International Journal on Advanced Science Engineering Information Technology

Vol.10 (2020) No. 3
ISSN: 2088-5334

Screening for Optimal Parameters of Nattokinase Synthesis by *Bacillus subtilis natto* in Solid-State Fermentation

Cuong Viet Bui[a,1], Minh Nguyet Thi Nguyen[a,2], Minh Hanh Thi Truong[a,3], Xuan Dong Bui[b]

[a] *Department of Food Technology, Faculty of Chemical Engineering, University of Science and Technology, The University of Danang, Danang City, 550000, Viet Nam*
E-mail: [1]bvcuong@dut.udn.vn; [2]ntmnguyet@dut.udn.vn; [3]ttmhanh@dut.udn.vn

[b] *Department of Bio-Technology,Faculty of Chemical Engineering, University of Science and Technology, The University of Danang, Danang City, 550000, Viet Nam*
E-mail: xdbui@dut.udn.vn

Abstract*— Nattokinase, which is an extracellular enzyme synthesized by *Bacillus subtilis natto*, is a medication for cardiovascular disease treatment. In this study, soybean seed was used as a substrate for culturing *Bacillus subtilis natto* in solid-state fermentation to produce nattokinase. The optimal culture parameters for solid-state fermentation to synthesize nattokinase by *Bacillus subtilis natto* were 2:50 (v:w) of the ratio of *Bacillus subtilis natto* pre-culture to the substrate, 42 h of fermentation time, and 3 cm of the thickness of the substrate. At this optimal culture parameter of solid-state fermentation by using *Bacillus subtilis natto*, the enzymatic activity of crude nattokinase was 7.13 ± 0.2, 16.24 ± 0.33, and 16.55 ± 0.06 (specific activity/mL), respectively. Furthermore, we aim to apply this study with large scale production initially. Thus, the ratio of reusing *Bacillus subtilis natto* in fermentation product to substrate for a new process of solid-state fermentation and circulation time of reusing *Bacillus subtilis natto* in new solid fermentation process were screened. The maximal enzymatic activity of crude nattokinase of 14.10 ± 0.18 (specific activity/mL) was found at 3:100 (w:w) of reusing *Bacillus subtilis natto* in fermentation product to substrate for a new process of solid-state fermentation. The suggestion for the circulation time of reusing *Bacillus subtilis natto* for new solid fermentation process was 2nd. The results of this study had provided the necessary information for further research on nattokinase.

Keywords*— nattokinase; *Bacillus subtilis natto*; optimal culture parameters; reusing *Bacillus subtilis natto*; circulation time; solid-state fermentation.

I. INTRODUCTION

Cardiovascular diseases: myocardial infarction, cerebral infarction, high blood pressure, etc. are the main causes of global death [1], [2]. According to statistical data of the World Health Organization, the diseases caused 17.3 million deaths, which were accounted for 30% of global death in 2008 [3]. The number of global deaths caused by cardiovascular diseases is projected to increase to 23.3 million in 2030. Global efforts have been made to prevent death from cardiovascular disease [4].

The turbidity of the blood vessel by thrombosis is a major cause of cardiovascular disease. Insoluble fibrin fibers form thrombosis, insoluble fibrin fibers are necessary protein, and the major component of thrombosis is insoluble fibrin fibers [5]. Pharmaceutical products: plasminogen activator (t-PA), urokinase, streptokinase, etc. have been used extensively to treat cardiovascular diseases caused by thrombosis. Nevertheless, these pharmaceutical products have side effects (blood loss, allergies), short duration time, high cost, and large doses, etc. as their disadvantages [6].

Recently, insoluble fibrin fibers-degrading enzymes have been discovered in traditional fermented foods: Natto of Japan [7], Cheonggukjang of Korea [1], [8], Douchi of China [9]. Nattokinase, one of the extracellular enzymes synthesized by *Bacillus subtilis natto*, is capable of degrading insoluble fibrin fibers of thrombosis and promoting the formation of t-PA to further enhance the efficiency of degrading insoluble fibrin fibers of thrombosis [10]. Highly purified microbial enzymes extracted from fermented food have a potential application as medication [11]-[13].

In comparison to submerged fermentation, solid-state fermentation has many advantages such as low initial substrate requirements, minimal contamination, no foaming during fermentation time, low operating costs, energy-saving, simple product acquisition process, etc. [14]-[16]. In addition, soybean seed contains a balanced composition in

nutrients [17]. Thus, it is always used for natto production with a traditional scale.

This study aims to explore the advantages of solid-state fermentation for producing nattokinase by using *Bacillus subtilis natto* with soybean seed as a substrate. Optimal culture parameters for *Bacillus subtilis natto* cultivation to produce nattokinase were determined to provide initial information for further study of large-scale production of nattokinase.

