sup>4</sup> CFU/g of *Bacillus subtilis*, an initial pH of 5.5, and a fermentation duration of 33 hours. Additionally, natto produced from black turtle beans exhibited remarkable antioxidant activity, as indicated by a DPPH activity of 81.21 µg/mL. These findings highlight the significant potential of black turtle bean-based natto in enhancing fibrinolytic activity and effectively combating free radicals. These attributes contribute to the treatment of atherosclerotic blood clots and hold promise for improving overall human health by nutritional interventions.

**Keywords:** *Bacillus subtilis*, black turtle beans, fibrinolytic activity, nattokinase, soy allergies.

## 1. Introduction

Natto, a traditional Japanese fermented product produced from soybeans and *Bacillus subtilis*, is renowned for its potent fibrinolytic activity, largely attributed to the presence of nattokinase. This enzyme is recognized for its important role in the prevention and treatment of cardiovascular diseases, which are a leading cause of death worldwide owing to blood clot formation (Roth et al., 2018; Wang et al., 2023). Despite these health benefits, soybeans are well-established allergens, posing challenges for individuals with soy allergy (Inomata et al., 2007).

To address this limitation, recent studies have investigated alternative legumes for natto production. Evidence suggests that some legumes, when fermented with *Bacillus subtilis*, can produce nattokinase with fibrinolytic activity comparable to that of conventional soy-based natto (Sumi et al., 2022). In Vietnam, black turtle beans (*Phaseolus vulgaris*) have emerged as a promising candidate to substitute for soybeans in natto production. These beans not only exhibit a protein profile similar to that of soybeans but also lack the major allergens Gly m4, Gly m5, and Gly m6, which are primarily responsible for allergic reactions to soy (Platteau et al., 2011; He et al., 2021). Therefore, black turtle beans represent a potential allergen-free substrate for natto fermentation while preserving the cardiovascular benefits of nattokinase.

This study aims to optimize the fermentation process of black turtle bean natto by identifying key fermentation parameters and applying Response Surface Methodology (RSM) to maximize

nattokinase production. RSM has proven effective for optimizing medium composition for recombinant nattokinase production from *Bacillus subtilis*, resulting in increased enzyme yields (Tran et al., 2014). It has also been widely used to optimize fermentation conditions for nattokinase across different settings, highlighting its utility as an optimization technique (Nguyen et al., 2022). Additionally, given the role of oxidative stress in abnormal blood clot formation, this study will evaluate the antioxidant properties of crude nattokinase extracts from black turtle bean natto (Wang et al., 2020).

By optimizing the fermentation process and examining the biofunctional properties of black turtle bean natto, this research aims to provide a safe alternative for individuals with soy allergy while maintaining its cardiovascular health benefits.

## 2. Materials and Methods

### Screening of Suitable *Bacillus sp.* For Black Turtle Bean Natto Fermentation Through Evaluation of Extracellular Fibrinolytic Enzyme Production Capability

Numerous prior studies have consistently demonstrated that *Bacillus subtilis*, during the fermentation process of natto, produces the nattokinase enzyme, which effectively dissolves blood clots (Hmood et al., 2016; Yanagisawa et al., 2010; Hmood & Aziz, 2016). Consequently, the primary objective of this study is to evaluate the fibrinolytic activity of four indigenous strains of *Bacillus sp.*, namely CO18, MO39, TO46, and MS05. These strains, derived from the strain collection at the Institute of Food and Biotechnology, Can Tho University, were carefully selected and subjected to in vitro assessments to determine their fibrinolytic potential. The ultimate aim is to identify the most suitable *Bacillus*

### Authors information:

<sup>1</sup>Institute of Food and Biotechnology, Can Tho University, Can Tho City 900000, VIETNAM. E-mail: lhlhuong@ctump.edu.vn<sup>1</sup>, E-mail: nvthanh@ctu.edu.vn<sup>2</sup>

<sup>2</sup>Can Tho University of Medicine and Pharmacy, Can Tho City 900000, VIETNAM. E-mail: lhlhuong@ctump.edu.vn<sup>1</sup>

\*Correspondence: lhlhuong@ctump.edu.vn

Received: July, 2024

Accepted: March, 2025

Published: March, 2026

strain for natto fermentation on a black turtle bean substrate, a prevalent bean variety in the Mekong Delta region of Vietnam.

The fermentation time for producing natto using *Bacillus* sp. was examined within a range of 6 to 30 hours to determine the optimal fermentation duration for further research.

In the natto fermentation process, black turtle beans were soaked in water at a ratio of 1:5 (g/mL) for 12 hours. The soaked beans were then steamed and sterilized at 121°C and 1 atm pressure for 15 minutes using an autoclave (Hirayama HVE-50, Japan) (Hui et al., 2004). Natto fermentation was carried out for 30 hours at 30°C and pH 8.0. Additionally, 0.25% (w/v) glucose and 0.3% (w/v) fungal extract were added to the fermentation mixture (Suwanmanon & Hsieh, 2014).

