Innovating Natto Production with Black Turtle Beans (*Phaseolus vulgaris*): A High Nattokinase, Soy-Free Alternative

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Abstract- This study investigates the feasibility of using black turtle beans (*Phaseolus vulgaris*) as an alternative substrate for natto fermentation to produce nattokinase, an enzyme with substantial fibrinolytic activity. Given the allergenic concerns associated with soybean-based natto, black turtle beans offer a potential hypoallergenic alternative. Four indigenous *Bacillus* sp. strains (CO18, MO39, TO46, and MS05) were evaluated for their fibrinolytic enzyme production. Results showed that *Bacillus subtilis* TO46 exhibited the highest enzyme activity, with optimized fermentation conditions being determined using Response Surface Methodology (RSM) and Central Composite Design (CCD). Key factors such as initial pH, peptone concentration, bacterial density, and fermentation time were optimized to enhance nattokinase production. Under optimal conditions, a remarkable nattokinase activity of 418.32 FU/mL was achieved, surpassing that of traditional soybean-based natto. The findings suggest that black turtle beans are a promising alternative substrate for natto fermentation, offering potential benefits for individuals with soybean allergies while maintaining high fibrinolytic activity.

Index Terms- *Bacillus subtilis*; black turtle bean natto; fermentation optimization; nattokinase activity; soy allergies

I. INTRODUCTION

Natto, a traditional fermented food often made from soybeans fermented with *Bacillus subtilis* bacteria, has been recognized for its fibrinolytic activity due to the presence of the enzyme nattokinase. This enzyme has been shown to be effective in preventing and treating cardiovascular diseases, which are a major cause of significant global mortality, mainly attributed to the formation of blood clots in the vascular system (Roth et al., 2018; Wang et al., 2023). However, many studies have shown that soybeans belong to a group of highly allergenic foods, raising concerns about the suitability of soybean-based natto as a nutritional therapy (Inomata et al., 2007). Recent research has shed light on the potential of certain legumes fermented with *Bacillus subtilis* to produce highly fibrinolytic nattokinase (Sumi et al., 2022). This discovery opens up the possibility of identifying suitable alternatives to soybeans in natto production.

We have identified a promising native candidate in Vietnam, black turtle bean (Phaseolus vulgaris). Black turtle beans have protein content similar to soybeans but contain about half the amount of protein (Hmood et al., 2016). Importantly, black turtle beans do not contain Glycine, the allergenic factor associated with Gly m4, Gly m5, and Gly m6 proteins commonly found in soybeans (Platteau et al., 2011; He et al., 2021). Therefore, black turtle beans are a feasible substitute for the natto fermentation process, offering a potential solution for individuals with soybean allergies seeking the health benefits associated with consuming natto.

Despite these promising findings, there is still a lack of comprehensive studies that systematically investigate the potential of alternative legumes, like black turtle beans, in producing natto with comparable fibrinolytic activity to soybean-based natto. Most existing literature has not fully explored the optimization of fermentation conditions, which are crucial for enhancing its health benefits. This study aims to fill these gaps by thoroughly examining the fermentation process, optimizing conditions for maximal nattokinase activity of black turtle bean natto.

In this study, the fermentation conditions will be systematically investigated, starting with the selection of suitable native bacterial strains for fermenting black turtle bean natto. The factors influencing the fermentation process will be screened to identify the ones that truly affect the fermentation process. These factors will then be optimized utilizing the Response Surface Methodology (RSM) approach in conjunction with Central Composite Design (CCD). By employing this experimental model, the optimal conditions will be determined for producing black turtle bean natto, maximizing its fibrinolytic activity.

The overall goal of this study is to determine whether black turtle bean can serve as a viable alternative to soybeans in natto production, ensuring high fibrinolytic activity while offering additional health benefits, particularly for those with soybean allergies. The hypothesis is that optimizing the fermentation process with *Bacillus subtilis* strains will yield a natto product with enhanced fibrinolytic. This research aims to contribute to the development of safe alternative natto products for individuals with soybean allergies while maintaining the beneficial characteristics associated with consuming natto.