II. Materials and Methods

A. Materials

1) Soybean seed preparation: Soybean seed was provided by Vinasoy Factory, Quang Ngai Sugar Company, Viet Nam. Soybean seed was soaked in distilled water for 12 h at a ratio of soybean seed to distilled water of 1:5 (w:v). For use as a pre-culture medium, the soybean seed was then grinded with distilled water by using a household grinder at a ratio of soaked soybean seed to distilled water of 1:5 (w:v). The grinded mixture (liquor) was filtered by using filter cloth with a pore size of 0.2 mm to remove large particle size. The filtered liquor of soybean was filled into a 500 mL Erlenmeyer flask and was sterilized at 121°C, 1 atm, and 15 min using autoclave machine (Hirayama HVE-50, Japan). The soybean liquor was used as a medium for *Bacillus subtilis natto* pre-culture. As a substrate for solid-state fermentation, the soaked soybean seed was transferred into a 500 mL Erlenmeyer flask and was sterilized.

2) Microorganism: Bacillus subtilis natto was provided by Microbiology Laboratory, Department of Food Technology, Faculty of Chemical Engineering, University of Science and Technology, The University of Danang, Viet Nam. *Bacillus subtilis natto* was preserved on a nutrient agar medium containing 1 g/L of peptone, 0.5 g/L of meat extract, and 0.5 g/L of NaCl at 0°C. The bacteria were activated monthly by transferring into a fresh nutrient agar medium to maintain the activity of *Bacillus subtilis natto* during the study.

B. Experimental setup

1) Effect of ratio of Bacillus subtilis natto pre-culture to substrate on the enzymatic activity of crude nattokinase: Solid-state fermentation to synthesize nattokinase by *Bacillus subtilis natto* was carried out at different ratios of *Bacillus subtilis natto* pre-culture to the substrate (1:50, 2:50, 3:50, 4:50, 5:50, v:w). The fermentation was done for 30 h at 37°C with a thickness of the substrate of 3 cm.

2) Effect of fermentation time on the enzymatic activity of crude nattokinase: Fermentation times from 24 h to 48 h (4 h of increment) were used to study the effect of fermentation times on enzymatic activity of crude nattokinase. The optimal ratio of *Bacillus subtilis natto* pre-culture to the substrate, the thickness of the substrate of 3 cm, temperature fermentation of 37°C was used.

3) Effect of thickness of substrate on the enzymatic activity of crude nattokinase: Thicknesses of steamed and sterilized soybean seed as a substrate in the range 2 cm to 5 cm (1 cm of increment) were studied to produce nattokinase. The optimal ratio of *Bacillus subtilis natto* pre-culture to substrate, optimal fermentation time, and 37°C of temperature fermentation was employed.

4) Effect of ratio of reusing Bacillus subtilis natto in fermentation product to substrate on the enzymatic activity of crude nattokinase: Ratios of reusing *Bacillus subtilis natto* in fermentation product to the substrate of 1:100, 2:100, 3:100, 3.5:100, and 4:100 (w:w) were used for starting a new process of solid-state fermentation for nattokinase synthesis. Optimal culture parameters, including fermentation time and thickness of the substrate, were used for this study.

5) Effect of circulation time of reusing Bacillus subtilis natto on the enzymatic activity of crude nattokinase: Circulation times (1, 2, 3, and 4) of reusing *Bacillus subtilis natto* were explored for a new process of solid-state fermentation. The cultivation was performed with the optimal ratio of reusing *Bacillus subtilis natto* to the substrate, optimal fermentation time, and optimal thickness of the substrate.

C. Methods

1) Pre-culture and growth profile of Bacillus subtilis natto: The filtered soybean seed liquor was used as a medium for the pre-culture of *Bacillus subtilis natto*. The pre-culture of *Bacillus subtilis natto* was carried out by using a shaking incubator (Daihan IS-30, Korea) operated at 120 rpm and 37°C. Cultured samples that contained *Bacillus subtilis natto* biomass were collected from the pre-culture medium every four h. The samples were diluted and spread on Petri dishes containing nutrient agar medium. Petri dishes were incubated in an incubator (Memmert BE-400, Germany) at 37°C for 24 h. Colonies of *Bacillus subtilis natto* on petri dishes were counted. The growth curve of *Bacillus subtilis natto* was constructed, which was based on the intensity of *Bacillus subtilis natto* in the sample. The intensity of *Bacillus subtilis natto* (CFU/mL) in the sample was calculated by using the following equation:

$$M_t = \frac{A_t \times D}{V} \text{ (CFU/mL)} \quad (1)$$

Where,

- M_t : Intensity of *Bacillus subtilis natto* biomass in a sample at time t,
- A_t : Average number of colonies of *Bacillus subtilis natto* on a petri dish at time t,
- D : Dilution factor,
- V : Volume of the sample (mL).