The fibrinolytic activity of the nattokinase enzyme (FU/mL) was defined as the quantity of enzyme necessary to elicit a 0.01 increase in absorbance at 275 nm within 1 minute, under specific reaction conditions. The evaluation of fibrinolytic activity on the thrombin-fibrinogen model was performed using the method described as "Degradation of artificial thrombus by nattokinase" (Suwanmanon & Hsieh, 2014).

#### Investigating The Media Components

This investigation sought to determine the most effective carbon and nitrogen sources for maximizing the fibrinolytic activity of natto during fermentation by *Bacillus* strains. To assess the effect of medium composition on fibrinolytic activity, several common sugar sources were investigated. Fermentation experiments were conducted using glucose, maltose, sucrose, and lactose were carried out varying from 2 to 6% of concentration to investigate the influence of carbon sources on natto fermentation (Suwanmanon & Hsieh, 2014). The effect of different nitrogen sources on enzyme activity was also examined. Peptone, yeast extract, and whey protein were utilized as nitrogen sources at concentrations ranging from 1.5% to 3.5% (Suwanmanon & Hsieh, 2014).

#### Identification of Fibrinolytic Enzyme in Black Turtle Bean Natto

The bacterial strain with the highest fibrinolytic activity was identified using a specific primer pair designed to amplify a segment of the *Bacillus subtilis* 16S rRNA gene. The primers Bsub5F (5'-AAGTCGAGCGGACAGATGG-3') and Bsub3R (5'-CCAGTTTCCAATGACCCTCCCC-3') (Bharose et al., 2017) were used for PCR amplification. The resulting amplicons were purified using the Isolate II PCR and Gel Kit (Bioline) prior to Sanger sequencing at Macrogen (South Korea). The sequences were processed and analyzed using BioEdit software, and BLAST searches against the NCBI gene database were conducted to confirm species identity.

The specific gene segment was amplified using the polymerase chain reaction (PCR) protocol described by Sambrook & Russell (2001). The amplified *Bacillus subtilis*-specific gene segment, approximately 600 bp in size, was analyzed by agarose gel electrophoresis (Bharose et al., 2017).

Extracellular enzymes exhibiting the highest fibrinolytic activity, obtained after fermentation of black turtle bean natto by *Bacillus* sp. strains, were purified and characterized using two protein

precipitation steps: step 1, 60% ammonium sulfate precipitation, followed by step 2, 80% ethanol precipitation (Yanagisawa et al., 2010). The protein fraction obtained after precipitation was separated, and the nattokinase enzyme was identified using ion exchange chromatography based on the enzyme's isoelectric point (pI) of 8.6 (Hmood & Aziz, 2016). Finally, the purified enzyme was analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Yanagisawa et al., 2010).

#### Evaluation of The Antioxidant Capacity of Nattokinase

The free radical scavenging activity was evaluated using the DPPH\* method (Barriada-Bernal et al., 2014). Nattokinase was diluted to various concentrations ranging from 10 to 60 µg/mL in separate Eppendorf tubes, maintaining a DPPH (2,2-diphenyl-1-picrylhydrazyl) concentration of 40 mg/L and a final volume of 1 mL in each tube. The reaction mixture comprised 630 µL of DPPH solution (4.0 mg/mL in methanol), 100 µL of samples at different concentrations of the fermented solution, and 270 µL of MeOH. The mixture was incubated in the dark at 25°C for 30 minutes, and the absorbance was measured at 517 nm using a spectrophotometer. A control sample comprising 630 µL of DPPH solution (4.0 mg/mL) and 370 µL of methanol was also measured under the same conditions. Double-distilled water was utilized as the negative control, and ascorbic acid was employed as the positive control. The IC<sub>50</sub> value of the sample, defined as the concentration at which 50% of DPPH free radicals are scavenged, was calculated from a plot of sample concentration versus percentage free radical scavenging activity.

#### Improvement of Fibrinolytic Activity of Enzyme in Black Turtle Bean Natto

##### Screening of Significant Components

A screening Blackett-Burman matrix design was conducted to investigate the influence of key parameters that affect nattokinase enzyme activity. The experimental design consisted of 13 runs with five levels for each parameter. Six factors were investigated, including concentration of carbon source, concentration of nitrogen source, *Bacillus* sp. initial density, fermentation temperature, substrate pH, and fermentation time (Deepak et al., 2008). The design was created using Stagraphics Centurion software, version XV, with nattokinase enzyme activity as the monitored response. Nattokinase production was recorded for each experiment. Analysis of variance (ANOVA) was performed to determine the significance of each factor, and significant factors were further optimized using response surface methodology (RSM).

