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Article

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Determination of factors affecting the protease content generated in fermented soybean by *Bacillus subtilis* 1423

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Abstract

The objective of the study to determine the factors affecting the protease content generated during soybean fermentation by *Bacillus subtilis*. In this study, four factors were investigated during soybean fermentation: (1) pH, (2) fermentation temperature, (3) fermentation time and bacterial density, and (4) added glucose content on steamed soybean. The experimental results showed that at 33 °C temperature, 48 h fermentation time, bacterial density 10⁴ CFU, pH 7, and supplementation of 1 g glucose into 50 g steamed soybean, *Bacillus subtilis* fermented soybean (another name is natto) had the highest protease activity. This suggests that the fermentation conditions in the study are consistent with the growth of *Bacillus subtilis* to produce proteases. Moreover, the optimization of fermentation conditions contributes to reducing the energy consumption to obtain the desired product, by increasing the yield in reaction. Therefore, this study is important at an industrial level, while contributing to the rational use of energy.

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Keywords: Soy; Soybean; Natto; Protease; Nattokinase; *Bacillus subtilis*

1. Introduction

Soybean is a very popular ingredient in Vietnam and around the world in the field of food production. Soybeans have many healthy nutrients. This protein is good for health because it has less fat and cholesterol. The essential amino acids that the body cannot produce are found in soybeans. Natto is a popular Japanese dish made from soybeans fermented with the *Bacillus Subtilis* bacteria. It is considered a healthy food because of its natural, unprocessed character, and is even consumed daily by many people in Japan. Natto may not only prevent blood clots from forming, but also dissolve them. In 1980, Dr. Hiroyuki Sumi analyzed 173 different foods capable of dissolving blood clots and found that natto had the most active “thrombolysis”.

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Currently, the incidence of arterial obstruction caused by thrombi such as myocardial infarction or cerebral infarction is increasing in Vietnam, as well as in the world. The total number of patients admitted to the Vietnam Institute of Cardiology has increased markedly in recent years, an increase of 53.5% of patients admitted to the Hospital within 5 years. In particular, the rate of ischemic heart disease tends to increase from 11.2% in 2003 to 24% in 2007 [1].

This present study covers the benefits that natto offer to users in preventing coronary artery disease. In addition to drug treatment and myocardial reperfusion, other treatments increase the quality of life, reduce risk factors, use non-pharmacological treatments, change lifestyle [2]. In this study, soybeans were fermented in the laboratory under different fermentation conditions. The factors affecting the fermentation process and the protease content investigated include: fermentation temperature, fermentation time, bacterial density, pH and added glucose content.

There are some remarkable related studies, such as “Isolating *Bacillus subtilis* from Japanese natto to breed natto production” [3]. In that research, two strains of *B. subtilis* N18 and N44 that have an ability to produce protease identified. [4], China, Korea [5] and South Korea [6]. Another study concludes that nattokinase (abbreviated: NK, also known as subtilisin NAT) (EC 3.4.21.62) is one of the most significant extracellular enzymes was produced by *Bacillus Subtilis* [7]. This enzyme causes fibrinolysis, the breakdown of the fibrin in blood clots, and is a useful agent for the treatment of oral fibrinolysis. Therefore, NK is considered a nutritious, safe and easy to mass produce dietary supplement. Other important health applications for NK include: hypertension, Alzheimer’s disease, and vitreoretinal disorders. In the study “Production, purification and characterization of nattokinase from *Bacillus subtilis*, isolated from tea garden soil samples of Dibrugarh, Assam” [8], the production of nattokinase from *Bacillus subtilis* was isolated from 5 soil samples from different tea gardens is located in Dibrugarh Assam district. The results describing the characteristics of enzyme purification show optimal conditions at 37 °C, pH 7, 5% substrate concentration and 1 ml of 10% zinc chloride. Enzymatic activity was found to decrease gradually when the temperature exceeded 70 °C. Pure enzymes were identified and the results found a promising future for research and commercial production. In “Purification and characterization of nattokinase from *Bacillus subtilis* natto B-12” [9], *Bacillus subtilis* natto B-12 was isolated from Natto, a traditional Japanese fermented soybean food. This study provides some important information about the factors affecting blood fibrinolytic activity, selective methods, and characteristics of nattokinase isolated from *B. subtilis* natto B-12, which enriches the theoretical information available for the development and research of nattokinase as a functional additive of food. Enzyme nattokinase is interested in production research and widely used to prevent arterial obstruction caused by blood clots [10]. Nattokinase: An oral antithrombotic to prevent cardiovascular disease [11]. The mechanisms of nattokinase activity directly cleave fibrin in the thrombus and, indirectly by activating the production of urokinase and plasmin in tissue [12]. Another study related to economic efficiency due to reduced energy costs in the production of fermented products “Evaluation of Energy Saving Using a New Yeast Combined with Temperature Management in Sparkling Base Wine Fermentation” [13], Heat removal accounts for ~90% of the total energy requirements of the winery and is mostly related to the temperature control of the wine tanks used for fermentation and maturation, the result shows that Fermentation required 21.6 Wh/Lgrape must at 15 °C and 7.7 Wh/Lgrape must at 19 °C, reducing energy use by ~65% at the higher temperature without affecting sensory characteristics of the wine. Besides, *Bacillus Subtilis* is also applied in many other fields such as Biosurfactants produced by *Bacillus subtilis* A1 and *Pseudomonas stutzeri* NA3 reduce longevity and fecundity of *Anopheles stephensi* and show high toxicity against young instars [14]; Reducing soil pollution caused by crude oils [15]; *Bacillus subtilis* HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, *Oreochromis niloticus* [16]; improved of hybrid Hulung grouper’ growth and health [17].

