OPEN ACCESS

International
Journal
Of Modern Engineering Research (IJMER)

Examining Some Probiotics Activities of Bacillus Subtilis Natto

Huynh Thi Hong Nhi[1]; Nguyen Thuy Huong[2]
[1]*Ho Chi Minh City University Of Sciences, Vietnam*
[2]*Department Of Biotechnology, Ho Chi Minh City University Of Technology, Vietnam*

Abstract: *The article presents the examining results of probiotics nature of Bacillus subtilis natto, which includes: adhesive, simulated gastric juice (SGJ) resistant, simulated intestinal fluid resistant, antimicrobial compounds creating, and digestive enzymes synthesis abilities. The results show that: The adhesive ability of Bacillus subtilis natto to organic solvent xylene is 24.04%, self-adhesive ability is 20.31%, well adhesive to chicken intestinal mucosa; Be able to exist in simulated gastric juice and simulated intestinal fluid with survival rates are 59.68% at pH 2 and 81.15% at pH 3; Be able to resist Salmonella sp. and E. coli, and has ability to synthesize digestive enzymes like protease, amylase.*

Keywords: *Bacillus subtilis, Bacillus subtilis natto, probiotics, adhesion, antibacterian*

I. INTRODUCTION

Probiotics has long been known in functional food and pharmaceutical products. Probiotic bacteria has important role in boosting and remaining human health, such as digestion supporting, reducing digestive disorders, improving immunity, preventing inflammation, reducing cholesterol, restraining harmful intestinal bacteria. To promote this usage, the probiotics bacteria must be able to tolerate the harsh condition in digestive system. In addition, they must well express the adhesive ability to create biological barrier, resist harmful bacteria, tolerate digestive enzymes, secrete supportive enzymes for digestion (amylase, protease,...) and functional enzymes (lastase, nattokinase,...).

Nattokinase is a searine protease which can dissolve thrombosis, hydrolyse fibrin (a kind of structural protein in thrombosis) derived from products fermented from soy beans by *Bacillus subtilis* natto. Moreover, nattokinase also promotes plasmin synthesis (enzyme created by the body to dissolve thrombosis on endothelium). Nattokinase is determined to be stronger than other ordinary thrombosis dissolving medicine like Urokinase, Streptokinase and tissue plasminogen (t-PA) [9]. Hence, nattokinase is being researched, produced and used widely to prevent arterial embolism.

In this research, we determined probiotic nature of *Bacillus subtilis* natto through: adhesive, simulated gastric juice (SGJ) resistant, simulated intestinal fluid resistant, antimicrobial compounds creating, and digestive enzymes synthesis abilities, which were used to appreciate the probiotic nature of *Bacillus subtilis* natto to ferment and produce probiotic products include both probiotic and nattokinase activities.

II. MATERIALS AND METHODS

1. Materials

Bacillus subtilis natto strain is provided by Biotechnology Department, Ho Chi Minh city University of Technology.

NA medium: used to store bacterial strain, determine *Bacillus subtilis* natto density.

NB medium: used to multiply bacterial strain, culture, examine *Bacillus subtilis* natto.

SGJ medium (Simulated Gastric Juice): NaCl 9 g/L, pepsin 3 g/L, pH = 2; SIF medium (Simulated Intestinal Fluid): NaCl 9 g/L, ox bile 3 g/L, pH = 6.5, used in examining SGJ and SIF resistant ability of *Bacillus subtilis* natto.

TSA medium: used to grow, culture *E. coli*, *Salmonella* sp.

2. Methods

a. Adhesive ability

Examining adhesive ability of Bacillus subtilis natto with organic solvent xylene

Multiply bacterial strain the second time and define the cell density of *Bacillus subtilis* natto. Pipet 24 mL culture liquid, centrifuge 5,000 rpm in 15 minutes, obtain cell biomass. Wash biomass two times with KNO_3

0.1 M and resuspend in 24 mL KNO_3 0.1 M (pH = 6.2). Pipet 6 mL experiment sample, measure OD value at 600 nm. 2 mL of xylene was added to the rest of the sample. Incubate in 10 minutes at room temperature. Vortex thoroughly and incubate in 20 minutes more until there appeared a separation. Remove solvent and measure OD value at 600 nm [10].
Cell density determination formula

$$Adhesiveability\ \% = \frac{A_0 - A_t}{A_0} \times 100$$

A_0: OD_{600} – no xylene solvent added
At: OD_{600} at the time after xylene solvent was added and incubated for 30 minutes.