2) Solid-state fermentation: Pre-culture of *Bacillus subtilis natto*/reusing *Bacillus subtilis natto* in fermentation product was thoroughly mixed with steamed and sterilized soybean seed as a substrate at a specific ratio of *Bacillus subtilis natto* pre-culture/reusing *Bacillus subtilis natto* in fermentation product to the substrate. Solid-state and fermentation were performed on a stainless steel tray (8×8 cm). An incubator (Memmert BE-400, Germany) was used to maintain solid-state fermentation temperature at 37°C. Solid-state fermentation product was mixed with Tris – HCl buffer (pH 9) at 4°C at a ratio of solid-state fermentation product to the Tris – HCl buffer of 1:3 (w:v). The mixture

was filtered by using filter cloth with a pore size of 0.2 mm to remove large particle size. The filtered liquor was centrifuged with a centrifugation machine (Hettich ROTINA 420R, Germany) at 4°C, 8,000 rpm. The supernatant containing crude nattokinase was kept in the refrigerator at 4°C for further analysis.

3) Proximate constituents of soybean seed determination: Proximate constituents of soybean seed (protein, lipid, total ash, moisture) were analyzed by using the standard method of Association of Official Analytical Chemists International [18]. Especially, protein content was determined by Kjeldahl method with a conversion factor of 6.25, lipid content was determined by Soxhlet extraction method, total ash content was determined by total ash method with 500 – 550°C of ash temperature, 8 – 12 h of ash time, moisture content was determined by drying method with 100°C of drying temperature, 18 h of drying time.

4) Enzymatic activity essay: Amano method was used to determine the enzymatic activity of crude nattokinase [19]. Casein 1% (1 mL) in the closed tube was incubated at 37°C for 15 min in an incubator (Memmert BE-400, Germany) and 1 mL of diluted crude nattokinase was added, and then the mixture thoroughly mixed. The mixture was incubated at 37°C for 60 min in an incubator (Memmert BE-400, Germany). Trichloroacetic acid 0.4 M (2 mL) was added to the mixture, and then the mixture (casein 1%, diluted crude nattokinase, and trichloroacetic acid 0.4 M) was shaken thoroughly and kept at 37°C for 25 min in an incubator (Memmert BE-400, Germany). Filter paper (Whatman No. 1) was used to remove the precipitate of the mixture. Filtered liquor (1 mL) was thoroughly mixed with 5 mL of Na_2CO_3 0.4 M (aq.) and 1 mL of folin reagent. The mixture (filtered liquor, Na_2CO_3 0.4 M and folin reagent) was stabilized at 37°C for 20 min in an incubator (Memmert BE-400, Germany). The absorption of the mixture was recorded at 660 nm by using UV-VIS spectrometer (Jenway spectrophotometer 6305, UK). Tyrosine solutions with different concentrations were used to construct a standard curve. Enzymatic activity of crude nattokinase was calculated by the equation.

$$\frac{(A_s - A_0) \times F \times n}{100} \text{ (specific activity/mL)} \quad (2)$$

Where,

- F: Generated tyrosine in hydrolysis reaction with casein as a substrate and crude nattokinase as a catalyst (µg),
- A_s: Absorption of the sample (ABS),
- A_0: Absorption of blank (ABS),
- n: Dilution coefficient of crude nattokinase (n)
- 100: Conversion coefficient.

5) Statistical analysis: A significant difference of obtained results was analyzed by ANOVA – One Way method [20].

III. Results and Discussion

A. Proximate constituents of soybean seed

Quality and proximate constituents of substrate were considered as an essential factor that affected the growth of microorganisms and products profile of solid-state fermentation. The major components found in solid-state fermentation medium were protein and carbohydrate [14], [21]. Table I have shown the proximate composition of soybean seed.

TABLE I
PROXIMATE CONSTITUENTS OF SOYBEAN SEED

Constituents	Percentage (%)
Protein	40.95 ± 0.08
Total carbohydrate	25.85 ± 0.12
Lipid	21.2 ± 0.09
Ash	5.2 ± 0.71
Moisture	6.8 ± 0.12

Soybean seed was rich in protein (40.95 ± 0.08%) and total carbohydrate (25.85 ± 0.12%), which were in agreement of the report by El-Shemy [17]. A high percentage of protein and total carbohydrate of soybean seed would be a perfect source for the growth of *Bacillus subtilis natto* and nattokinase synthesis in solid-state fermentation.