II. MATERIALS AND METHODS

Screening of suitable *Bacillus* sp. for black turtle bean natto fermentation through evaluation of extracellular fibrinolytic enzyme production capability

To determine the most suitable *Bacillus* strain for natto fermentation on a black turtle bean substrate, variety widely cultivated in Mekong Delta, four indigenous *Bacillus* sp. strains, namely CO18, MO39, TO46, and MS05, were selected from the strain collection at the Institute of Food and Biotechnology, Can Tho University. These strains were subjected to in-vitro evaluations to assess their fibrinolytic potential.

The fermentation period for natto using *Bacillus* sp. was examined over a range from 6 to 30 hours to establish the optimal fermentation duration for further research. In preparing the black turtle beans for fermentation, they were soaked in water at a 1:5 (g/mL) ratio for 12 hours. Post soaking, the beans were steamed and sterilized using an autoclave (Hirayama HVE–50, Japan) at 121°C and 1 atm pressure for 15 minutes, as described by Hui et al. (2004). The fermentation process was then conducted over 30 hours at a controlled temperature of 30°C and a pH of 8.0. Moreover, 0.25% (w/v) glucose and 0.3% (w/v) fungal extract were incorporated into the fermentation mixture (Suwanmanon & Hsieh, 2014).

The fibrinolytic activity of the nattokinase enzyme (FU/mL) was defined by the amount of enzyme required to induce a 0.01 increase in absorbance at 275 nm within a 1-minute period under specific reaction conditions. The fibrinolytic activity was further evaluated using the thrombin-fibrinogen model, following the methodology outlined as "Degradation of artificial thrombus by nattokinase" (Suwanmanon & Hsieh, 2014).

Investigating the media components

In order assess the influence of media composition on enhancing fibrinolytic activity, various sugar and nitrogen sources were systematically tested. Fermentation trials were conducted using glucose, maltose, sucrose, and lactose at concentrations ranging from 2% to 6%, as recommended by Suwanmanon & Hsieh (2014) based on their findings regarding optimal sugar levels for *Bacillus* fermentation. In parallel, peptone, yeast extract, and whey protein were employed as nitrogen sources at concentrations from 1.5% to 3.5%, following the concentration ranges suggested by the same study for maximizing enzyme production (Suwanmanon & Hsieh, 2014). Each test was performed in triplicate to ensure the reliability of the results. This investigation aimed to identify the optimal nutrient conditions that maximize fibrinolytic enzyme production.

Identification of fibrinolytic enzyme in black turtle bean natto

The bacterial strain demonstrating the highest fibrinolytic activity was identified using a specific primer pair targeting a segment of the 16S rRNA gene sequence of *Bacillus subtilis*. The primers employed were Bsub5F (5′-AAGTCGAGCGGACAGATGG-3′) and Bsub3R (5′-CCAGTTTCCAATGACCCTCCCC-3′) (Bharose et al., 2017). The amplification of this gene segment, approximately 600 bp in size, was performed via the polymerase chain reaction (PCR) following the method outlined by Sambrook & Russell (2001). The resulting PCR products were analyzed through agarose gel electrophoresis (Bharose et al., 2017).

Subsequently, the PCR amplicons were purified using the Isolate II PCR and Gel Kit (Bioline), and sequencing was conducted by Macrogen (South Korea). The obtained sequences were then processed and analyzed using BioEdit software, and a comparison was made with the NCBI gene database using the BLAST tool.

Following the identification process, the extracellular enzyme exhibiting the highest fibrinolytic activity, derived from the fermentation of black turtle bean natto by *Bacillus* sp. strains, underwent purification. This was achieved through a two-step protein precipitation process: initially with 60% ammonium sulfate, followed by 80% ethanol precipitation (Yanagisawa et al., 2010). The resulting protein product was subsequently separated, and the nattokinase enzyme was identified via ion exchange chromatography, utilizing its isoelectric point of 8.6 (Hmood & Aziz, 2016). Finally, the purified enzyme was analyzed using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Yanagisawa et al., 2010).