##### Optimization of Conditions for Natto Fermentation Using RSM

In evaluating the clot-dissolving potential of black turtle bean natto, the combined effects of fermentation factors were found to influence nattokinase enzyme activity. To examine this multivariable relationship, response surface methodology (RSM) was employed as an experimental statistical modeling technique. A central composite design (CCD) with seven factors and five levels was used to determine the optimal process parameters for

maximizing nattokinase production. The experimental design was generated using Statgraphics Centurion software, version XV. The relationship between the independent variables and the response variable was described using a second-order polynomial model. The coefficients of this polynomial equation were estimated using statistical methods, and response surface plots were generated to illustrate the relationships among the variables and the response (Deepak et al., 2008). This approach enabled the researchers to identify optimal conditions for maximizing the clot-dissolving capacity of nattokinase in the black turtle bean natto fermentation process.

### Statistical Analysis

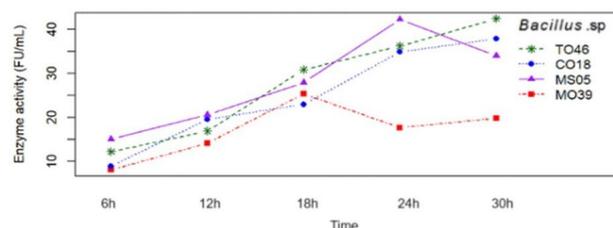
The results are presented as mean values with associated standard deviations, calculated from at least three independent measurements. Statistical analysis was conducted using the Statgraphics Centurion software version XV and Microsoft Excel 2016. Analysis of variance (ANOVA) and the least significant difference test (LSD) were utilized to assess the statistical significance of any differences observed, with a significance threshold set at  $p < 0.05$ .

## 3. Results and Discussion

### Suitable *Bacillus* sp. For Black Turtle Bean Natto Fermentation

The fibrinolytic effect was assessed by examining the ability of the crude extracellular enzyme derived from natto fermentation to dissolve artificial blood clots composed of thrombin and fibrinogen. The results of these evaluations are presented in Figure 1. The relationship between independent factors associated with natto fermentation time and different strains of *Bacillus*, in terms of fibrinolytic activity, was analyzed using a two-way ANOVA. The findings depicted in Figure 1 indicate a strong association between the bacterial strains used in fermentation and the enzymatic activity generated during the process. The bacterial strains utilized in natto fermentation exhibit varying capacities to dissolve fibrin, with the ascending order of activity being CO18, MO39, MS05, and TO46. Among these, both TO46 and MS05 exhibited similar and significant effectiveness in dissolving blood clots, with a confidence level of  $p < 0.05$ .

The strong association, with a  $p$ -value below 0.05, highlights the critical role of time and specific *Bacillus* species in the fermentation process. The statistical findings provide evidence that natto fermented for 30 hours using the *Bacillus* sp. TO46 strain exhibited the highest fibrinolytic activity at 42.36 FU/mL. Following closely, natto fermented for 24 hours with the *Bacillus* sp. MS05 strain showed an enzyme activity of 42.23 FU/mL. These two bacterial strains have been selected for further investigation into suitable nutritional supplementation for natto fermentation.



**Figure 1.** Coefficients of *Bacillus* sp. strains and fibrinolytic activity.

Figure 1 also suggests that the optimal fermentation time for black turtle bean natto is 24 hours. The difference in fibrinolytic activity between 24 hours and 30 hours among most bacterial strains is not statistically significant at the 95% confidence level. However, the fibrinolytic activity of natto fermented with *Bacillus* sp. MS05 tends to decrease when fermentation time exceeds 24 hours.

### Effect of Carbon and Nitrogen Sources on Nattokinase Enzyme Activity

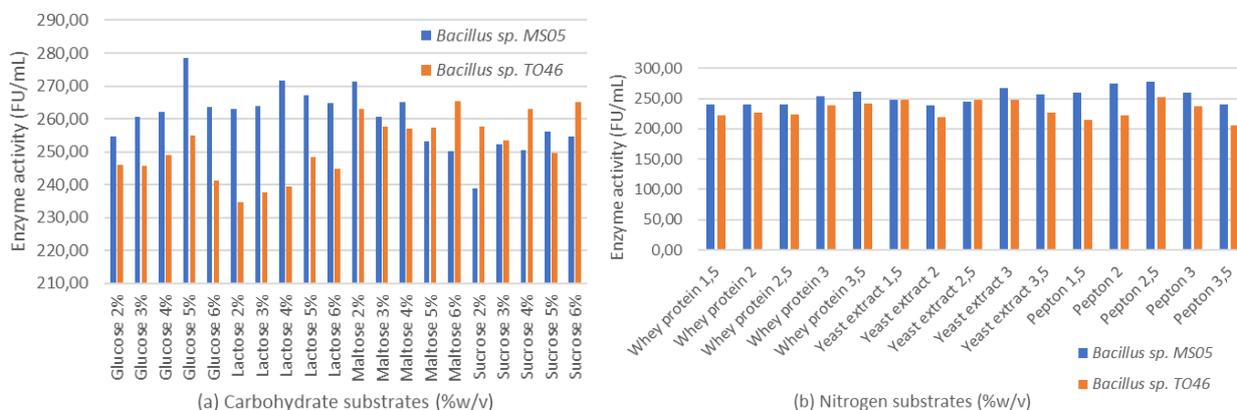
The study examined various sugar sources, including glucose, lactose, maltose, and sucrose, as well as different nitrogen sources, consisting of peptone, yeast extract, and whey protein. Figure 2a illustrates that, among the tested carbon sources, glucose supplementation at a concentration of 5% yielded the highest nattokinase activity, reaching  $278.38 \pm 1.11$  FU/mL during fermentation with *Bacillus* sp. MS05. Lactose at 4% and maltose at 2% also showed substantial enzyme activity when fermented with *Bacillus* sp. MS05. In contrast, sucrose at 2% yielded the lowest nattokinase activity relative to the other carbon sources. Additionally, sucrose at 3% and 4%, together with maltose at 5%, showed comparatively lower enzyme activity. These findings suggest that sucrose is not a suitable carbon source for the growth of *Bacillus subtilis* during black turtle bean natto fermentation, thereby reducing nattokinase production. This observation aligns with previous studies conducted on soybean natto (Suwanmanon & Hsieh, 2014; Deepak et al., 2008).