B. Growth profile of Bacillus subtilis natto

The growth curve of microorganisms had wide application in a different model of fermentation and large scale production. The sharpness of the growth curve of microorganisms was different toward a variety of substrates [15]. Fig. 1 showed a growth curve of *Bacillus subtilis natto* cultivated with extracted soybean seed liquor.

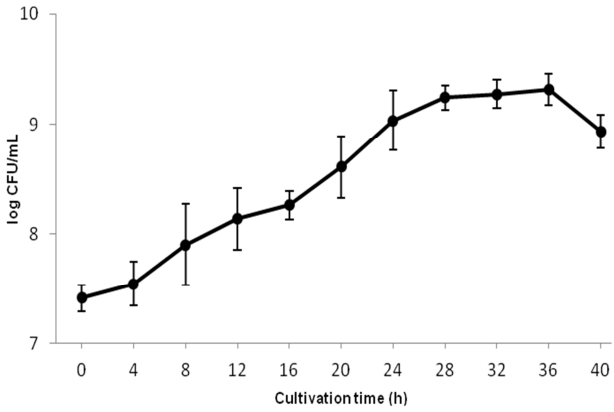

Fig. 1 The growth curve of *Bacillus subtilis natto*

According to Fig. 1, *Bacillus subtilis natto* grew slowly during 4 hours of cultivation time, and *Bacillus subtilis natto* biomass slightly increased to 7.54 log CFU/mL. Then, the biomass of *Bacillus subtilis natto* exponentially increased and reached the maximal value of 9.24 log CFU/mL corresponding to 1.08×10^8 CFU/mL of *Bacillus subtilis natto* intensity in the pre-culture medium at 28th hour of cultivation time. At the end of the log phase (28th hour), the quality and quantity of *Bacillus subtilis natto* were satisfied the requirements for solid-state fermentation. The duration time of the stationary phase was in 8 h from 28th of cultivation time to 36th of cultivation time. Then the death phase occurred afterward. Thus, the intensity of *Bacillus subtilis natto* of 1.08×10^8 CFU/mL at 28th hour of cultivation time with extracted soybean seed liquor was selected for solid-state fermentation.

C. *The effect of the ratio of Bacillus subtilis natto pre-culture to substrate on the enzymatic activity of crude nattokinase*

Generally, the ratio of *Bacillus subtilis natto* pre-culture to substrate significantly affected enzymatic activity of crude nattokinase. Enzymatic activity of crude nattokinase decreased with increasing ratio of *Bacillus subtilis natto* pre-culture to substrate in general (Fig. 2).

Enzymatic activity of crude nattokinase increased and reached the highest activity of 7.13 ± 0.20 (specific activity/mL) at 2:50 (v:w) of the ratio of *Bacillus subtilis natto* pre-culture to substrate. With increasing ratio of *Bacillus subtilis natto* pre-culture to substrate, the enzymatic activity of crude nattokinase decreased because the amount of substrate provided for nattokinase synthesis did not correspond to a number of *Bacillus subtilis natto* in pre-culture [22]. This would inhibit nattokinase synthesis by using *Bacillus subtilis natto* in solid-state fermentation. The result of this study can be compared to Kapoor *et al.* in 2013. They determined at 2.5:50 (v:w) of the ratio of *Bacillus sp.* pre-culture to substrate the maximal value of crude nattokinase was obtained in solid-state fermentation [22].

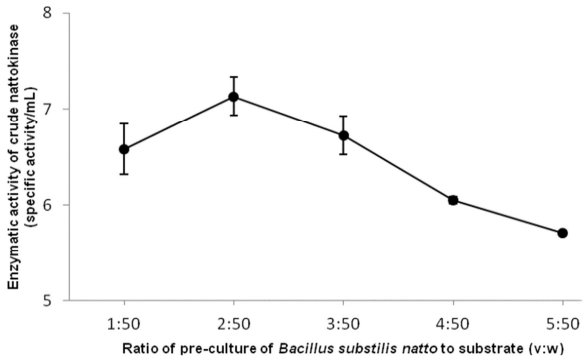

Fig. 2 The effect of the ratio of *Bacillus subtilis natto* pre-culture to substrate (v:w) on the enzymatic activity of crude nattokinase

TABLE II
SIGNIFICANT DIFFERENCE ANALYSIS OF THE ENZYMATIC ACTIVITY OF CRUDE NATTOKINASE FOR THE EFFECT OF THE RATIO OF *BACILLUS SUBTILIS NATTO* PRE-CULTURE TO SUBSTRATE (V:W)

The ratio of *Bacillus subtilis natto* pre-culture to the substrate (v:w)	Enzymatic activity of crude nattokinase (specific activity/mL)
1:50	$6.58^{b} \pm 0.262$
2:50	$7.13^{a} \pm 0.200$
3:50	$6.72^{b} \pm 0.200$
4:50	$6.05^{c} \pm 0.036$
5:50	$5.71^{d} \pm 0.026$

Significant difference analysis of the enzymatic activity of crude nattokinase for different ratios of *Bacillus subtilis natto* pre-culture to the substrate was performed. A ratio of *Bacillus subtilis natto* pre-culture to the substrate of 2:50 (v:w), nattokinase activity was significantly different to other ratios of *Bacillus subtilis natto* pre-culture to substrate. Thus, 2:50 (v:w) was considered as an optimal ratio of *Bacillus subtilis natto* pre-culture to the substrate (v:w) for further study.