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 truly influence on nattokinase enzyme activity. The experimental design consisted of 13 runs with five levels for each parameter. There were 6 investigated factors, 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 is created using Stagraphics Centurion software, version XV, with nattokinase enzyme activity as the monitored response. The nattokinase production was recorded for each experiment. Analysis of variance (ANOVA) was performed to determine the significance of each factor, and the significant factors were further optimized using response surface methodology (RSM).

Optimization of conditions for natto fermentation using RSM

In the evaluation of the clot-dissolving potential of black turtle bean natto, the simultaneous interaction of fermentation factors was found to impact the nattokinase enzyme activity. To analyze this multi-variable relationship, the experimental statistical modeling technique of response surface methodology (RSM) was employed. A central composite design (CCD) with seven factors and five levels was utilized to determine the optimal process parameters for maximizing nattokinase production. The experimental design was generated using Stagraphics Centurion software, version XV. The association between the independent variables and the response variable was described by a second-order polynomial model. The coefficients of this polynomial equation were calculated using statistical tools, and response surface plots were generated to visualize the relationship between the variables and the response (Deepak et al., 2008). This approach enabled the researchers to identify the optimal conditions for maximizing the clot-dissolving capacity of the nattokinase enzyme in the black turtle bean natto fermentation process.

Statistical analysis

The results are presented as mean values along with their corresponding standard deviations, calculated from a minimum of three independent measurements to ensure accuracy. Statistical analysis was performed using the Statgraphics Centurion XV software and Microsoft Excel 2016, providing a comprehensive evaluation of the data. Specifically, Analysis of Variance (ANOVA) was employed to identify significant differences among the experimental groups, while the Least Significant Difference (LSD) test was applied to pinpoint where those differences occurred. The Statgraphics Centurion XV software was instrumental in conducting these analyses, ensuring robust and precise computations. A significance level was determined, with p-values less than 0.05 considered statistically significant, indicating that the observed differences were unlikely due to random chance.

III. RESULTS AND DISCUSSIONS

Suitable *Bacillus* sp. for black turtle bean natto fermentation

The fibrinolytic activity was assessed by examining the ability of the crude extracellular enzyme derived from natto fermentation to dissolve artificial blood clots composed of thrombin-fibrinogen. A two-way ANOVA analysis was conducted to evaluate the relationship between independent factors such as fermentation time and different *Bacillus* strains regarding fibrinolytic activity. As shown in Figure 1, there is a significant correlation between the bacterial strains used in fermentation and the resultant enzymatic activity. The results indicate an increasing order of fibrinolytic activity among the *Bacillus* strains CO18, MO39, MS05, TO46 respectively.

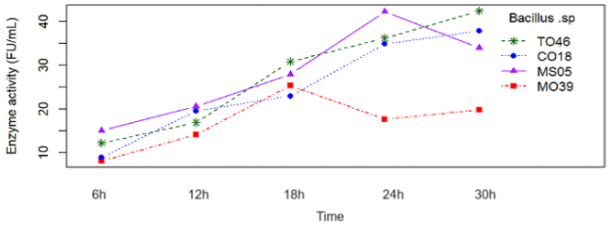

Figure 1. Coefficients of *Bacillus* sp. strains and fibrinolytic activity.

Figure 1 highlights that the *Bacillus* sp. TO46 strain exhibited the highest fibrinolytic activity, reaching 42.36 FU/mL after 30 hours of fermentation. In comparison, *Bacillus* sp. MS05 achieved a peak activity of 42.23 FU/mL at 24 hours, with no statistically significant difference observed between the two strains at their respective peak times. However, *Bacillus* sp. TO46 maintained a consistently higher fibrinolytic activity from 24 to 30 hours, surpassing all other strains during this period. These findings suggest that *Bacillus* sp. TO46 is more suitable for prolonged fermentation processes, demonstrating both high and stable fibrinolytic activity over time. Conversely, *Bacillus* sp. MS05, despite its slightly lower peak activity, reached its maximum enzyme production in a shorter time frame (24 hours), indicating its potential for shorter fermentation cycles.

