Vol.7, No.2 pages 47-57 Science Technology and Engineering Journal (STEJ) Research Article

Inhibitory Effect of *Bacillus subtilis* p5-6 Against *Staphylococcus aureus* on Different States of Medium

Hoang Truc Anh To[1*] and Cheunjit Prakitchaiwattana[1, 2]

[1] *Department of Food Technology, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok, 10300, Thailand*
Tel.: (+66) 2218 3280, E-mail: int.off@chula.ac.th

[2] *The Development of Foods and Food Additive from Innovative Microbial Fermentation Research Group, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok, 10300, Thailand*
Tel.: (+66) 22185515-6, E-mail: Cheunjit.P@chula.ac.th

[*] *Corresponding author. Email: trucanh.tohoang@gmail.com*

(Received: 5th July 2021, Revised: 7th July 2021, Accepted: 9th July 2021)

Abstract-The aim of this research was to investigate the inhibitory effect of *Bacillus subtilis* P5-6 against *Staphylococcus aureus* on different states of standard medium with the presence of sodium chloride at 5% (v/v), to develop an effective protective culture in food. Halophile *Bacillus subtilis* P5-6 isolated from Plara (Thai traditional salt fermented fish) contained both the genes encoding subtilin (spaS) and subtilosin (sboA). Only subtilosin gene expression was detected along with housekeeping gene BA-rpoB when co-cultured with *Staphylococcus aureus*. With the spot on lawn assay, cell suspension from the P5-6 culture showed inhibitory effect against *Staphyloccus aureus*, while no inhibition was observed when cell free supernatant was used. In liquid co-culture, the inhibitory effect of P5-6 on *Staphylococcus aureus* was observed when its inoculum size (population density of 8 log CFU/ mL) was double that of *S.aureus*. In solid medium, *Bacillus subtilis* P5-6 could exert higher antagonistic action against this target pathogen. *Bacillus subtilis* P5-6 displayed an inhibitory effect even when its population was 2 log CFU/ mL lower than that of *Staphylococcus aureus*. Solid state cultivation with the presence of sodium chloride could enhance production and/or activity of bacteriocin of P5-6. The observation reflects an importance of the self-inhibitory effect as found in liquid and solid medium cultivation system. The data obtained could be fundamental importance in bacteriocin production development and application of protective culture from P5-6 to protect food against *Staphyloccus aureus*-one of the most common cause of foodborne disease.

Keywords: Bacteriocin, solid medium, liquid medium

1. Introduction

Staphylococcus aureus is a pathogen that commonly associated with food poisoning as a result of food contamination. It has been found in various types of food such as meat, dairy products, fish and ready-to-eat food (Castro *et al*., 2018). The first case of food poisoning caused by *S.aureus* was reported by Vaughan (1984). Because *S.aureus* tolerates up to 15% of NaCl, grows in broad range of temperature (7°C to 48.5°C) and pH (4.2 to 9.3), it can survive and reproduce in numerous types of food (Kadariya *el al*., 2014). Moreover, enterotoxins produced by *S.aureus* are highly stable, which cannot be destroyed by cooling (Balaban & Rasooly, 2000). Thus, *S.aureus* has been causing concern and challenge in food industry. Due to the urge of dealing with foodborne disease caused by food pathogens, several researches on natural antimicrobial substances have been conducted as an environmental-friendly approach protecting consumer's health and reducing food loss. There are many food grade bacteria, namely *Bacillus* can possess antimicrobial activity as a natural mechanism to protect themselves and compete for nutrients with other microorganism. They can be used as protective culture, which is defined as live microbes added in foods to inhibit the growth of pathogens and spoilage microbes without interfering technological and sensory qualities of food (Ben Said *et al*., 2000). Chhetri *et al*. (2019) investigated the inhibitory effect of halophilic *Bacillus subtilis* P5-6 against *S.aureus*. The *B.subtilis* strain was prepared in skim milk powder and applied in cheese. The data showed that *B.subtilis* P5-6 could be a potential protective culture, which helps to significantly reduce viable count of *S.aureus* and helps prolong freshness of the cheese. Since this isolate is halophilic, its growth and inhibitory effect could be induced at high concentration of sodium chloride. In addition, the antagonistic effect (inhibitory function) of bacteriocin generated from cell (i.e migration bacteriocin from cell to target cell) have not been reported. The aim of this study is to investigate the inhibitory effect of *B.subtilis* P5-6 against *S.aureus* on different state of standard medium with the presence of sodium chloride at high level. Thereby, the growth and antagonistic effect of *B.subtilis* P5-6 against *S.aureus* will be improved, in order to be developed as an effective protective culture in food.