Examining Adhesive Ability Of Bacillus Subtilis Natto With Animal Epithelial Cell

Bacillus subtilis natto strain was grew to secondary level in 20 mL NB medium fluid, incubated at 37°C in 24 hours. Chicken intestinal epithelium sample was cut into short parts about 1 mL, immersed in PBS buffer for 30 minutes at 4°C to remove viscous liquid. The samples were then immersed in bacterial medium liquid, incubated at 37°C in 30 minutes. The bacterial liquid was removed and the samples were immobilized in formalin. Formalin was then removed by increasing ethanol concentration gradually. The samples were cut in half, Gram stained and observed with microscope to compare the adhesion of nattokinase [7].

Examining Self-Adhesive Ability Of Bacillus Subtilis Natto

Bacillus subtilis natto strain was grew to secondary level in 30 mL NB medium fluid, incubated at 37°C in 24 hours. Pipet 4 mL bacterial culture, centrifuge 5,000 rpm in 15 minutes, wash with PBS buffer two times and resuspend in 4 mL PBS buffer. To 0h sample: pipet 0.1 mL resuspended liquid to 3.9 mL PBS buffer and measure OD value at 600 nm. To the rest of resuspended liquid, incubate for 5 hours, pipet 0.1 mL resuspended liquid per hour to 3.9 mL PBS buffer, measure OD value at 600 nm [4].
Self-adhesive ability of *Bacillus subtilis* natto determination formula:

$$Self - adhesiveability\ \% = \frac{A_0 - A_t}{A_0} \times 100$$

A_0: OD_{600} at t = 0 h
A_t: OD_{600} at t = 1, 2, 3, 4, 5 h.

b. Simulated Gastric Juice (SGJ) And Simulated Intestinal Fluid Resistant Ability

Prepare simulated gastric juice (SGJ) and simulated intestinal fluid medium at pH 2, pH 3. Centrifuge 2 mL 24-hour liquid bacterial culture. Wash biomass with physiological saline. Add 1 mL SGJ pH 2, pH 3. Incubate at 37°C, 200 rpm. After 0, 30, 60, 90, 120 minutes, spread the bacteria on Petri dishes contained NA medium to determine cell density [3].

c. Antibacterial Ability

Bacteria were cultured for 24 hours in liquid NB medium to get the cell density of 10^8 CFU/mL. The bacterial liquid was centrifuged to remove bacterial cells completely. *E. coli*, *Salmonella* sp. was grown to 10^8 CFU/mL and spread on TSA agar with the volumn of 100 µL. Sterile steel pipes were used to pierce some holes on the agar with diameter of 5 mm. Filtered fluid of *Bacillus subtilis* natto was added to the holes with the volumn of 100 µL. The Petri dishes were incubated overnight at 37°C (12 hours) [1].

d. Digestive Enzymes Synthesis Ability

Determine Protease Activity In Casein Medium

Prepare 10 mL phosphate buffer Na_2KHPO_4 and KH_2PO_4 (pH 6.2), add 1% casein with distilled water added to 100 mL and boil. Add 1% agarose, boil and then pour into Petri dishes, put at room temperature for 1 hour. Create holes with diameter of 4 mm on agarose layer, 80 µL fluid to test activity. Incubate dishes for 24 hours at 37°C. Drip 5-time diluted Folin onto agar layer. Observe the activity according to the diameters of casein dissolving circles.

Determine Amylase Activity In Starch

Prepare 10 mL phosphate buffer Na_2KHPO_4 and KH_2PO_4 (pH 6.2), add 1% starch with distilled water added to 100 mL and boil. Add 1% agarose, boil and then pour into Petri dishes, put at room temperature for 1 hour. Create holes with diameter of 4 mm on agarose layer, 80 µL fluid to test activity. Incubate dishes for 24 hours at 37^{o}C. Drip iodine onto the agar layer and observe the diameters of dissolving circle.

