http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 17 (8): 1015-1022, 2014
ISSN 1028-8880 / DOI: 10.3923/pjbs.2014.1015.1022

Low Cost Medium for Spore Production of *Bacillus* KKU02 and KKU03 and the Effects of the Produced Spores on Growth of Giant Freshwater Prawn (*Macrobrachium rosenbergii* de Man)

[1,2]Charkrit Wangka-Orm, [3]Sirirat Deeseenthum and [4,5,6]Vichai Leelavatcharamas
[1]Graduate School, Khon Kaen University, Khon Kaen, 40002, Thailand
[2]Department of Environmental Science, Faculty of Science,
Udon Thani Rajabhaj University, Udon Thani, 41000, Thailand
[3]Department of Biotechnology, Faculty of Technology, Mahasarakham University,
Maha Sarakham, 44150, Thailand
[4]Agricultural Biotechnology Research Center for Sustainable Economy, Faculty of Agriculture,
[5]Fermentation Research Center for Value Added Agricultural Products, Faculty of Technology,
[6]Department of Biotechnology, Faculty of Technology, Khon Kaen University,
Khon Kaen, 40002, Thailand

Abstract: In order to extend the shelf life of 2 high potential *Bacillus* probiotic isolates which were *Bacillus* KKU02 and *Bacillus* KKU03, the spore forms of these 2 *Bacillus* isolates were studied for using as probiotic instead. The low cost medium for spore production of these 2 *Bacillus* isolates was examined in order to produce probiotic spores for feeding the shrimps. It was found that cassava at 100 g L^{-1} and supplemented with 20.0 g L^{-1} dextrose, 0.1 g L^{-1} $MgSO_4$ and 2.0 g L^{-1} $(NH_4)_2SO_4$ showed the highest spore concentration at about 1×10^8 CFU mL^{-1}. The effects of feeding these 2 *Bacillus* spores on the growth of giant freshwater prawns were further examined. The spores of *Bacillus* KKU02 and *Bacillus* KKU03 (~10^7 spore mL^{-1}) in pure and mixed culture forms were mixed with commercial prawn feed (200 mL kg^{-1}) to give six feed treatments. Body length and weight of the prawns in mixed spore culture tanks after rearing for 90 days (13.5 cm and 59.8 g, respectively) were significantly higher ($p = 0.05$) than other treatments. The treated prawns were further challenged with *Aeromonas hydrophila* for 7 days. The percentages of survival after the challenge in the prawns fed with the mixed spores (46.8%) were also found significantly higher ($p = 0.05$) than others groups, except the mixed live cell treatment (60%). These results indicated that the spores of *Bacillus* KKU02 and *Bacillus* KKU03 had a high potential for using as commercial probiotics.

Key words: Giant freshwater prawn, probiotic, *Bacillus* sp., spore production

INTRODUCTION

Giant freshwater prawn (*Macrobrachium rosenbergii* de Man) is commonly found in the nature among Southeast Asian countries. This prawn is used to be caught along the major rivers in Thailand and is an alternative source of protein, especially for the North-eastern area of Thailand where the productivity of agricultural crop is low. Because of its good taste and texture, the freshwater shrimp has become a very popular food which, consequently, resulting in an over fishing and destroying of its natural habitat. The natural catching, thus, has been reduced dramatically. The production of post larvae in hatcheries with potential for pond culture and number of prawn farms, thus, has significantly increased. The fast growing of shrimp farming and the continuous use of the farming land led to the neglect of good husbandry and environmental management. Under these damaging environmental conditions, shrimps were stressed and weakened and, thus, could cause the development of shrimp diseases. Thus, the major problem for the shrimp farming industry is the shrimp disease. Vaccines and antibiotics are the important disease control measures for the shrimp farming. Vaccines alone, however, could not be used in controlling all shrimp diseases. Thus it is not an economical measure for shrimp protection. Antibiotic and chemotherapeutics treatments are, hence, the important measures for disease controlling

Corresponding Author: Vichai Leelavatcharamas, Faculty of Technology, Khon Kaen University, 123, Mitrapap Rd., Khon Kaen, 40002, Thailand Tel/ Fax: +66-43-362-122

in aquaculture. Bacterial resistance to antibiotics development has been well documented. The fear of the spread of this resistance to human pathogens has led to the banning of several antibiotics as so-called growth promoters in animal husbandry within the European Union and the year 2006 is the date proposed for a complete ban of antibiotics in animal feed within Europe (Cartman and La Ragione, 2004; Hong *et al*., 2005). This concern has also been raised in the aquaculture industry and has led to suggestions for other disease control measures. The use of probiotic is an alternative measure and many species have been used and produced (Westerdahl *et al*., 1991; Smith and Davey, 1993; Gatesoupe, 1994; Austin *et al*., 1995; Bly *et al*., 1997; Gram *et al*., 1999; Sanders *et al*., 2003; Hong *et al*., 2005; Cutting, 2011). However, the fear of antibiotic resistant gene transfer among the bacterial species is another concern. Thus, new isolates of probiotic which could not transfer antibiotic resistance gene are still needed.