2. Materials and Methods

2.1 Bacteriocin Gene, the Gene Expression, Growth Curve and Inhibitory Effect Assay

Bacillus subtilis P5-6 isolated from Plara (Thai traditional salt fermented fish) were grown in Nutrient broth (NB) supplemented with 5% NaCl (Chhetri *et al*., 2019) at 37°C in shaking incubator with speed of 150 rpm for 24h. Cell culture was then subjected to DNA extraction using commercial kit (GF01-1, Vivantis, Malaysia). Primers of subtilin (spa) and subtilosin (sbo) encoding genes were used to perform PCR. Gel electrophoresis was followed by running PCR products on 1.5% agarose gel stained with ethium bromide (Velho *et al*., 2013).

The expression of the gene during cultivation was investigated by rRNA extracted at 6th hour of cultivation in the same condition mentioned above, using commercial kit (GF-1 Total RNA extraction kit, Vivantis, Malaysia). RNA was then

converted into complementary DNA (cDNA) using reverse transcriptase reaction (Rio, 2014). The expression of bacteriocin encoding genes were confirmed along with housekeeping gene (HKG-1389472946 rpoB) (Ko *et al*., 2004). The PCR products were checked on 1.5% agarose gel electrophoresis.

Growth curve of *B.subtilis* P5-6 was constructed by cultivating this isolate in NB supplemented with 5% NaCl in shaking incubator at 37°C, 150 rpm for 24 hours. Inhibitory effect of *B.subtilis* P5-6 against pathogens and food spoilage microbes was screened by investigating its cell suspension and cell free supernatant from cultivated broth at optimum period, which was chosen according to growth curve. Cell free supernatant was obtained by centrifugation and filtration with 0.45 µm. Antimicrobial activity was determined by using spot on lawn assay as described by Tagg and Mc Given (1971). Pathogens and spoilage microbes including target *Staphylococcus aureus* ATCC 25922 and non-target ones including Samonella typhimurium ATCC 1331, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* TISTR2370 were prepared at selected cell population in 0.5% peptone water. One mL of each cell suspension was spreaded onto Nutrient agar plate. Twenty µL of *B.subtilis* P5-6 cell suspension was spotted directly on each seeded plate. Antimicrobial activity of cell free supernatant from *B.subtilis* P5-6 was checked by conducting spot on lawn with the same procedure as cell suspension. Inhibitory effect was qualitatively determined by measuring the clear zone generated on each lawn.

2.2 Inhibitory Effect of *Bacillus Subtilis* P5-6 on *Staphylococcus Aureus* in Liquid Medium

Bacillus subtilis P5-6 and *Staphylococcus aureus* with initial concentration 8 log CFU/ mL were co-cultured in NB supplemented with 5% NaCl. The inoculum size of target pathogen was fixed at 10% (v/v) while *B.subtilis* P5-6 inoculum size was varied at 2, 10 and 20% (v/v). The mixed cultures were incubated for 8 hours before counting population of each bacterium by spread plate technique on Nutrient agar (NA) (Sanders, 2012).

2.3 Inhibitory Effect of *Bacillus Subtilis* P5-6 on *Staphylococcus Aureus* on Solid Medium

Inhibitory action in solid medium was investigated based on spot on lawn assay. *Staphylococcus aureus* lawn on plate were prepared from 0.1 mL of the cultures containing 6, 7 and 8 log CFU/ mL. *Bacillus subtilis* P5-6 cell suspension was diluted to obtain 1 to 7 log CFU/ mL then spot on the *S.aureus* lawn and incubated at 37°C for 24 hours. Inhibitory effect was qualitatively determined by measuring the inhibition zone on each lawn. Diameter of inhibition zone was measured from the edge of cell suspension colonized to the end edge of clear zone.