D. *The effect of fermentation time on the enzymatic activity of crude nattokinase*

Products profile of solid-state fermentation was complicated [15] and fermentation time was another important parameter which was necessary to be screened for desirable product. Fig. 3 showed the effect of fermentation time (h) on the enzymatic activity of crude nattokinase.

With the results in Fig.3, fermentation time in the range of 24 h to 42 h had a positive effect on crude nattokinase activity. In this range of fermentation time, enzymatic activity of crude nattokinase significantly increased and reached the highest value of 16.24 ± 0.33 (specific activity/mL) at 42 h of fermentation time. A negative effect of fermentation time on enzymatic activity of crude nattokinase was found after 42nd hour. Auto-hydrolysis of protease occurred in normal condition [23] and fermentation products accumulation inhibited nattokinase synthesis by *Bacillus subtilis natto* in solid-state fermentation [15], thus, enzymatic activity of crude nattokinase would decreased with longer fermentation time. Liu *et al.* in 2013 also reported that fermentation at 40 h was optimal fermentation time for *Bacillus subtilis SBS* to synthesize nattokinase in solid-state fermentation [24].

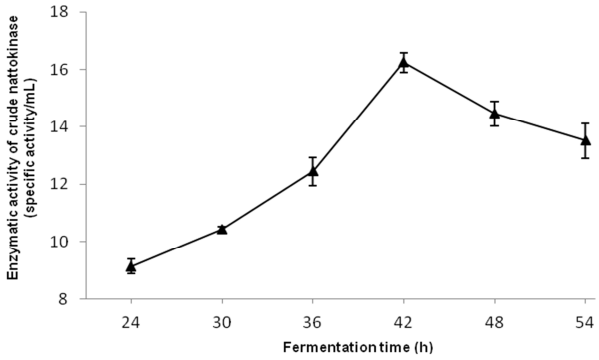

Fig. 3 The effect of fermentation time (h) on the enzymatic activity of crude nattokinase

TABLE III
SIGNIFICANT DIFFERENCE ANALYSIS OF THE ENZYMATIC ACTIVITY OF CRUDE NATTOKINASE FOR THE EFFECT OF FERMENTATION TIME (H)

Fermentation time (h)	Enzymatic activity of crude nattokinase (specific activity/mL)
24	$9.13^{e} \pm 0.251$
30	$10.43^{d} \pm 0.085$
36	$12.45^{c} \pm 0.491$
42	$16.24^{a} \pm 0.330$
48	$14.46^{b} \pm 0.430$
54	$13.51^{bc} \pm 0.600$

The obtained result identified that at 42 h of fermentation time enzymatic activity of crude nattokinase had a a significant difference in comparison to others fermentation time, and at this fermentation time, the enzymatic activity of crude nattokinase was the highest enzymatic activity (16.24 ± 0.330, specific activity/mL). Thus, 42 h of fermentation time was selected for further screening of other parameters that affected solid-state fermentation for nattokinase synthesis by *Bacillus subtilis natto*.

E. The effect of thickness of substrate on the enzymatic activity of crude nattokinase

The thickness of steamed and sterilized soybean seed as substrate holds an important role insolid-state fermentation because it affected diffusion of oxygen into the medium. Using the same size of solid-state fermentation tray, four-levels of thickness of substrate was screening for the highest enzymatic activity of crude nattokinase synthesized by *Bacillus subtilis natto*. In general, the thickness of substrate had considerably affected the enzymatic activity of crude nattokinase (Fig. 4).

In stationary phase, *Bacillus subtilis natto* still grew to maintain the balance between the growth rate and the death rate. At the low thickness of the substrate of 2 cm, the substrate would enough for *Bacillus subtilis natto* to grow and synthesize nattokinase. However, at the high thickness of the substrate (4 cm, 5 cm), the thickness of substrate would inhibit the diffusion of oxygen into medium, and thus nattokinase synthesis ability of *Bacillus subtilis natto* would reduce. The optimal thickness of the substrate in the range of 2 cm to 5 cm (1 cm of increment) was 3 cm. At this thickness of the substrate, the enzymatic activity of crude nattokinase was the highest value of 16.55 ± 0.06 (specific activity/mL), and it was significantly different in comparison to the enzymatic activity of crude nattokinase at others thicknesses of the substrate (Table IV).