Among the nitrogen sources examined in Figure 2b, peptone at a concentration of 2.5% exerted the strongest effect on nattokinase activity, reaching  $277.15 \pm 1.2$  FU/mL. In addition, peptone at 2% produced significantly higher enzyme activity than other organic nitrogen sources, such as yeast extract and whey protein, which yielded enzyme activity 10-20% lower than the maximum observed activity. These findings are consistent with previous studies on nitrogen sources for natto fermentation (Suwanmanon & Hsieh, 2014; Deepak et al., 2008).

It is worth noting that the glucose concentration used in this study was higher than in previous reports, whereas the peptone concentration was higher or lower depending on the study. These variations may be attributed to differences in source materials, as black turtle beans contain less glycine compared to soybeans.

Therefore, a higher supplementary nutritional requirement may be necessary for the synthesis of nattokinase with high activity in black turtle bean natto fermentation (Pradhananga, 2019; Amin et al., 2020). The results shown in Figure 2 also indicate that *Bacillus* sp. strain MS05 exhibits higher fibrinolytic enzyme activity

than that produced by *Bacillus* sp. strain TO46, and this difference is statistically significant. Therefore, *Bacillus* sp. MS05 was selected for use in the fermentation process of black turtle bean natto.

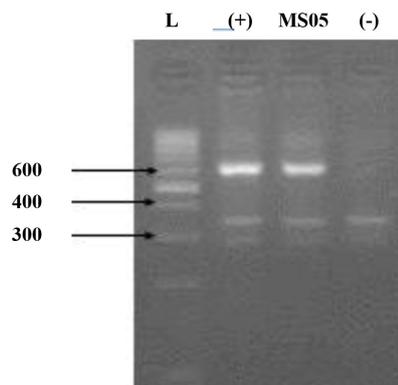


**Figure 2.** The influence of different nutrient sources on enzyme activity. (a) Carbohydrate sources; (b) Nitrogen sources

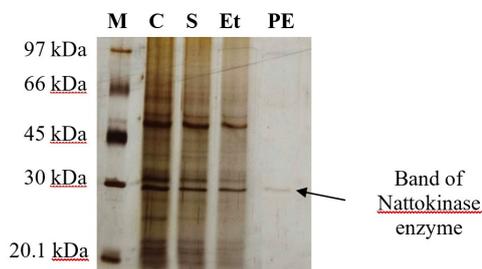
**Identification of Nattokinase from Black Turtle Bean Natto**

The sequencing of the 16S rRNA gene after PCR using the specific primer pair Bsub5F and Bsub3R revealed a 602 bp gene segment specific to *Bacillus subtilis* in the *Bacillus* sp. MS05 bacterial strain (Figure 3). The 16S rRNA gene sequence of this strain was compared with 16S rRNA sequences of bacteria in the NCBI database using the BLAST tool. The sequencing results for the isolated strain showed 100% coverage and 99.5% similarity to the 16S rRNA sequence of *Bacillus subtilis* var *subtilis*, with accession number MN888748.1 in the NCBI database (Table 1).

Numerous studies have shown that the nattokinase enzyme, which dissolves blood clots, is produced by *Bacillus subtilis* during natto fermentation (Hmood et al., 2016; Yanagisawa et al., 2010; Hmood & Aziz, 2016). Enzymes extracted from black turtle bean natto fermented by *Bacillus* sp. MS05 were subsequently purified to determine the presence of nattokinase. SDS-PAGE analysis demonstrated that the precipitation steps effectively removed specific extracellular proteins. The ion exchange chromatography results for the purified enzyme (PE) revealed a protein band of approximately 27 kDa (Figure 4). Previous studies have indicated that nattokinase is a single polypeptide chain consisting of 275 amino acids, with a molecular weight of 27,724 Da (Yanagisawa et al., 2010). Hence, the clot-dissolving enzyme in this study, with a molecular weight of 27 kDa, was identified as nattokinase.