The distinct characteristics of both strains—TO46's sustained enzyme activity and MS05's rapid enzyme production—make them ideal candidates for further research. By selecting these two strains, the study ensures that both efficiency (MS05) and productivity (TO46) are explored in optimizing black turtle bean natto fermentation.

Effect of carbon and nitrogen sources on nattokinase enzyme activity

This study investigated the influence of different sugar sources (glucose, lactose, maltose, and sucrose) and nitrogen sources (peptone, yeast extract, and whey protein) on nattokinase activity during fermentation. According to Figure 2a, glucose supplementation at a concentration of 5% demonstrated the highest nattokinase activity, reaching 278.38±1.11 FU/mL with *Bacillus* sp. TO46. This result aligns with findings by Suwanmanon & Hsieh (2014), emphasizing glucose's efficacy as a carbon source. In contrast, lactose at 4% and maltose at 2% showed significant enzyme activity when fermented with *Bacillus* sp. MS05, indicating that different strains might respond distinctively to carbon sources. Notably, sucrose at 2% yielded the lowest nattokinase activity, confirming that sucrose is not an ideal carbon source for *Bacillus subtilis* in black turtle bean natto fermentation.

Similarly, Figure 2b shows that peptone at a concentration of 2% had the most substantial effect on nattokinase activity, reaching 277.15±1.2 FU/mL. This is consistent with earlier studies on natto fermentation (Suwanmanon & Hsieh, 2014; Deepak et al., 2008), reinforcing peptone's role as an optimal nitrogen source. These results indicate that *Bacillus* sp. TO46, when supplemented with glucose and peptone, can produce high levels of nattokinase, suggesting the efficacy of using black turtle beans as an alternative substrate.

It is noteworthy that the glucose concentration employed in this study is higher compared to previous reports, while the peptone concentration fluctuates, being either higher or lower, contingent upon the specific study. These discrepancies can be ascribed to variations in the source materials, as black turtle beans contain a reduced amount of glycine compared to soybeans. Consequently, a more substantial supplementary nutritional input may be requisite for the synthesis of nattokinase with elevated activity during black turtle bean natto fermentation (Pradhananga, 2019; Amin et al., 2020). Furthermore, the results depicted in Figure 2 reveal that the *Bacillus* sp. strain TO46 manifests superior fibrinolytic enzyme activity relative to that produced by the *Bacillus* sp. strain MS05. Accordingly, *Bacillus* sp. TO46 was selected for the fermentation process of black turtle bean natto.

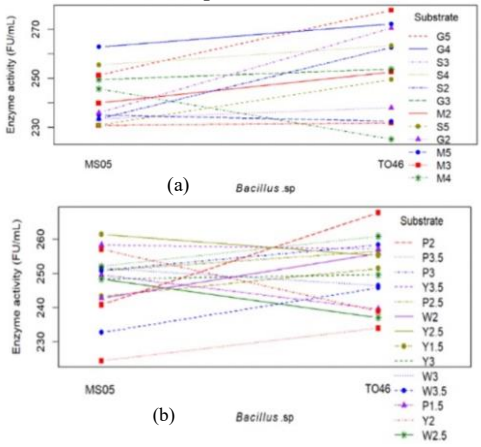

Figure 2. The influence of different nutrient sources on enzyme activity. (a) Carbon source: G-glucose, S-sucrose, M-maltose; (b) Nitrogen source: P-peptone, Y-yeast extract, W-whey protein.

Identification of *Bacillus* sp. TO46 and nattokinase from black turtle bean natto

The specific primer pair Bsub5F and Bsub3R successfully amplified a 602 bp gene fragment of *Bacillus* sp. TO46 (Figure 3). The sequence of this fragment was then compared with bacterial 16S rRNA gene sequences available in the NCBI database. The comparative results, visualized through a phylogenetic tree (Figure 4), confirmed that *Bacillus* sp. TO46 (GenBank accession number PQ328223.1) is genetically closely related to *Bacillus subtilis* subsp. *subtilis* EPP65 (MK560071.1) and *Bacillus subtilis* BC210917 (OR381485.1)

However, the *Bacillus* sp. TO46 strain occupies a distinct branch,of genetic divergence from other *Bacillus subtilis* strains, including those typically involved in soybean natto production. This suggests that TO46 possesses unique genetic characteristics compared to other *Bacillus subtilis* strains. Such genetic distinctiveness implies that *Bacillus subtilis* TO46 might have specific biological properties, offering a promising foundation for further research on its potential application in natto production using alternative substrates beyond soybeans.