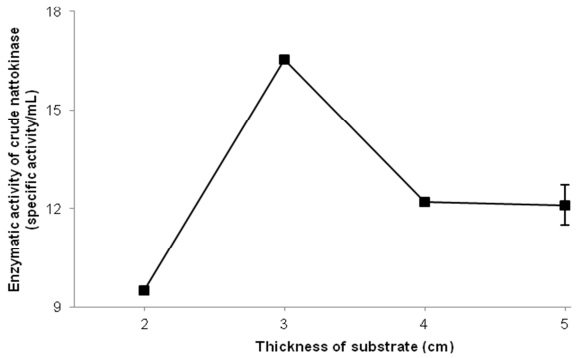

Fig. 4 The effect of thickness of substrate on the enzymatic activity of crude nattokinase

TABLE IV
SIGNIFICANT DIFFERENCE ANALYSIS OF THE ENZYMATIC ACTIVITY OF CRUDE NATTOKINASE FOR THE EFFECT OF THICKNESS OF THE SUBSTRATE (CM)

Thickness of substrate (cm)	Enzymatic activity of crude nattokinase (specific activity/mL)
2	$9.51^{c} \pm 0.020$
3	$16.55^{a} \pm 0.062$
4	$12.21^{b} \pm 0.066$
5	$12.11^{b} \pm 0.624$

Thus, 3 cm of steamed and sterilized soybean seed as substrate was used as the optimal thickness of substrate for further study.

F. The effect of the ratio of reusing Bacillus subtilis natto in fermentation product to substrate for a new process of solid-state fermentation on the enzymatic activity of crude nattokinase

Reusing microorganism in fermentation product for a new fermentation process is one of economic strategies with industrial scale. Since reusing microorganism in fermentation product would reduce time and substrate requirements for preparing pre-culture of microorganism. Thus, the production process would be more economical. In this study, we aim to initially apply this fashion in large scale, thus, reusing *Bacillus subtilis natto* in fermentation product for new process of solid-state fermentation was necessary. Fig. 5 showed the effect of reusing *Bacillus subtilis natto* in fermentation product to substrate (w:w) for new process of solid-state fermentation on enzymatic activity of crude nattokinase.

A positive effect of the ratio of reusing *Bacillus subtilis natto* in fermentation product to the substrate (w:w) for a new process of solid-state fermentation on the enzymatic activity of crude nattokinase was found in the range of 1:100 to 3:100 (w:w). Enzymatic activity of crude nattokinase dramatically increased and reached a maximal value of 14.10 ± 0.18 (specific activity/mL). Afterwards, the enzymatic activity of crude nattokinase drastically decreased with increasing ratio of reusing *Bacillus subtilis natto* in fermentation product to the substrate (w:w) for a new process of solid-state fermentation because substrate provided for the cultivation was not enough for *Bacillus subtilis natto* to grow and synthesize nattokinase.

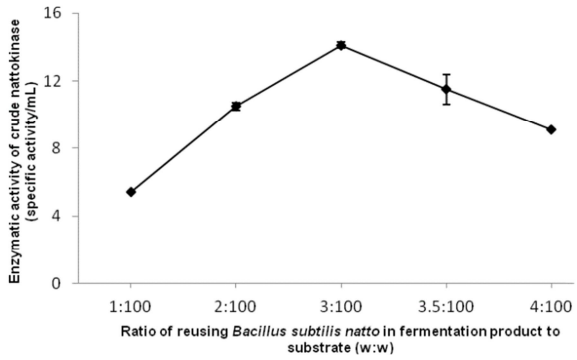

Fig. 5 The effect of the ratio of reusing *Bacillus subtilis natto* in fermentation product to the substrate (w:w) for a new process of solid-state fermentation on the enzymatic activity of crude nattokinase

TABLE V
SIGNIFICANT DIFFERENCE ANALYSIS OF THE ENZYMATIC ACTIVITY OF CRUDE NATTOKINASE FOR THE EFFECT OF THE RATIO OF REUSING *BACILLUS SUBTILIS NATTO* IN FERMENTATION PRODUCT TO THE SUBSTRATE (W:W) FOR A NEW PROCESS OF SOLID-STATE FERMENTATION