2.4 Statistical Analysis

Data were presented as mean ± standard deviation from experiments conducted in triplicates. Analysis of variance (ANOVA) and multiple comparisons by Duncan's test were performed using IBM-SPSS statistics

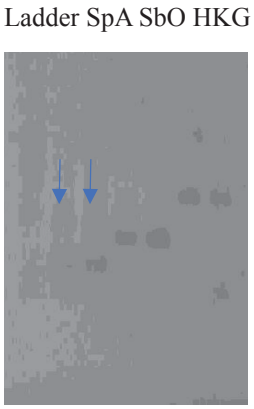
package version 22 (SPSS Inc., Chicago, IL, USA). Statistically significant difference was calculated at significant level of $p<0.05$

3. Results and Discussion

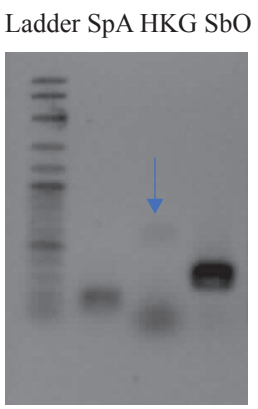
3.1 Bacteriocin Gene, the Gene Expression, Growth Curve and Inhibitory Effect Assay

According to figure 1a, the target bacteriocin genes including sboA (734 bp) encodes subtilosin and spaS (566 bp) encodes subtilin were observed in *B.subtilis* P5-6. However, when the gene expression tested only the sboA was observed (figure 1b). Thus the subtilosin might be the key bacteriocin playing inhibitory effect on the competitive/specific microbes.

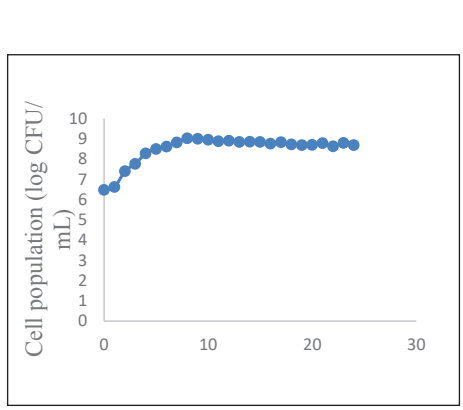
The spaS gene was not detected. It could be because this gene did not function properly and/or the cultivation condition in this study might not favor the expression of spaS gene. It is clearly shown in figure 1c that during cultivation, log phase ranged from 1 to 10th hour, which associated with the significant increase in bacterial population and then changed to stationary phase (10th-25th hour). The mid-stationary phase involving the production of secondary metabolites, particularly antimicrobial substances, which are not necessary for bacterial growth will be generated (Oakley, 2017). Therefore, in order to check for antagonistic effect, 16th hour was chosen to grow *B.subtilis* P5-6 to obtain optimum antimicrobial activity of bacteriocin. Antimicrobial test demonstrated that cell suspension of *B.subtilis* P5-6 could effectively inhibit *S.aureus*. Based on the gene expression result, this inhibitory effect could be due to subtilosin encoded by sboA gene as stated above. However, when bacterial cells were removed, cell free supernatant did not show any inhibitory effect against *S.aureus*. It could be because of the low concentration of bacteriocin secreted in broth or bacteriocin produced might attatch to *B.subtilis* P5-6 cell surface. Therefore, when cells were removed, bacteriocin was also taken out, resulting in no inhibition zone on *S.aureus* lawn. When cell suspension was spotted on lawn, bacteriocin generated during cultivation might still attach to protect the cell from *S.aureus* without regenerating. It might not generate at too high concentration in broth system to prevent self-inhibitory effect (to kill its cells). In solid matrix, bacteriocin generated could diffuse through the solid matrix and was re-genrated to futher kill *S.aureus*. Since the bacteriocin would migrate away from bacterial cell, the self-inhibitory effect is unlikely to occur on this medium state.