**Figure 3.** Electrophoresis to assess *B. subtilis*. (L) Ladder 100bp, (+) positive control, (MS05) *Bacillus* sp. MS05, (-) Negative control



**Figure 4.** SDS-PAGE electrophoresis to assess nattokinase. (M) Prestained protein LMW marker, (C) Crude enzyme, (S) Salt precipitated enzyme, (Et) Ethanol precipitated enzyme, (PE) Purified enzyme.

**Sequencing the *Bacillus sp.* Using in Black Turtle Bean Natto Fermentation**

The MS05 bacterial strain was sequenced using the Bsub5F and Bsub3R primers, which were designed to target a conserved region of the bacterial 16S rRNA gene, with the aim of amplifying a 600 bp segment. This region is highly conserved and is suitable

for the identification of *Bacillus subtilis*. The obtained sequence was compared with bacterial 16S rRNA sequences in the NCBI database using the BLAST tool. The sequencing results for the isolated strain showed 100% coverage and 99.5% similarity to the 16S rRNA sequence of *Bacillus subtilis* var *subtilis*, accession number MN888748.1, in the NCBI database (Table 1).

**Table 1.** The similarity between the isolated strain MS05 and the sequence in NCBI database

Name of bacteria	Nomenclature	Size of 16S rRNA sequence	Coverage	Similarity	Accession number
<i>Bacillus subtilis</i> strain NTB-107 16S ribosomal RNA gene, partial sequence	<i>Bacillus subtilis</i> var <i>subtilis</i>	1512 bp	100%	99,5%	MN888748.1

**Antioxidant Property of Fibrinolytic Enzyme**

The antioxidant properties of the crude enzyme found in natto were evaluated using the DPPH free radical scavenging activity assay. The absorption peak observed at 517 nm signifies the interaction between the DPPH free radical and an unpaired electron. Determination of the IC<sub>50</sub> value is a widely adopted method for assessing the free radical scavenging capacity of bioactive substances. In this study, the IC<sub>50</sub> value, defined as the concentration of nattokinase required to convert 50% of the DPPH free radicals into their reduced form (DPPHH), was used to assess the extent of free radical scavenging activity. A lower IC<sub>50</sub> value indicates a higher antioxidant activity.

The IC<sub>50</sub> values are presented in Table 2. The standard curve equation, derived from regression analysis with an R<sup>2</sup> value of 0.9794, confirms the suitability of the linear regression model. A coefficient above 0.75 indicates a satisfactory fit; in this case, the model explains 97.94% of the variability in the response.

**Table 2.** The similarity between the isolated strain MS05 and the sequence in NCBI database

Sample	IC <sub>50</sub> (µg/mL)
Negative control	-
Fibrinolytic enzyme	81.21
Positive control (acid ascorbic)	19.36
The regression equation: $y = 0.0016x + 0.0391$ R <sup>2</sup> = 0.9794	

The IC<sub>50</sub> value of nattokinase was determined to be 81.21 µg/mL, indicating a relatively weaker free radical scavenging capacity compared with the reference standard, ascorbic acid (19.36 µg/mL). However, it is important to note that the IC<sub>50</sub> value of the crude enzyme from black turtle bean natto in this study is

lower than the IC<sub>50</sub> value reported for crude nattokinase, which was 380 µg/mL (Amin et al., 2020). This finding suggests a higher potential free radical scavenging activity of the fibrinolytic enzyme in black turtle bean natto than that of the previously examined nattokinase enzyme. Overall, these findings indicate substantial antioxidant activity of the fibrinolytic enzyme in black turtle bean natto.

**Nattokinase Activity Improvement by Optimization of Conditions for Natto Fermentation**

*Screening the Factors That Affect the Fermentation Process*

By employing a one-factor-at-a-time approach based on existing literature reports, the fermentation process was performed with *Bacillus subtilis* MS05 using 5% glucose, 2% peptone, and the specified culture conditions, including initial bacterial density, temperature, and fermentation time (Table 3). The optimal conditions for achieving the highest nattokinase activity included a bacterial density of 10<sup>4</sup> CFU/g, a fermentation temperature of 30°C, an initial pH of 6.0, and a fermentation time of 36 hours. The corresponding activities at a confidence level of p<0.05 for each surveyed factor were 226.4±1.37 FU/mL, 223.8±6.5 FU/mL, 254.6±4 FU/mL, and 248.4±16.5 FU/mL, respectively.

It is important to note that the activity observed under these optimal conditions may vary compared to previous experiments; this highlights the role of fermentation factors in either promoting or hindering nattokinase production. During the fermentation of *Bacillus subtilis* in natto production, both positive and negative influencing factors have optimal thresholds. Exceeding these thresholds inhibits bacterial growth and subsequently reduces nattokinase activity (Yang et al., 2021). Therefore, a systematic examination of nattokinase activity variations under different fermentation conditions is crucial for improving overall activity.