2. Materials and methods

2.1. Materials

Bacillus Subtilis (isolated from the biological laboratory of the Department of Agriculture and Aquaculture, Tra Vinh University), soybeans (purchased from the Department of Plant Breeding, Department of Agriculture and Applied Biology, University of Can Tho). Glucose purchased from Dong Hanh Phat Technology Co., Ltd, HCMC

2.2. Research methods

Bacterial isolation: *Bacillus* strains are isolated from commercial Natto by spreading on pepton (MPA) gypsum agar at 37 °C for 24 h. The colonies are purified. The colony morphology is then observed and noted, and biochemical reactions are tested in order to select specific strains of interest. The microorganisms are thereafter identified by sequencing method and the chosen strains are preserved in inclined agar at 4 °C.

Soybeans are soaked overnight to expand the outer shell and facilitate its separation. After separating the bean pods, they are rinsed several times to remove the overnight water soaked beans and put in a 45-min autoclave cycle until the beans are tender, then cool. Afterward, they are put into a triangle bottle at a quantity of 50 g of beans per bottle. The previously isolated *Bacillus* spp. strains are then cultured in seeded soybeans at a rate of 1% of 2 cm environmental thickness and incubated at 37 °C. A 24-h fermentation time is elapsed before the protease and nattokinase enzymes are collected. Crude enzyme products are obtained into vacuum presses and cold storage. When conducting experiments to determine the parameters' values for optimized protease activity (pH, fermentation temperature, bacterial density and fermentation time, the amount of glucose added to the fermentation medium) in each experiment will change the corresponding parameters, remaining parameters are fixed.

Qualitative and quantitative analysis of protease and nattokinase: Total protease includes nattokinase and other proteases. If the total protease activity is high, nattokinase activity is also likely high. When testing for nattokinase preparations, nattokinase is obtained and accounts for the majority of the total protease (over 80%) [18]. Because of the very expensive cost of chemical analysis of nattokinase, this experiment focuses on identifying protease content, which we believe is a good proxy for assessing overall nattokinase activity. In this study, quantification of protease activity by improved Anson method.

2.3. Physical and chemical analysis

Content of generated protease, protid, lipid, glucid content and DPPH. Only presented generated protease content in this paper.

2.4. Methods of data processing

All methods of analysis in this study are done via the Stagraphic Centurion software

3. Results and discussion

Results of physical and chemical analysis:

Determination of fermentation conditions to generate high protease content: pH, fermentation temperature, added glucose content into fermentation medium

As we can see from Table 1, in the experiment (1) to identify pH (column 1 and 2 from left side) the protease activity was the lowest (169.29 U/g) at pH = 6.3 (the natural pH of soybean) while it had the highest value (280,897 U/g) at pH 7. This result is consistent with the study of Nguyen Duc Luong and Nguyen Thi Thuy Duong [19] and Slepecky and Hemphill [20]. These authors suggested that *Bacillus Subtilis* has the ability to synthesize protease, amylase and catalase enzymes at a suitable pH of 7.0–7.4 [19,20]. At a fermentation temperature of 28 °C of the experiment (2), protease activity was quite high. However, the highest protease activity was found to be at 33 °C (178.64 U/g) and gradually decreased with increasing temperature. At the two remaining fermentation temperatures, and especially at 38 °C, protease activity is considered to be low (122.64 U/g). In accordance with these results, the optimal natto fermentation temperature was chosen to be 33 °C. In the study of Nguyen Anh Tuan [21] the highest nattokinase yield was 69.3 ± 0.2 FU/mL of a substrate in an optimized medium after 20 h of fermentation in 37 °C and pH 7.5. The fermentation temperature was higher in our study but fermentation time was shorter than ours. Besides, Fermentation medium components of our studies were not quite the same [21]. Another study, Natto is a fermented traditional soybean product in Japan. When forming natto, the soybeans are first soaked and cooked, then mixed with the *Bacillus subtilis* to allow fermentation up to 24 h at 40 °C. Then the natto is cooled and aged in a refrigerator for up to 1 week to allow the development of stringiness [22]. Generally, the fermentation time in these studies was shorter than in our study. However, the fermentation process depends on many factors as fermentation medium, temperature, bacterial density, and oxygen content.