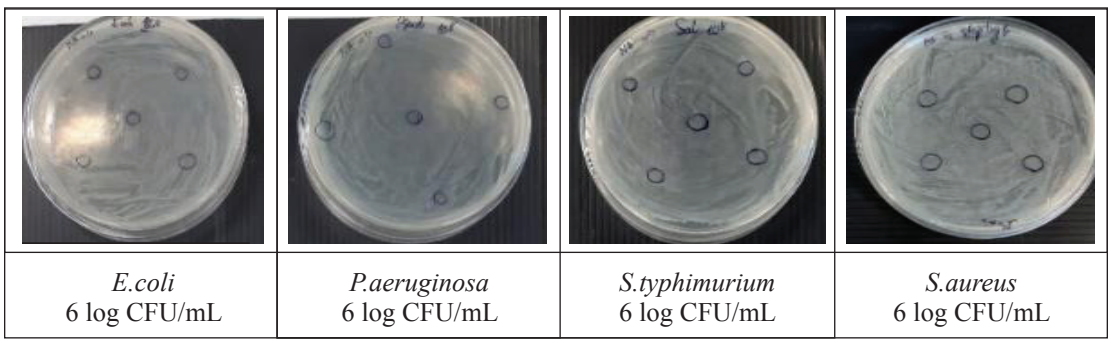
a)



b)

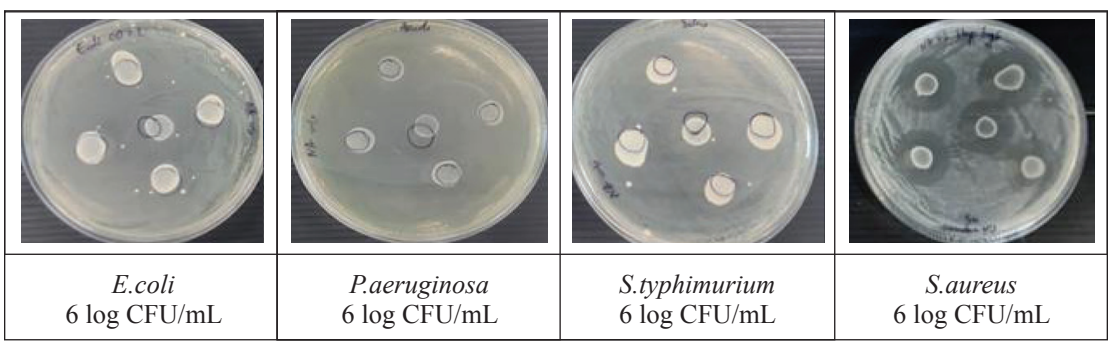


c)



E.coli 6 log CFU/mL	*P.aeruginosa* 6 log CFU/mL	*S.typhimurium* 6 log CFU/mL	*S.aureus* 6 log CFU/mL

d)



E.coli 6 log CFU/mL	*P.aeruginosa* 6 log CFU/mL	*S.typhimurium* 6 log CFU/mL	*S.aureus* 6 log CFU/mL

e)

Figure 1. (a) bacteriocin gene (DNA) by PCR analysis
(b) bacteriocin gene expression (RNA) by reverse transcriptase PCR analysis, lane 1: DNA ladder, lane 2: SpA-gene encoding subtilin, lane 3: SbO-gene encoding subtilosin, lane lane 4: HKG-housekeeping gene used as internal control
(c) growth curve of *B.subtilis* P5-6
(d) spot on lawn of *B.subtilis* P5-6 cell-free supernatant on *Pseudomonas aeruginosa* TISTR2370, *Escherichia coli* ATCC 25922, *Samonella typhimurium* ATCC 1331 and *Staphylococcus aureus* ATCC 25922 on Nutrient agar

Aside from *S.aureus*, *B.subtilis* P5-6 could not inhibit those non-target microbes. This helped to comfirm that bacteriocin generated from *B.subtilis* P5-6 inhibiting *S.aureus* was subtilosin. This result is in agreement with the finding of Liu *et al*. (2012) that subtilosin A from B.amyloliquefaciens did not perform any antibacterial activity against Samonella Typhimurium, *Escherichia coli* and *Pseudomonas aeruginosa*. Beside, the susceptibility in term of cell membrane property (Tagg & Mc Given, 1971), it could be because of quorum sensing system, which is a way of communication between cells to adjust gene expression according to cell density. Quorum sensing system are different in gram-positive and gram-negative bacteria (Rutherford & Bassler, 2012). While signaling molecules of gram-positive bacteria are peptides, gram-negative use small molecules such as acylated homoserine lactones or others as autoinducers (Wei *et al*., 2011). Because *B.subtilis* and *S.aureus* are both gram-positive bacteria, *B.subtilis* can sense the cell density of its competitor and express the gene encoding bacteriocin by producing bacteriocin. *Staphylococcus aureus* was inhibited by subtilosin so the clear zone around cell suspension droplet was observed. On the other hand, Pseudomonas, E.coli and Salmonella are gram-negative bacteria. Therefore, the quorum sensing that they use is different from the one used by *B.subtilis*. Their cell density might not be detected by *B.subtilis* to possess any inhibitory effect against them.