III. RESULTS AND DISCUSSION

1. Adhesive Ability

Examining Adhesive Ability Of Bacillus Subtilis Natto With Organic Solvent Xylene

The results in Table I show that the average rate of adhesive ability of *Bacillus subtilis* natto is 24.04%.

Table I. Adhesive Rate Of Bacillus Sibtilis Natto With Xylene

Adhesive rate (%) when added xylene	***Adhesive rate with xylene (%) after 30 minutes***
12.92 ± 0.99	24.04± 1.77

Xylene is a kind of liquid, transparent, colorless and insoluble in water. Due to its insolubility in water, its density is low (0.864), therefore, after being mixed with bacterial fluid and incubated for 30 minutes, xylene floated on the surface and separated. The bacteria which had linked with xylene would be risen together. This implied that *Bacillus subtilis* natto cells are hydrophobic, and adhesive to intestinal epithelium cells. According to Lemke et al. (1995), hydrophobicity of cell surface affects the adhesion and the ability to use nutrition, so affects the distribution of bacteria. Moreover, the hydrophobicity also increases the adhesive ability of bacterial cell surface.

Examining Adhesive Ability Of Bacillus Subtilis Natto With Animal Epithelial Cell

The bacterial adhesive ability was checked with ileum cells derived from fresh chicken small intestine, followed the research of improved Jensen method [2,6]. After being incubated with epithelial membranes of chicken small intestine and washed three times, the results show that *Bacillus subtilis* natto has adhesive ability on intestinal epithelium, with formed cell colonies.

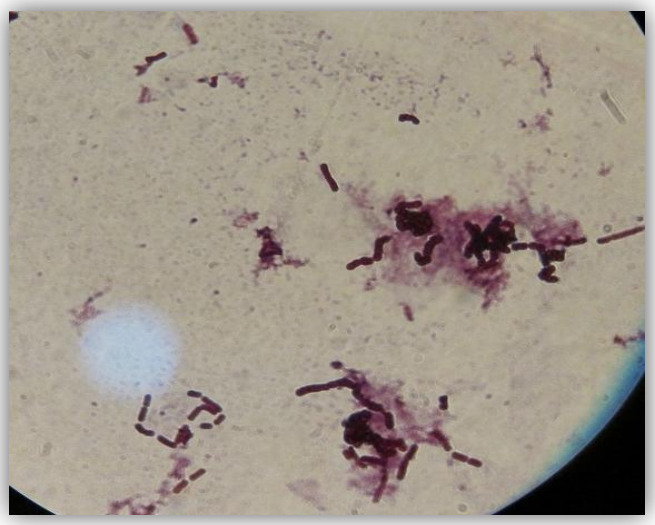

Figure 1. Adhesive ability of *Bacillus subtilis* natto on epithelial cells

Examining Self-Adhesive Ability Of Bacillus Subtilis Natto

To survive in digestive system areas, probiotic bacteria must have well competition. One of the factors which helps the bacteria not to be rejected is the self-adhesive ability.

The examining self-adhesive ability of *Bacillus subtilis* natto results show that the adhesive rate among *Bacillus subtilis* natto cells tends to increase by time. After being incubated at room temperature for an hour, the adhesive rate reached 6.76 ± 2.12%. After being incubated for 2 hours, the adhesive rate increased to 10.27 ± 2.91%. The adhesive rate continued to rise up to 15.12 ± 4.04% and 20.21 ± 1.16%. After being incubated for 5 hours, the adhesive rate reached 20.31 ± 1.21%.

2. The Ability To Sustain The Condition Of Simulated Gastric Juice And Simulated Intestinal Fluid

The ability to sustain the condition of simulated gastric juice and simulated intestinal fluid are the characteristics that must be had in the probiotic bacteria. In the gastric medium with low pH (pH ~2), due to the gastric secretion per day, the number of bacteria reduced prominently [3]. Hence, they are said to be two harsh medium of the digestive system, creating a major obstacle to the number of probiotic bacteria in the gut. Therefore, the survival ability of *Bacillus subtilis* natto on the simulated gastric juice medium at pH 2 and pH 3 with the addition of simulated intestinal fluid at 0.3% concentration in the ingredient was researched. The results of microbial density during the researching time are shown in Table II.