Table 1. Protease content at different fermentation conditions.

pH (1)	Protease content (U/g)	Fermentation temperature (°C) (2)	Protease content (U/g)	Additional glucose content (g/50 g soybeans) (3)	Protease content (U/g)
6.0	184.897ab	28	162.167b	0.25	164.653e
6.3	169.29c	33	178.64a	0.5	175.143de
7.0	280.897a	35	138.487c	0.75	181.97d
8.0	209.077b	38	122.64d	1.0	221.68c
				1.25	311.58a

Note: In the same column, the numbers with the same following letters are not significant at 5%.

In an experiment (3), glucose added to the fermenting medium at 6 different levels (0.25–1.25 g/50 g soybean). When supplemented with 1 g glucose/50 g, soybeans showed high protease enzyme and antioxidant activity while in addition also exhibiting the high content value of other nutrients. Statistical results showed that increasing the amount of glucose added to the fermenting medium beyond 1 g glucose/50 g of soybeans, did not significantly increase the nutrient content value. The supplementation of 1 g glucose/50 g of soybeans thus proved to be sufficient and was therefore chosen as the optimal value for the nattokinase production process.

Bacterial density and natto fermentation time

The changes in fermentation bacteria density did not significantly affect the concentration of nutrients and antioxidant activities at a 5% significance level ($p > 0.05$). However, when analyzing the interactions between bacterial density and fermentation time, there were significant differences (Tables 2 and 3). At density 10^4 and 48 h fermentation time, significant differences were found compared to the rest. In Fig. 1, we see that the yellow area, where the bacterial density ≥ 4 and the fermentation time is situated between 44 and 52 h, effectively shows the highest protease activity. Moreover, at 48 h fermentation time the protease activity was higher than the remaining time (this is an important indicator in determining the result). Based on the statistical results and sampling criteria so that the highest enzyme activity should choose the density of 10^4 , fermentation time 48 h for the next experiment.

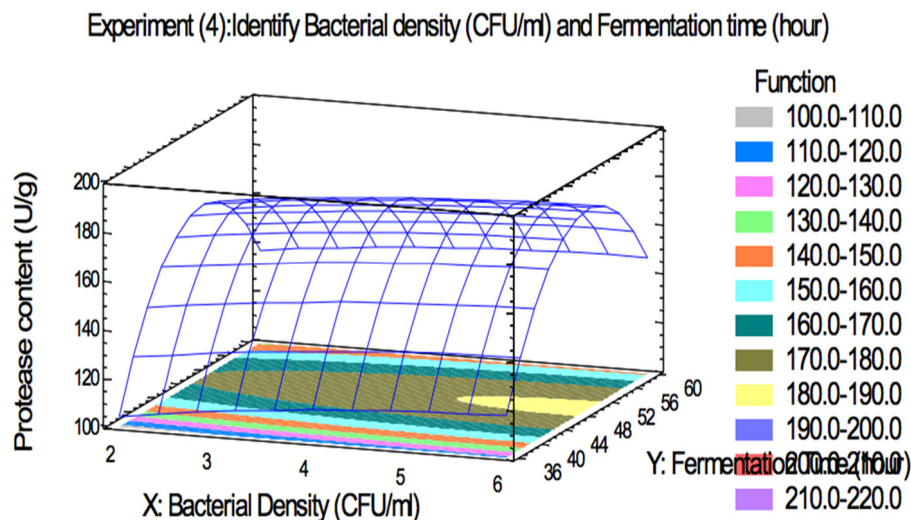


Fig. 1. Changes in protease content in natto according to bacteria density and fermentation time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

The results showed that at 33 °C, fermentation time of 48 h, bacterial density 10^4 , pH 7 and supplementation of 1 g glucose/50 g of steamed soybeans for natto had the highest protease activity. We hope that this study will be applied in practice to diversify soy food products, bringing healthy food, promote functional products and contribute

Table 2. Protease content change according to bacterial density and fermentation time.

Bacterial density CFU/ml (4)	The average value of the protease (U/g)		
	Fermentation time (h)		
	36	48	60
10 ²	162.78c	141.26a	177.37b
10 ³	124.86c	156.05a	160.42b
10 ⁴	105.08c	173.36a	151.11b
10 ⁵	95c	185.45a	135.52b
10 ⁶	71.76c	223.58a	96.05b

Note: In the same column, the numbers with the same following letters are not significant at 5%.

Table 3. Protease content changes significantly at 5% level ($p < 0.5$).

	Count	Mean	Homogeneous groups
Bacterial density	45	4.0	X
Fermented time	45	48.0	X

to a new look for human health friendly food lines. On the other hand, this research will be able to connect with many other sciences to optimize production processes. All researches are in high-temperature conditions suitable for high humid tropical conditions. This is a new step that needs to be paid attention to by many multi-field scientists. Challenges and prospects of renewable technology to save energy cost based on natto processing in a tropical area.

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