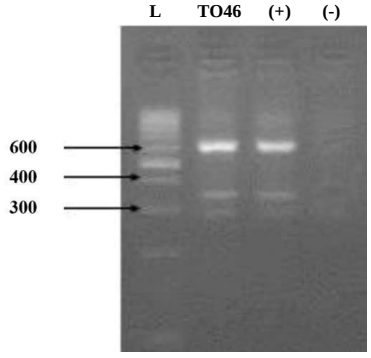

Figure 3. Electrophoresis to assess *Bacillus* sp. TO46. (L) Ladder 100bp, (TO46) *Bacillus* sp. TO46 strain, (+) positive control, (-) negative control

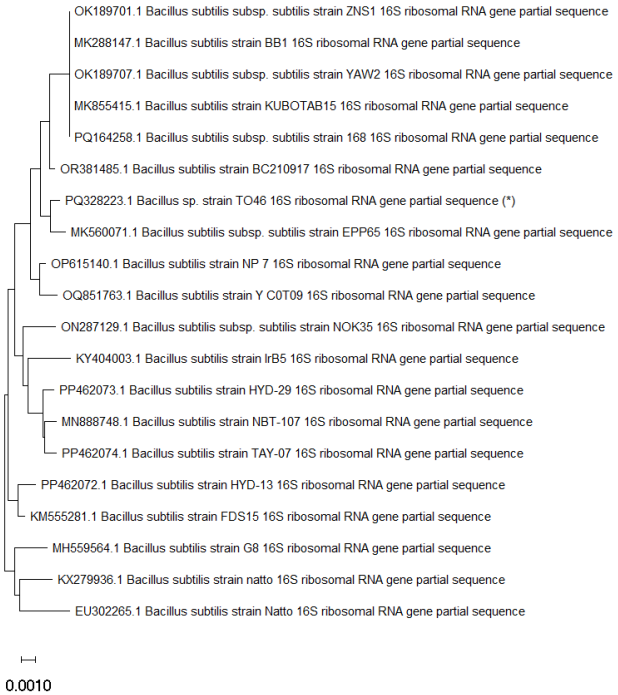

Figure 4. The similarity between the *Bacillus subtilis* TO46 and the sequence in NCBI database

Numerous studies have shown that the nattokinase enzyme, which dissolves blood clots, is produced by the *Bacillus subtilis* bacteria during the natto fermentation (Hmood et al., 2016; Yanagisawa et al., 2010; Hmood & Aziz, 2016). SDS-PAGE analysis (Figure 5) demonstrated that the precipitation steps effectively removed specific extracellular proteins and confirmed the presence of nattokinase in the crude enzyme extracted from black turtle bean natto, with a molecular weight of approximately 27 kDa, consistent with previous reports (Yanagisawa et al., 2010). This suggests that *Bacillus subtilis* TO46 is capable of producing nattokinase with fibrinolytic properties.

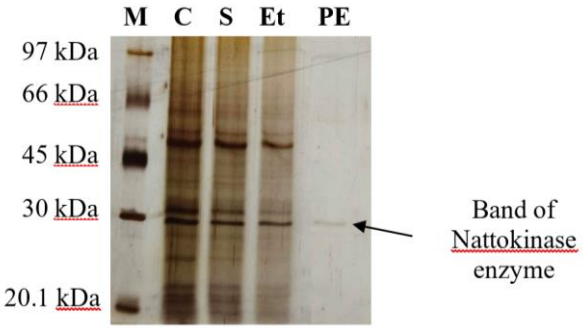

Figure 5. SDS-PAGE electrophoresis to assess nattokinase. (M) Prestained protein LMW marker, (C) Crude enzyme, (S) Salt precipitated enzyme, (Et) Ethanol precipitated enzyme, (PE) Purified enzyme