Table II. Assess the ability to sustain the condition of simulated gastric fluid and bile salts

	The survival of Bacillus subtilis natto (log CFU/ml)				
pH	***0 min***	***30 min***	***60 min***	***90 min***	***120 min***
2.0	8.06± 0.11	7.02± 0.1	6.72± 0.15	5.60± 0.12	4.81± 0.14
3.0	8.01± 0.05	7.83± 0.07	7.14± 0.12	6.52± 0.14	6.50± 0.13
7.0	8.18± 0.02	7.99± 0.09	8.15± 0.1	8.18± 0.11	8.22± 0.02

After being researched in the condition of simulated gastric juice and simulated intestinal fluid for 120 minutes, *Bacillus subtilis* natto bacteria is still capable of surviving with high density. The survival rate of environmental viability in simulated gastric juice and simulated intestinal fluid was 59.68% and 81.15% at correspondingly pH 2 and pH 3. Comparing with probiotic strains which are commonly used like *Lactobacilus acidophilus*, *Bacillus subtilis* natto is capable of better survival at pH 2 and pH 3. In the study by Xiaodong et al. (2009), after 60 minutes, the survey shows that only 25% and 50% of cells survived at pH 2 and pH3 [11].

3. Antibacterialability

When the probiotics arrive and developing localize in the small intestine, they must reflect the characteristics of their functions. One of these features includes the ability to resist diseases causing by harmful microorganisms. Therefore, in this study, a research of the antibacterial ability in the products produced by two strains of microorganisms tested *Salmonella* sp. and *E.coli* was conducted. The results are shown in Table III.

Table III. The antibacterial ability of*Bacillus subtilis* natto

Strain	***The diameter of antibacterial ability (mm)***	
	Salmonella sp.	***E. coli***
***Bacillus subtilis* natto**	10.64 ± 0.16	9.42 ± 0.12

The data above shows that *Bacillus subtilis* natto is resistant with *Salmonella* sp. and *E.coli*. This result is appropriate with reports of the ability to produce bacteriocin and similar bacteriocin compounds of *Bacillus subtilis* MA139 to prevent the growth of harmful strains such as *Salmonella* sp. (42.5mm), *E. coli* (32mm) [5].

4. The Ability To Produce Digestive Enzymes

Determination Of Protease Activity On Casein Medium

The protease enzyme catalyzes the hydrolysis of peptide bonds in the peptide or protein. *Bacillus subtilis* natto and *Bacillus subtilis* are capable of synthesizing neutral protease and alkaline as well as synthesize protease proteolysis and other polymeric substrates in nutrient medium to low molecular forms for easy absorption micro-organisms [10]. Experimental results in the ability to hydrolyze casein of *Bacillus subtilis* natto is shown in Figure 2.