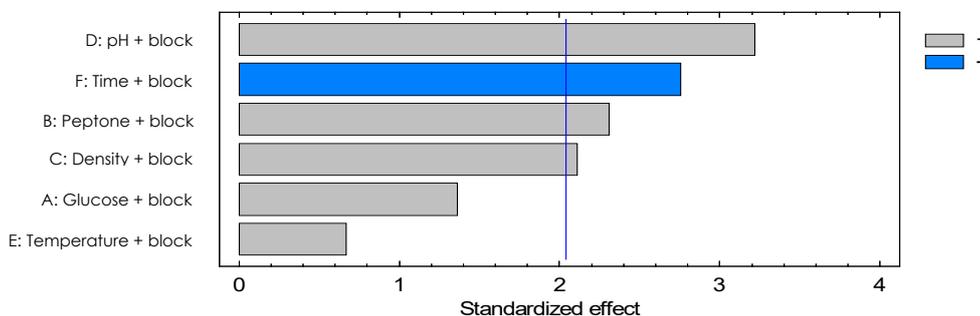
**Table 3.** Nattokinase enzyme activity under single-factor surveys.

Run	Starter Density (CFU/ g)	Enzyme Activity (FU/mL)	Temperature (°C)	Enzyme Activity (FU/mL)	pH	Enzyme Activity (FU/mL)	Time (Hrs)	Enzyme Activity (FU/mL)
1	10 <sup>3</sup>	219.4±0.97 <sup>b</sup>	25	202.8±7.1 <sup>bf</sup>	5.0	254.6±4 <sup>d</sup>	12	210.1±1.1 <sup>b</sup>
2	10 <sup>4</sup>	226.4±1.37 <sup>d</sup>	30	223.8±6.5 <sup>d</sup>	6.0	248.6±2.5 <sup>a b</sup>	24	235.8±12 <sup>a b</sup>
3	10 <sup>5</sup>	193.3±1.57 <sup>bf</sup>	35	203.9±3.9 <sup>bf</sup>	7.0	221.4±11.3 <sup>bf</sup>	36	248.4±16.5 <sup>d</sup>
4	10 <sup>6</sup>	180.5±1.5 <sup>c</sup>	40	191.4±2.7 <sup>c</sup>	8.0	236.7±6.5 <sup>bf</sup>	48	236.3±8.9 <sup>a b</sup>
5	10 <sup>7</sup>	214.5±1.69 <sup>b</sup>	45	196±5.4 <sup>c</sup>	9.0	222.6±17.5 <sup>bf</sup>	60	228.3±5.8 <sup>b</sup>
	CV%= 4.63		CV%= 8.04		CV%= 6.87		CV%= 8.65	

Note: Results of a 95% Least Significant Difference (LSD) test on the fibrinolytic activity. Letters in homogenous groups column denote significantly different groups.

To gain a comprehensive understanding of the combined effects and interactions among these factors, a screening process was conducted using an enzyme activity based approach. The Blackett-Burman matrix was used to evaluate the independent effects and interactions of the selected factors, which encompassed a range of values, including glucose concentration, peptone concentration, bacterial density, fermentation temperature, pH, and fermentation time. Factors exceeding the standardized effect line were deemed to have a significant impact on nattokinase activity, whereas those below the line were considered insignificant ( $p > 0.05$ ). The results illustrated in Figure 5 indicated that the initial pH of the substrate exerted the strongest influence on nattokinase activity. The subsequent factors, ranked in terms of their impact, were fermentation time,

peptone concentration, initial bacterial density, glucose concentration, and fermentation temperature. Notably, an increase in fermentation time resulted in a decrease in nattokinase activity, whereas the other factors had positive effects, indicating that increases in these variables would enhance enzyme activity. The study also showed that glucose concentration and fermentation temperature had negligible effects on enzyme activity in black turtle bean natto, suggesting differences in fermentation conditions compared with soybean natto. This finding aligns with previous studies that also reported an insignificant influence of fermentation temperature and initial glucose concentration on enzyme activity (Suwanmanon & Hsieh, 2014; Wang et al., 2009).



**Figure 5.** Influence of factors on nattokinase enzyme activity

**Optimization of Conditions for Black Turtle Bean Natto Fermentation Using RSM**

Based on the above screening results, only four factors, including X<sub>1</sub>, initial pH of the substrate (5.0 to 9.0); X<sub>2</sub>, fermentation time (12 to 60 hours); X<sub>3</sub>, peptone concentration (1.5 to 3.5%); and X<sub>4</sub>, initial bacterial density (10<sup>3</sup> to 10<sup>7</sup> CFU/mL), were identified as significant contributors to nattokinase activity. Therefore, these factors were selected for optimization. The experimental ranges for these factors were established, and a 26-experiment RSM-CCD model was designed using Statgraphics Centurion optimization software.