Nattokinase activity improvement by optimization of conditions for natto fermentation

Screening the factors that truly affect the fermentation process

A one-factor-at-a-time approach, guided by existing literature, was employed to screen the factors affecting the fermentation process of *Bacillus subtilis* TO46 using 5% glucose, 2% peptone, and specific culture conditions such as bacterial density, temperature, and fermentation time (Table 1). The optimal conditions for attaining the highest nattokinase enzyme activity were determined to be a bacterial density of 10^4 CFU/g, a fermentation temperature of 30°C, an initial pH of 6.0, and a fermentation time of 36 hours. The corresponding enzyme activities for each 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, with a confidence level of $p<0.05$.

It is important to note that the enzyme activity observed under these optimal conditions may vary compared to previous experiments, highlighting the critical role fermentation factors play in enhancing or inhibiting nattokinase production. During the fermentation process of *Bacillus subtilis* bacteria in natto production, both positive and negative influencing factors have optimal thresholds. Exceeding these thresholds inhibits bacterial growth and subsequently reduces nattokinase enzyme activity (Yang et al., 2021). This indicates the necessity for careful optimization of fermentation conditions to achieve high nattokinase activity.

Table 1. Nattokinase enzyme activity under single-factor surveys.

Run	Starter Density (CFU/ g)	Enzyme Activity (FU/mL)	Tempera -ture (°C)	Enzyme Activity (FU/mL)	pH	Enzyme Activity (FU/mL)	Time (Hrs)	Enzyme Activity (FU/mL)
1	10^3	219.4±0.97^{ab}	25	202.8±7.1^{bc}	5.0	254.6±4^{a}	12	210.1±1.1^{b}
2	10^4	226.4±1.37^{a}	30	223.8±6.5^{a}	6.0	248.6±2.5^{ab}	24	235.8±12^{ab}
3	10^5	193.3±1.57^{bc}	35	203.9±3.9^{bc}	7.0	221.4±11.3^{bc}	36	248.4±16.5^{a}
4	10^6	180.5±1.5^{c}	40	191.4±2.7^{c}	8.0	236.7±6.5^{abc}	48	236.3±8.9^{ab}
5	10^7	214.5±1.69^{ab}	45	196±5.4^{c}	9.0	222.6±17.5^{bc}	60	228.3±5.8^{ab}
	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.

The observed optimal pH and temperature conditions were consistent with studies by Suwanmanon & Hsieh (2014), which reported similar ranges for maximizing nattokinase production. However, our findings regarding the impact of glucose concentration differed, as glucose concentration showed a minimal effect on enzyme activity in this study. This contrasts with reports on soybean natto fermentation, where glucose played a more substantial role, suggesting inherent differences between black turtle beans and soybeans in their fermentation requirements.

The Blackett-Burman matrix was employed to evaluate the independent effects and interactions of factors such as glucose concentration, peptone concentration, bacterial density, fermentation temperature, pH, and time. Figure 6 illustrates that the initial pH of the substrate had the most significant impact on nattokinase activity, followed by fermentation time, peptone concentration, initial bacterial density, glucose concentration, and temperature. Notably, an increase in fermentation time resulted in decreased enzyme activity, whereas other factors had a positive impact.

Interestingly, the negligible impact of glucose concentration and fermentation temperature on enzyme activity in black turtle bean natto is consistent with certain findings in soybean natto studies (Suwanmanon & Hsieh, 2014; Wang et al., 2009). This indicates that, despite different substrate sources, certain fermentation parameters might exhibit universal trends in influencing nattokinase activity.