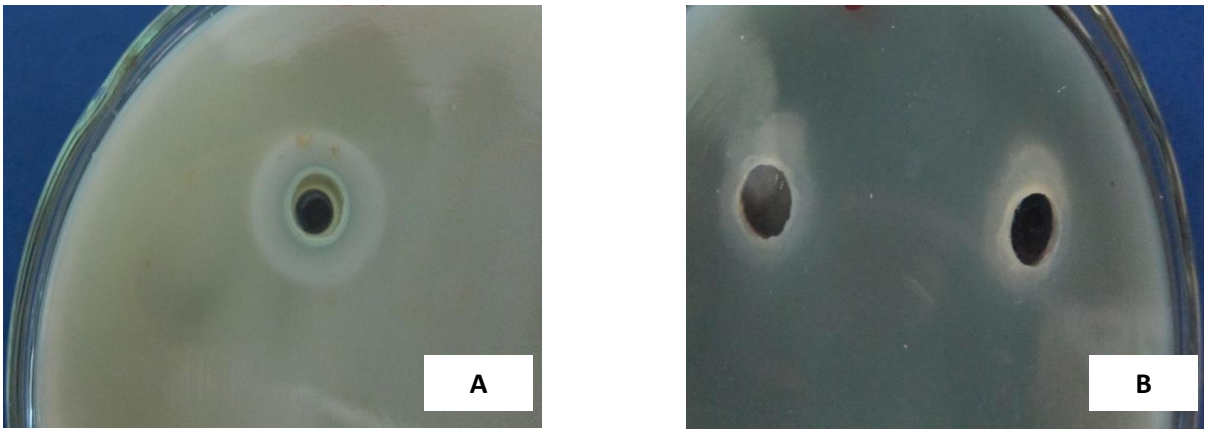

Figure 2. Casein (A) and starch(B) hydrolization of *Bacillus subtilis* natto

Determination Of Amylase Activity On Starch Medium

Bacillus subtilis and *Bacillus subtilis* nattoare capable of creating an α-amylase bulk. α-amylase of *Bacillus subtilis* and *Bacillus subtilis* natto hydrolyzes starch to form dextrin having long chain 6-8 of glucose. The final product of substrate hydrolysis by amylase is glucose and maltose [10]. Experimental results in the ability to hydrolyze starch of *Bacillus subtilis* natto is shown in Figure 2.

The survey showed that *Bacillus subtilis* natto is capable of hydrolyzing protein and starch. This is an important characteristic for probiotic production application supporting digestion, increasing the ability of food digestion and absorption nutrients by the host.

IV. CONCLUSION

The research in probiotic characteristics of *Bacillus subtilis* natto showed that *Bacillus subtilis* natto potentially exists in the condition of simulated gastric juice and simulated intestinal fluid after 120 minutes with a survival rate is 59.68% at pH 2 and 81.15% at pH 3; resist to *Salmonella* sp. and the *E. coli*; has the capable of good adhesion to the intestinal mucosa gastrointestinal tract, hydrolysis of protein and starch, and characteristics of bio-safety. These are suitable to evaluate *Bacillus subtilis* natto bacteria is potentially in use as probiotic.

ACKNOWLEDGEMENTS

We would like to thank teachers in Biotechnology Department of Ho Chi Minh City University of Technology for the helps and technical assistance.

REFERENCES

[1]. Aslim B and Kilic E. (2006),"Some probiotics properties of vaginal Lactobacilli isolated from healthy women",Japanese Journal of Infectious Diseases, 59, p. 249-253.

[2].B Kos et al. (2003), "Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92", Journal of Applied Microbiology, 94(6), p. 981-987.

[3]. Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1998), "Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract", Journal of Applied Microbiology, 84, p. 759-768.

[4].Del Re B et al (2000), "Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum", Lett Appl Microbiol., 31(6), p. 438-442.

[5]. Guo, X., D. F. Li, W. Q. Lu, X. S. Piao, and X. L. Chen. (2006), "Screen-ing of Bacillus strains as potential probiotics and subsequent confirmation of the in vivo effectiveness of Bacillus subtilis MA139 in pigs", Antonie Van Leeuwenhoek, 90, p. 139-146.

[6]. Jacek Piatek et al. (2012), "The viability and intestinal epithelial cell adhesion of probiotic strain combination in vitro study", Annals of Agricultural and Environmental Medicine, 19(1), p. 99-102.

[7].Mayra-Makinen, A., Manninen, M., and Gyllenberg, M. 1983, "The adherence of lactic acid bacteria to the columnar epithelium cells of pigs and calves", J.Appl. Bacteriol, 55, p. 241-245.

[8]. McNamara, Christopher J., Lemke, Michael J., Leff, Laura G (1997), "Brief note: Characterization of hydrophobic stream bacteria based on adhesion to n-Octane", Ohio Journal of Science, 97, p. 59-61.

[9]. Milner, M. and K. Makise (2002), "Natto and its active ingredient nattokinase", Alternative & Complementary therapies, 8, p.157-164.

[10]. Rosenberg et al (1980), "Adherence of bacteria to hydrocarbons a simple method for measuring cell-surface hydrophobicity", *FEMS Microbiol. Lett*, 9, p. 29-33.

[11]. XiaodongPan (2009), "The acid, bile tolerance and antimicrobial property of *Lactobacillus acidphilus* NIT", *Food Control*, 20, p. 598- 602.