3.2 Inhibitory Effect of *Bacillus Subtilis* P5-6 on *Staphylococcus Aureus* in Liquid Medium

Because *B.subtilis* P5-6 could only inhibit *S.aureus* among those selected pathogens, it was used to further investigate the antimicrobial action on different state of culture medium. According to the growth curve, mid-stationary phase at 16th hour, which involves bacteriocin production was chosen to harvest the cultivation broth. Yan *et al*. (2016) indicated that inhibition was affected by both the growth stage and the amount of surrounding B. subtilis cells. In co-culture for 16 hours, *S.aureus* could not grow well as in single culture. The presence of *B.subtilis* may interfere the growth of *S.aureus*. According to Gonzalez *et al*. (2011), B. subtilis can inhibit neighboring S. aureus by releasing lipopeptide antibiotics. When co-culture with *S.aureus*, the amount of those peptides secreted is increased due to microbial competition.

Table 1. Bacterial population in co-culture after 16h of cultivation in nutrient broth supplemented with 5% NaCl

Inoculum size % (v/v) of 8 log CFU/ mL		**Cell population (log CFU/ mL)**	
B.subtilis P5-6	*S.aureus*	*B.subtilis* P5-6	*S.aureus*
2 (6 log CFU/ mL)	10 (7 log CFU/ mL)	8.30±0.18^{b}	8.49±0.48^{b}
10 (7 log CFU/ mL)	10 (7 log CFU/ mL)	8.77±0.09^{a}	8.41±0.12^{b}
20 (>7 log CFU/ mL)	10 (7 log CFU/ mL)	8.83±0.04^{a}	7.93±0.03^{c}
10 (7 log CFU/ mL) *single culture	Not added	8.73±0.32^{a}	ND
Not added	10 (7 log CFU/ mL) *single culture	ND	8.85±0.09^{a}

Based on these previous studies, *B.subtilis* was co-cultured with *S.aureus* at different ratio in order to induce bacteriocin production in broth. In co-culture of 2% (v/v) *B.subtilis* with 10% (v/v) of *S.aureus* for 16 hours, although inoculum size of *B.subtilis* was 5 times less than *S.aureus*, they both reached equal cell counts after 16 hours. Increasing *B.subtilis* P5-6's inoculum size from 2 to 10% (v/v), the change in *S.aureus* population was not much differen t. *Bacillus subtilis* population at 10% of inoculum size was nearly equal to that in single culture. When doubled the inoculum size of *B.subtilis* P5-6 from 10 to 20% (v/v), *S.aureus* population decreased approximately 1 log CFU/ mL (from 8.41 to 7.93 log CFU/ mL) and it's 1 log CFU/ mL less than that in single culture (8.85 log CFU/ mL). It seems like *B.subtilis* P5-6 dominated *S.aureus* in that culture. Because its initial population was double that of *S.aureus*, it could occupy more nutrient and grow faster than its competitor. This demonstrated that the P5-6 might have no inhibitory effect on S. aureus in the liquid culture.