The influence of each factor on nattokinase enzyme activity was analyzed, with pH found to have the strongest effect, followed by peptone concentration, initial bacterial density, and fermentation time. The overall second-degree polynomial equation for

nattokinase enzyme activity,  $Y$  (FU/mL) = -1178.07 + 275.355\*X<sub>1</sub> + 19.7741\*X<sub>2</sub> + 220.015\*X<sub>3</sub> + 104.913\*X<sub>4</sub> - 26.7765\*X<sub>1</sub><sup>2</sup> - 1.32823\*X<sub>1</sub>\*X<sub>2</sub> + 10.7808\*X<sub>1</sub>\*X<sub>3</sub> + 9.76781\*X<sub>1</sub>\*X<sub>4</sub> - 0.122106\*X<sub>2</sub><sup>2</sup> - 0.514241\*X<sub>2</sub>\*X<sub>3</sub> - 0.784647\*X<sub>2</sub>\*X<sub>4</sub> - 41.0529\*X<sub>3</sub><sup>2</sup> - 6.33031\*X<sub>3</sub>\*X<sub>4</sub> - 15.1853\*X<sub>4</sub><sup>2</sup>, with an R-squared value of 0.6575, indicates that 65.75% of the variation in nattokinase enzyme activity can be explained by the fermentation conditions. Regression analysis showed that the two most influential factors, pH and peptone concentration, had positive coefficients, whereas the constant term was -1178.07, indicating a negative intercept. This finding suggests the presence of additional unexamined factors that could affect the production of nattokinase and its blood clot-dissolving capability. According to previous studies, many other factors can influence the natto fermentation process beyond those investigated (Suwanmanon & Hsieh, 2014; Deepak et al.,

2008). Therefore, further research is required to examine factors affecting nattokinase enzyme activity during the fermentation of black turtle bean natto to enhance its blood clot-dissolving activity.

The model predicted that the optimal fermentation conditions would include a peptone concentration of 2.8%, bacterial density of  $10^4$  CFU/g, an initial pH of 5.5, and a fermentation time of 33 hours. These conditions were expected to yield a nattokinase enzyme activity ( $Y_{max}$ ) of 436.212 FU/mL. Experimental verification under these optimized conditions resulted in an observed enzyme activity ( $Y'_{max}$ ) of 418.32 FU/mL. The accuracy of the optimized model was confirmed by the  $Y'_{max}/Y_{max}$  coefficient of 0.9760, indicating high agreement between predicted and observed values (approximately 97.6%). These results support the applicability of the RSM-CCD experimental matrix model for optimization studies.

Compared with previous studies on soybean natto, the optimized black turtle bean natto exhibited higher nattokinase enzyme activity (Suwanmanon & Hsieh, 2014; Liu et al., 2005). This finding indicates the potential of black turtle bean natto to promote blood clot dissolution. However, further optimization of fermentation conditions remains necessary to increase enzyme activity.

#### 4. Conclusion

This study demonstrated the feasibility of utilizing black turtle beans as an alternative fermentation substrate for natto production, thereby addressing concerns related to soybean allergies. Using a systematic experimental approach to optimize the fermentation conditions for *Bacillus subtilis* MS05, a strain known for producing the fibrinolytic enzyme nattokinase, we identified key factors that significantly influenced the fermentation process. A peptone concentration of 2.8%, a bacterial density of  $10^4$  CFU/g of *Bacillus subtilis*, an initial pH of 5.5, and a fermentation duration of 33 hours were found to be the optimal conditions for maximizing fibrinolytic activity with 418.32 FU/mL of nattokinase activity.

Additionally, notable antioxidant activity of black turtle beans natto was indicated by a DPPH activity of 81.21  $\mu\text{g/mL}$ . This highlights the potential of black turtle bean-based natto to enhance fibrinolytic activity and effectively eliminate free radicals. These properties have implications for the treatment of atherosclerotic blood clots and offer opportunities to improve overall human health through appropriate nutritional interventions.

#### 5. Acknowledgement

This study was facilitated and supported by Can Tho University and Can Tho University of Medicine and Pharmacy, Vietnam, in terms of infrastructure and research conditions. The authors sincerely appreciate their invaluable assistance.