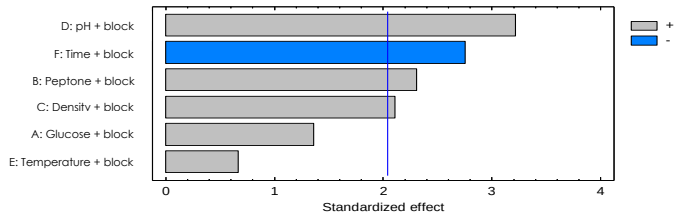

Figure 6. Influence of factors on nattokinase enzyme activity

Optimization of conditions for black turtle bean natto fermentation using RSM

Based on the screening results, four critical factors—initial pH (5.0 - 9.0), fermentation time (12 - 60 hours), peptone concentration (1.5 - 3.5%), and initial bacterial density (10^3 to 10^7 CFU/mL)—were selected for optimization using the Response Surface Methodology (RSM) with a Central Composite Design (CCD) model. The experimental model consisted of 26 trials designed using Statgraphics Centurion optimization software.

The influence of each factor was analyzed, with pH being the most influential, followed by peptone concentration, initial bacterial density, and fermentation time. The polynomial equation for nattokinase enzyme activity Y (FU/mL) = $-1178.07 + 275.355*X_1 + 19.7741*X_2 + 220.015*X_3 + 104.913*X_4 - 26.7765*X_1^2 - 1.32823*X_1*X_2 + 10.7808*X_1*X_3 + 9.76781*X_1*X_4 - 0.122106*X_2^2 - 0.514241*X_2*X_3 - 0.784647*X_2*X_4 - 41.0529*X_3^2 - 6.33031*X_3*X_4 - 15.1853*X_4^2$, with R-squared value of 0.6575, indicating that 65.75% of the variation in nattokinase activity could be explained by these fermentation conditions. the constant value of the regression equation was -1178.07, a negative value, suggests that additional factors not accounted for in this study could also contribute to nattokinase production.

The optimal conditions predicted by the model included 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, yielding an expected enzyme activity (Ymax) of 436.212 FU/mL. Experimental verification under these optimized conditions resulted in observed enzyme activity (Y'max) of 418.32 FU/mL, with a Y'max/Ymax coefficient of 0.9760, indicating a high level of model accuracy.

Comparing the results to previous studies on soybean natto (Suwanmanon & Hsieh, 2014; Liu et al., 2005), the optimized conditions for black turtle bean natto produced higher nattokinase activity, underscoring the potential of black turtle beans as a promising alternative substrate. This reinforces the significance of using black turtle beans, particularly for individuals seeking soy-free options without compromising on nattokinase benefits.

Potential Limitations and Future Considerations

While this study effectively demonstrated the feasibility of using black turtle beans for nattokinase production, certain limitations must be acknowledged. The use of specific *Bacillus subtilis* strains, which may not fully capture the potential variability in enzyme activity that could be achieved with other strains. Additionally, the optimization model accounted for only a limited number of factors, suggesting that other variables, such as humidity, agitation speed, or oxygen levels, could further influence enzyme activity.

Future studies should aim to explore a wider range of *Bacillus* strains and fermentation conditions to optimize nattokinase production further. Investigating the scalability of these findings in industrial fermentation settings is also crucial to ensure the practical application of black turtle bean natto as a viable alternative to traditional soybean-based natto products.

IV. CONCLUSION

The findings of this study demonstrate that black turtle beans serve as a viable alternative substrate for natto fermentation, producing a nattokinase enzyme with high fibrinolytic activity comparable to traditional soybean-based natto. The *Bacillus subtilis* TO46 strain exhibited superior enzyme production, especially under optimized fermentation conditions identified using Response Surface Methodology. These results are particularly significant for the development of hypoallergenic natto products, expanding the potential market to individuals with soybean allergies. Moreover, the enhanced enzyme activity

achieved using black turtle beans underscores the substrate's potential for commercial application, contributing to the diversification of natto products.

Despite the promising outcomes, further research is necessary to explore the scalability of this process in industrial settings and to investigate other *Bacillus* strains that may enhance nattokinase production even further. Additionally, optimizing other fermentation parameters, such as humidity, agitation speed, and oxygen levels, could improve enzyme yield. Overall, this study provides a solid foundation for the potential commercialization of black turtle bean natto, offering a novel and allergen-friendly product for the health food industry.

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