3.3 Inhibitory Effect of *Bacillus Subtilis* P5-6 on *Staphylococcus Aureus* on Solid Medium

According to bacteriocin production properties and the result of co-culture of *B.subtilis* P5-6 and *S.aureus*, antimicrobial activity in liquid culture was not significant in compared with that in solid medium. Since the hypothesis is that bacteriocin produced was attatched to cell membrane of this *Bacillus* strain to protect itself from *S.aureus* and the concentration of bacteriocin

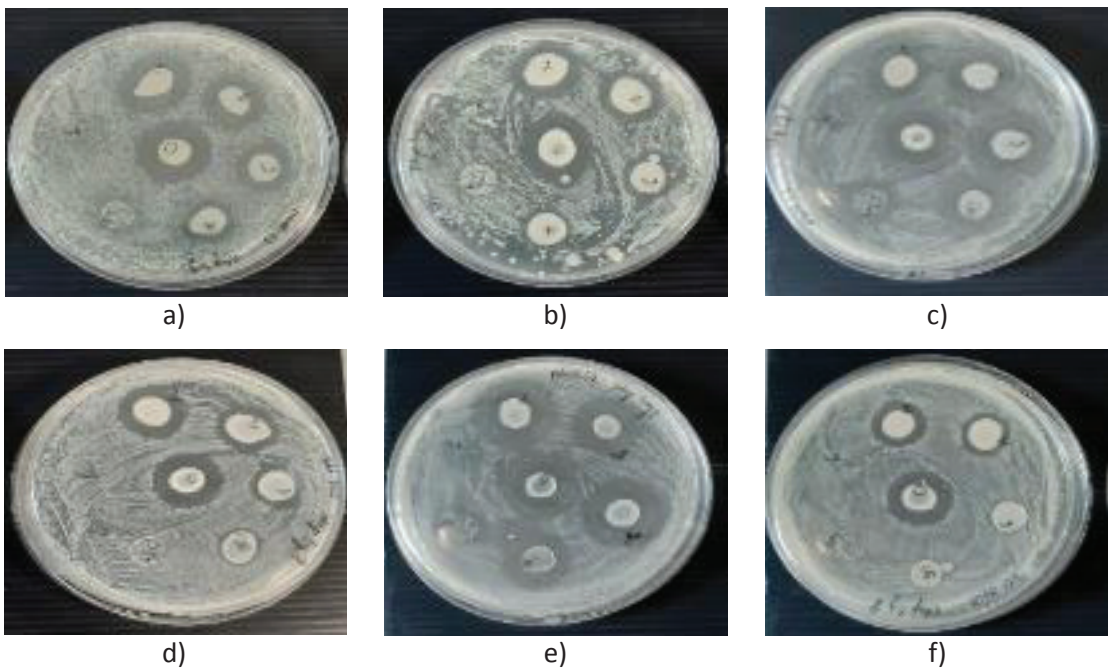


Figure 4. Spot on lawn on different population of *B.subtilis* on *S.aureus* lawn 6 log CFU/ mL on NA with 5% NaCl (a), and without NaCl (b), 7 log CFU/ mL on NA with 5% NaCl (c) and without NaCl (d), 8 log CFU/ mL on NA with 5% NaCl (e) and without NaCl (f),

generated was not too high to not harm itself but still sufficient to kill *S.aureus*, it may need to be in close contact to *S.aureus* to perform inhibitory effect. The presence of liquid could disperse *B.subtilis* P5-6 and *S.aureus* cell a part and also dilute bacteriocin, if it was secreted into broth. Therefore, solid medium could support the close contact between *B.subtilis* P5-6 and the target strain. These two strains were varied in concentration to screen for the specific cell concentration of *B.subtilis* P5-6 that can effectively inhibit *S.aureus*.

It can be seen in table 2 that at the same population of *B.subtilis* P5-6, the inhibition zone increased gradually as *S.aureus* population decreased. *B.subtilis* P5-6 can inhibit *S.aureus* even when its population was much lower in compared with *S.aureus* population. However, the inhibition effect was not significant at low population of this isolate. This could be due to the mechanism of quorum sensing. When cell density is low, signalling molecules diffuse away so there is no detection and response between cell (Kaplan & Greenberg, 1985). Thus, at the low amount of *B.subtilis* P5-6 cell, signal molecules (autoinducers) were insufficient for cells to communicate with each other, to perform any antimicrobial activity against *S.aureus*.