The ratio of reusing *Bacillus subtilis natto* in fermentation product to the substrate (w:w) for a new process of solid-state fermentation	Enzymatic activity of crude nattokinase (specific activity/mL)
1:100	$5.42^{e} \pm 0.021$
2:100	$10.51^{c} \pm 0.230$
3:100	$14.10^{a} \pm 0.180$
3.5:100	$11.5^{b} \pm 0.900$
4:100	$9.1^{d} \pm 0.036$

Significant difference analysis was carried out for selecting ratio of reusing *Bacillus subtilis natto* in fermentation product to substrate for new process of solid-state fermentation. At 3:100 (w:w) of ratio of reusing *Bacillus subtilis natto* in fermentation product to substrate

for new process of solid-state fermentation, enzymatic activity of crude nattokinase was the highest level and had the significant difference in comparison to others ratio of reusing *Bacillus subtilis natto* in fermentation product to substrate for new process of solid-state fermentation. Thus, 3:100 (w:w) was selected as optimal ratio of reusing *Bacillus subtilis natto* in fermentation product to substrate for new process of solid-state fermentation.

G. *The effect of circulation time of reusing Bacillus subtilis natto in fermentation product on the enzymatic activity of crude nattokinase*

The microorganism is important for fermentation toward quality and quantity. The quality of microorganism will reduce with increasing circulation time of reusing. Thus, the effect of circulation time of reusing *Bacillus subtilis natto* in fermentation product on the enzymatic activity of crude nattokinase was employed to determine optimal circulation time of reusing *Bacillus subtilis natto* in fermentation product. Overall enzymatic activity of crude nattokinase decreased with increasing circulation time of reusing *Bacillus subtilis natto* in fermentation product (Fig. 6).

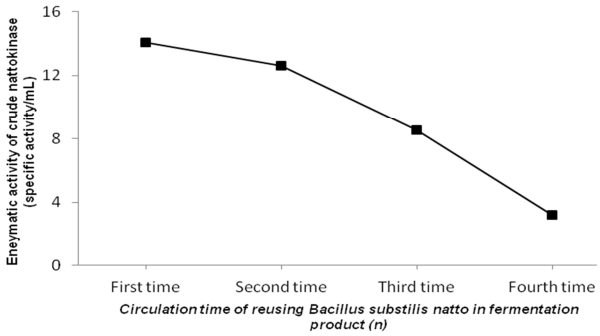

Fig. 6 The effect of circulation time of reusing *Bacillus subtilis natto* (n) in fermentation product on enzymatic activity of crude nattokinase

At the first circulation time, enzymatic activity of crude nattokinase was 14.20 ± 0.18 (specific activity/mL), which was not significantly decreased in comparison to initially enzymatic activity of crude nattokinase of 16.55 ± 0.06 (specific activity/mL). This pattern was found in the second circulation time which showed enzymatic activity of crude nattokinase of 12.62 ± 0.07 (specific activity/mL). However, the significant decrease of enzymatic activity of crude nattokinase was found at the third circulation time and the fourth circulation time which presented 8.53 ± 0.02 (specific activity/mL) and 3.2 ± 0.06 (specific activity/mL), respectively, of nattokinase activities.

TABLE VI
SIGNIFICANT DIFFERENCE ANALYSIS OF ENZYMATIC ACTIVITY OF CRUDE NATTOKINASE FOR THE EFFECT OF CIRCULATION TIME OF REUSING *BACILLUS SUBTILIS NATTO* IN FERMENTATION PRODUCT (N)

Circulation time of reusing *Bacillus subtilis natto* in fermentation product (n)	Enzymatic activity of crude nattokinase (specific activity/mL)
First time	$14.10^{a} \pm 0.180$
Second time	$12.62^{b} \pm 0.070$
Third time	$8.53^{c} \pm 0.020$
Fourth time	$3.20^{d} \pm 0.060$

Therefore, second reusing of *Bacillus subtilis natto* in fermentation product may be preferred for large scale production process.

IV. CONCLUSIONS

In this study, the proximate constituents of soybean seed as a substrate for solid-state fermentation to synthesize nattokinase by *Bacillus subtilis natto* was determined. Based on analysis result, soybean seed was a perfect substrate for nattokinase synthesis by *Bacillus subtilis natto* in solid-state fermentation. The optimal culture parameters for solid-state fermentation was 2:50 (v:w) of ratio of *Bacillus subtilis natto* pre-culture to substrate, 42 h of fermentation time, and 3 cm of thickness of substrate. Enzymatic activity of crude nattokinase corresponding to optimal parameters were 7.13 ± 0.2, 16.24 ± 0.33, and 16.55 ± 0.06 (specific activity/mL), respectively. This study also provided necessary information for large scale production with 3:100 (w:w) of reusing *Bacillus subtilis natto* in fermentation product to substrate for new process of solid-state fermentation and second time of suggested circulation time of reusing *Bacillus subtilis natto* in fermentation product. The results in this study can be applied for further research of nattokinase synthesized by *Bacillus subtilis natto* in solid-state fermentation and large-scale production.