#### 7. References

- Amin, K., Zeng, X., You, Y., Hu, Y., Sun, H., Lyu, B., ... & Yu, H. (2020). Enhanced thermostability and antioxidant activity of Nattokinase by biogenic enrichment of selenium. *Journal of Food Measurement and Characterization*, 14(4), 2145-2154.
- Barriada-Bernal, L. G., Almaraz-Abarca, N., Delgado-Alvarado, E. A., Gallardo-Velázquez, T., Ávila-Reyes, J. A., Torres-Morán, M. I., ... & Herrera-Arrieta, Y. (2014). Flavonoid composition and antioxidant capacity of the edible flowers of *Agave durangensis* (Agavaceae). *CyTA-Journal of Food*, 12(2), 105-114.
- Bharose, A. A., Gajera, H. P., Hirpara, D. G., Kachhadia, V. H., & Golakiya, B. A. (2017). Molecular identification and characterization of bacillus antagonist to inhibit aflatoxigenic *Aspergillus flavus*. *Int. J. Curr. Microbiol. Appl. Sci*, 6(3), 2466-2484.
- Deepak, V., Kalishwaralal, K., Ramkumarpandian, S., Babu, S. V., Senthilkumar, S. R., & Sangiliyandi, G. (2008). Optimization of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology. *Bioresource Technology*, 99(17), 8170-8174.
- He, S., Zhao, J., Zhang, Y., Zhu, Y., Li, X., Cao, X., ... & Sun, H. (2021). Effects of low-pH treatment on the allergenicity reduction of black turtle bean (*Phaseolus vulgaris* L.) lectin and its mechanism. *Journal of agricultural and food chemistry*, 69(4), 1379-1390.
- Hmood, S. A., & Aziz, G. M. (2016). Optimum conditions for fibrinolytic enzyme (Nattokinase) production by *Bacillus* sp. B24 using solid state fermentation. *Iraqi Journal of Science*, 1391-1401.
- Hmood, S. A., & Aziz, G. M. (2016). Purification and characterization of nattokinase produced by local isolate of *Bacillus* sp. B24. *Iraqi journal of Biotechnology*, 15(2).
- Hui, Y. H., Meunier-Goddik, L., Josephsen, J., Nip, W. K., & Stanfield, P. S. (Eds.). (2004). *Handbook of food and beverage fermentation technology* (Vol. 134). CRC Press.
- Inomata, N., Osuna, H., Kawano, K., Yamaguchi, J., Yanagimachi, M., Matsukura, S., & Ikezawa, Z. (2007). Late-onset anaphylaxis after ingestion of *Bacillus Subtilis*-fermented soybeans (Natto): clinical review of 7 patients. *Allergology international*, 56(3), 257-261.
- Liu, J., Xing, J., Chang, T., Ma, Z., & Liu, H. (2005). Optimization of nutritional conditions for nattokinase production by *Bacillus* natto NLSSE using statistical experimental methods. *Process Biochemistry*, 40(8), 2757-2762.

- Nguyen, V. T., Tran, L. H., & Le, Q. H. (2022). Optimization of Nattokinase Production from *Bacillus subtilis* Using Response Surface Methodology. *International Journal of Science, Engineering and Applied Science*, 8(8), 1-10.
- Platteau, C., Cucu, T., De Meulenaer, B., Devreese, B., De Loose, M., & Taverniers, I. (2011). Effect of protein glycation in the presence or absence of wheat proteins on detection of soybean proteins by commercial ELISA. *Food Additives and Contaminants*, 28(2), 127-135.
- Pradhananga, M. (2019). Effect of processing and soybean cultivar on natto quality using response surface methodology. *Food Science & Nutrition*, 7(1), 173-182.
- Roth, G. A., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., ... & Borschmann, R. (2018). Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The lancet*, 392(10159), 1736-1788.
- Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* 3rd ed. Chapter 6. Isolation of DNA Fragments from Polyacrylamide Gels by the Crush and Soak Method.
- Sumi, H., Naito, S., Yatagai, C., Mitsuo, T (2022). Strong Fibrinolysis with New Fermented Foods (Mung Bean Natto and Natto Miso). *The International Society on Thrombosis and Haemostasis 2022 Congress in London, England, U.K.* Abstract number: VPB0639.
- Suwanmanon, K., & Hsieh, P. C. (2014). Isolating *Bacillus subtilis* and optimizing its fermentative medium for GABA and nattokinase production. *CyTA-Journal of Food*, 12(3), 282-290.
- Tran, Q. T., Nguyen, T. T. K., Le, T. T. A., Dinh, M. H., & Tran, C. D. (2014). Optimization of medium composition for recombinant nattokinase production from *Bacillus subtilis* by using response surface methodology. *Academia Journal of Biology*, 36(1se), 130-137.
- Wang, C., Chen, J., Tian, W., Han, Y., Xu, X., Ren, T., ... & Chen, C. (2023). Natto: A medicinal and edible food with health function. *Chinese Herbal Medicines*.
- Wang, J. K., Chiu, H. H., & Hsieh, C. S. (2009). Optimization of the medium components by statistical experimental methods to enhance nattokinase activity. *Fooyin Journal of Health Sciences*, 1(1), 21-27.
- Wang, Q., & Zennadi, R. (2020). Oxidative stress and thrombosis during aging: the roles of oxidative stress in RBCs in venous thrombosis. *International journal of molecular sciences*, 21(12), 4259.
- Yanagisawa, Y., Chatake, T., Chiba-Kamoshida, K., Naito, S., Ohsugi, T., Sumi, H., ... & Morimoto, Y. (2010). Purification, crystallization and preliminary X-ray diffraction experiment of nattokinase from *Bacillus subtilis* natto. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*, 66(12), 1670-1673.
- Yang, Y., Lan, G., Tian, X., He, L., Li, C., Zeng, X., & Wang, X. (2021). Effect of fermentation parameters on natto and its thrombolytic property. *Foods*, 10(11), 2547.