Table 2. Inhibitory effect of different concentration of *B.subtilis* P5-6 on different concentration of *S.aureus* on solid medium (*statistical analysis was based on the same column)

***Bacillus subtills* P5-6**	*Staphylococcus aureus*					
	6 Log CFU/ mL on plate		**7 Log CFU/ mL on plate**		**8 Log CFU/ mL on plate**	
Population on plate (Log CFU/ mL)	**Inhibition zone on NA w/o NaCl (mm)**	**Inhibition zone on NA+5% NaCl (mm)**	**Inhibition zone on NA w/o NaCl (mm)**	**Inhibition zone on NA+5% NaCl (mm)**	**Inhibition zone on NA w/o NaCl (mm)**	**Inhibition zone on NA+5% NaCl (mm)**
7	6.06±0.47^{a}	6.20±0.17^{a}	5.58±0.33^{a}	5.13±0.31^{a}	4.32±1.36^{a}	4.55±0.13^{a}
6	4.45±0.13^{b}	5.75±0.35^{a}	4.50±0.56^{b}	4.07±0.38^{b}	3.03±0.45^{b}	3.70±0.01^{b}
5	3.88±0.53^{b}	4.75±0.08^{b}	3.27±0.33^{c}	3.22±0.28^{c}	1.90±0.25^{c}	2.73±0.32^{c}
4	2.43±0.40^{c}	3.63±0.20^{c}	2.18±0.10^{d}	2.25±0.34^{d}	0.87±0.27^{cd}	2.45±0.22^{c}
3	1.68±0.35^{d}	2.73±0.51^{d}	1.25±0.17^{e}	1.43±0.05^{e}	ND	1.12±0.12^{d}
2	ND	1.20±0.03^{e}	ND	0.80±0.34^{ef}	ND	0.93±0.0^{d}
1	ND	ND	ND	ND	ND	ND

In the presence of 5% NaCl, the inhibition zones were detected at the second lowest cell concentration of P5-6 isolate (2 log CFU/ mL) among tested concentration whereas no inhibitory effect was observed at the same cell concentration when there is no NaCl supplemented. Because *B.subtilis* P5-6 is halophile while *S.aureus* is halotolerant, the supplementation of NaCl could facilitate *B.subtilis* P5-6 cell growth and inhibit the growth of *S.aureus*. It was shown that in solid medium, *B.subtilis* P5-6

could exert higher antagonistic action against *S.aureus* in compared with that in liquid medium. In liquid culture, because there was low amount of bacteriocin generated and it still attached to cells of producer strain, in order to achieve a considerable inhibition effect, *B.subtilis* P5-6 must be used in the same population and double inoculum size in compared with that of *S.aureus*. On the other hand, solid culture might facilitate cell-cell contact between two bacteria, which might induce the antimicrobial activity of *B.subtilis*. In addition, antimicrobial substance produced by *B.subtilis* P5-6 could be more concentrated than that in liquid culture. Because the solid medium might support the migration of bacteriocin, they could diffuse away from cell and be regenerated. The concentration of bacteriocin was higher in compared with that in liquid culture but it was not harmful for producer strain because it did not attach to its cells. Thus, inhibitory effect could be detected at low population of *B.subtilis* P5-6. Solid state cultivation could be a suitable method for up-scale production of *B.subtilis* P5-6. According to several researches, solid state fermentation could enhance the production of interested product and reduce production cost (Zhao *et al*., 2007 ; Muslim, 2013 ; Chuayjum *et al*., 2020)

4. Conclusion

Bacillus subtilis P5-6 showed no or less inhibitory effect on *S.aureus* when co-culture in liquid medium. In solid medium, the P5-6 significantly expressed an inhibitory effect on *S.aureus* even when its population was 2 log CFU/ mL lower than that of this pathogen strain. The results obtained in this study demonstrated that P5-6 could express the subtilosin gene having an inhibitory effect on *S.aureus*. The solid state cultivation with The supplementation of NaCl could enhance production and/or activity of bacteriocin of P5-6 as well as bacterial cell growth. This oservation could help to prove the mechanism of bacteriocin of *B.subtilis* P5-6 and develope the technology for production and application of protective culture from this bacterial strain to protect food against *S.aureus*-one of the most common cause of foodborne disease.

5. References

Balaban, N. & Rasooly, A. (2000). Staphylococcal enterotoxins. *International Journal of Food Microbiology, 61* (1), 1-10.

Ben Said, L., Gaudreau, H., Dallaire, L., Tessier, M. & Fliss, I. (2019). Bioprotective Culture: A New Generation of Food Additives for the Preservation of Food Quality and Safety. *Industrial Biotechnology, 15*, 138-147.