ACKNOWLEDGEMENT

This project was funded by Research Budget of University of Science and Technology, The University of Danang, Viet Nam. We would like to thank Dr. Issara Poljungreed, a lecturer of Faculty of Agro-Industry, Panyapiwat Institute of Management, Thailand for helpful suggestion and critical revision to accomplish this paper.

REFERENCES

[1] Jeong, S.-J., et al., *Characterization of AprE176, a fibrinolytic enzyme from Bacillus subtilis HK176*, J. Microbiol. Biotechnol, 2015, 25(1), pp. 89-97.
[2] Afifah, D.N., M. Sulchan, and D. Syah, *Purification and characterization of a fibrinolytic enzyme from Bacillus pumilus 2. g isolated from Gembus, an Indonesian fermented food*, Preventive nutrition and food science, 2014, 19(3), pp. 213.
[3] WHO., *Global status report on noncommunicable diseases 2010*, Geneva, Switzerland, World Health Organization, 2011.
[4] Mathers CD, L.D., *Projections of global mortality and burden of disease from 2002 to 2030*, PLoS Med, 3: e442., 2006.
[5] Voet D, V.J., *Biochemistry*, 2nd ed, New York, USA: John Wiley and Sons, 1990.
[6] Killer, M., et al., *Current endovascular treatment of acute stroke and future aspects*, Drug discovery today, 2010, 15(15), pp. 640-647.
[7] Wang, C., et al., *Purification and characterization of nattokinase from Bacillus subtilis Natto B-12*, Journal of agricultural and food chemistry, 2009, 57(20), pp. 9722-9729.
[8] Jo, H.-D., et al., *Cloning and overexpression of aprE3-17 encoding the major fibrinolytic protease of Bacillus licheniformis CH 3-17*, Biotechnology and Bioprocess Engineering: BBE, 2011, 16(2), pp. 352.
[9] Peng, Y., et al., *Purification and characterization of a fibrinolytic enzyme produced by Bacillus amyloliquefaciens DC-4 screened from douchi, a traditional Chinese soybean food*, Comparative biochemistry and physiology part b: biochemistry and molecular biology, 2003, 134(1), pp. 45-52.
[10] Sumi, H., et al., *Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinases*, Acta haematologica, 1990, 84(3), pp. 139-143.

[11] Sizer, I.W., *Medical Applications of Microbial Enzymes*, Advances in Applied Microbiology, 1972, 15, pp. 1-11.
[12] Underkofler, L., R. Barton, and S. Rennert, *Production of microbial enzymes and their applications*, Applied microbiology, 1958, 6(3), pp. 212.
[13] Chand, S. and P. Mishra, *Research and application of microbial enzymes, India's contribution*, in Biotechnology in India II, 2003, pp. 95-124.
[14] Pandey, A., et al., *Solid-state fermentation for the production of industrial enzymes*, Current science, 1999, pp. 149-162.
[15] Stanbury, P.F., A. Whitaker, and S.J. Hall, P*rinciples of fermentation technology*, Elsevier, 2013.
[16] Subramaniyam, R. and R. Vimala, *Solid-state and submerged fermentation for the production of bioactive substances: a comparative study*, Int J Sci Nat, 2012, 3, pp. 480-486.
[17] El-Shemy, H., *Soybean and nutrition*, Tech. Publish, Rijeka, Croatia, 2011.
[18] A.O.A.C, *Official Methods of Analysis of AOAC International*, ed. 17th, Maryland: AOAC International, 2000.
[19] Inc, A.E., *Protease (Aspergillus llkaline proteinase, EC 3.4.21.63)*, Amano, pp. 77-78.
[20] Montgomery, D.C. and G.C. Runger, *Applied statistics and probability for engineers*, John Wiley & Sons, 2010.
[21] Saxena, R. and R. Singh, *Amylase production by solid-state fermentation of agro-industrial wastes using Bacillus sp*, Brazilian Journal of Microbiology, 2011, 42(4), pp. 1334-1342.
[22] Kapoor, R. and B.P. Panda, *Production of nattokinase from Bacillus sp. by solid-state fermentation*, International Journal of Medical Sciences and Biotechnology, 2013, I, pp. 61-65.
[23] Kellner, R., F. Lottspeich, and H.E. Meyer, *Microcharacterization of proteins*. 2008: John Wiley & Sons.
[24] Liu, C., Y. Zhao, and W. Cao, *Optimization of fermentation process on a fibrinolytic enzyme from Bacillus subtilis SBS*, Biotechnology, 2003, 14(6), pp. 52-54.