Castro, A., Silva, J. & Teixeira, P. (2018). *Foodborne Diseases.* Academic Press.

Chhetri, V., Prakitchaiwattana, C. & Settachaimongkon, S. (2019). A potential protective culture ; halophilic *Bacillus* isolates with bacteriocin encoding gene against *Staphylococcus aureus* in salt added foods. *Food Control, 104*, 292-299.

Chuayjum, C., Onsaard, W. & Prakitchaiwattana, P. (2020). Sugar Conversion of Immature Green Rice Syrup under Solid State Fermentation. *Science Technology and Engineering Journal, 6* (1), 46-56.

Gonzalez, D. J., Haste, N. M., Hollands, A., Fleming, T. C., Hamby, M., Pogliano, K., Nizet, V. & Dorrestein, P. C. (2011). Microbial competition between *Bacillus subtilis* and *Staphylococcus aureus* monitored by imaging mass spectrometry. *Microbiology, 157*, 2485-2492.

Kadariya, J., Smith, T.C. & Thapaliya, D. (2014). Staphylococcus aureus and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health. *BioMed Research International*.

Kaplan, H.B. & Greenberg, E.P. (1985). Diffusion of autoinducer is involved in regulation of the Vibrio fischeri luminescence system. *Journal of Bacteriology, 163*, 1210-1214.

Ko, K.S., Kim, J.W., Kim, J.M., Kim, W., Chung, S.I. & Kim, I.J. (2004). Population structure of the *Bacillus* cereus group as determined by sequence analysis of six housekeeping genes and the plcR gene. *Infection and Immunity, 72*, 5253-5261.

Liu, Q., Gao, G., Xu, H. & Qiao, M. (2012). Identification of the bacteriocin subtilosin A and loss of purL results in its high-level production in *Bacillus* amyloliquefaciens. *Research in Microbiology, 163*, 470-8.

Muslim, S. (2013). Comparative study between liquid state fermentation and solid state fermentation for production of endo-1, 4-beta-xylanase by *Bacillus* licheniformis. *Journal of American Arabic Academy for Sciences and Technology, 4* (11), 131-146.

Oakley, B.R. (2017). *Aspergillus nidulans*, *Reference Module in Life Sciences*. Elsevier.

Rio, D.C. (2014). Reverse transcription-polymerase chain reaction. *Cold Spring Harbor Protocols, 11*, 1207-1216.

Rutherford, S.T. & Bassler, B.L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine, 2* (11), a012427.

Sanders, E.R. (2012). Aseptic laboratory techniques: plating methods. *Journal of Visualized Experiment*, (63), e3064.

Tagg, J.R. & Mc Given, A.R. (1971). Assay system for bacteriocins. *Journal of Applied Microbiology, 21*, 943-949.

Vaughan, V.C. (1984). Poisonous or "sick" cheese. *Public Health, 10*, 241-245.

Velho, R.V., Basso, A.P., Segalin, J., Costa-Medina, L.F. & Brandelli, A. (2013). The presence of sboA and spaS genes and antimicrobial peptides subtilosin A and subtilin among *Bacillus* strains of the Amazon basin. *Genetics and Molecular Biology, 36* (1), 101-104.

Wei, Y., Perez, L.J., Ng, W.L., Semmelhack, M.F. & Bassler, B.L. (2011). Mechanism of Vibrio cholerae autoinducer-1 biosynthesis. *ACS Chemical Biology, 6*, 356-365.

Yan, Z., Ai, X., Su, Y., Liu, X., Shan, X. & Wu, S. (2016). Intracellular biosynthesis of fluorescent cdse quantum dots in *Bacillus subtilis*: a strategy to construct signaling bacterial probes for visually detecting interaction between *Bacillus subtilis* and *Staphylococcus aureus*. *Microscopy and Microanalysis, 22* (1), 13-21.

Zhao, S., Hu, N., Huang, J., Liang, Y. & Zhao B. (2008). High-yield spore production from *Bacillus* licheniformis by solid state fermentation. *Biotechnology Letters, 30* (2), 295-297. doi: 10.1007/s10529-007-9540-1.