Most probiotic, generally found normally in the Gastrointestinal Tract (GIT) of humans and animals, are supplied as live supplements in feed which must have the ability to survive passage through the intestinal tract. However, microorganisms which are not normally found in the GIT, such as the spore forming bacteria, are alternative interesting probiotic sources. One disadvantage of using live non-spore forming bacteria is the stability of the probiotic product. If the spore form is used, thus it can be stored longer on the shelf (Hong *et al*., 2005; Cutting, 2011).

Bacillus KKU02 and *Bacillus* KKU03 have been isolated from the intestine of the giant freshwater prawns. It was found that these 2 isolates of *Bacillus* showed a high potential for using as prawn probiotic (Deeseenthum *et al*., 2007). However, when these 2 isolates were applied in the field by the farmers, their efficiencies were declined because of the reduction of their viabilities. The advantage of the *Bacillus* sp. was they could form spores which could survive in some stress conditions, such as heat and dry conditions. In order to use the spore for testing the probiotic efficiency, the mass production of spore need to be examined. Thus, the low cost medium for spore production and the effects of the produced spores of *Bacillus* KKU02 and *Bacillus* KKU03 as probiotic on growth of giant freshwater prawn after feeding with the probiotic were the aims of these studies.

MATERIALS AND METHODS

Low cost media formulation for *Bacillus* KKU02 and KKU03 spores production

Microorganism: Two *Bacillus* sp. isolated from the intestine of the giant freshwater prawn which were *Bacillus* KKU02 and *Bacillus* KKU03 as reported earlier (Deeseenthum *et al*., 2007), were used in this study.

Media formula for spore production: Four cheap agricultural substrates which were sweet potato (*Impomoea batatasil*), cassava root (*Manihot esculenta*), rice (*Oryza sativa*) and sticky rice (*Oryza sativa* var. glutinosa), were added to the tested media 200 g L^{-1} as carbon source, comparison to Nutrient Broth (NB). Each agricultural substrate medium was boiled for a given time to obtain the aqueous extract which was then supplemented with 20.0 g L^{-1} dextrose and use as spore production medium. Control medium was consisted of 20.0 g L^{-1} dextrose as a sole carbon source. The initial pH of the medium was adjusted to 7.0 prior to sterilization at 121°C for 15 min. The sterilized culture medium (150 mL in 250 mL Erlenmeyer flasks) was inoculated with 1.0% of 12 h culture of *Bacillus* KKU02 and KKU03 and cultivated at 37°C on a rotary shaker (150 rpm). The obtained optimum carbon source medium was further examined for the mineral salt supplementation by adding the aqueous extract medium with $MgSO_4$ 0.1 g L^{-1} and $(NH_4)_2SO_4$ 2.0 g L^{-1} which were used in the previous cell production medium (Deeseenthum *et al*., 2007). In order to obtain the optimum concentration of the carbon source, the concentrations of the obtained carbon source medium were then further varied to 50, 100 and 200 g L^{-1}. The culture condition was the same as described earlier.

Analysis: Spore concentration was determined by viable plate count technique. Samples were taken every 4-6 h and boiled at 80°C for 10 min for eliminating of the vegetative cells. The remaining spore sample was then used for viable plate counting.

Giant freshwater prawn production using *Bacillus* KKU02 and *Bacillus* KKU03 in spore form as probiotic

Preparation of experimental animals: Post larvae of giant freshwater prawns (PL-15) were obtained from a hatchery located in Supanburi province, Thailand. The prawns were acclimatized in the tanks for 60 days and fed with only the commercial feed. Two hundred giant freshwater prawns of uniform size (12.0-14.0 g) were kept in each tank. The prawns were reared with probiotic bacteria in six feed treatments until reach to 90 days.

Experimental conditions: The experiment was conducted for 150 days at the Department of Biotechnology, Khon Kaen University, Khon Kaen, Thailand. The experiment was divided into six triplicates experimental groups in

18 concrete tanks (1.50×2.00×1.00 m^3 at 0.5 m water height). The total water in each tank was maintained at 1,500 L and aeration was continuously provided. About 50% of water was replaced with fresh water once a week. The details of the experimental groups were as follows:

Treatment 1: Commercial diet+spore of *Bacillus* KKU03
Treatment 2: Commercial diet+spore of *Bacillus* KKU02
Treatment 3: Commercial diet+spores of *Bacillus* KKU02 and KKU03
Treatment 4: Commercial diet+vegetative cells of *Bacillus* KKU02 and KKU03 (Deeseenthum *et al*., 2007)
Treatment 5: Commercial diet (control)
Treatment 6: Commercial diet+commercial probiotic (reference control)

The commercial feed used was purchased from Charoen Pokphand Company, Samutsakhon, Thailand (CP No. 9041). The feed was autoclaved at 110°C for 28 min before mixing with the cultured spore for eliminating of the contamination.

Bacterial strains and feeding regime: The *Bacillus* KKU03 and *Bacillus* KKU02 were grown in cooked cassava chip aqueous extract medium as described earlier for 48 h at 37°C on a shaker at 150 rpm. The final spore concentration of about 10^7 spore mL^{-1} was mixed with the feed at the ratio of 200 mL to 1 kg feed (the expected concentration in the feed was 2×10^6 spore g^{-1}). After acclimatization, the prawns were fed twice daily, at 08.00 am and 06.00 pm. The daily feeding rate was about 10% of total body weight.

Analysis of samples: Ten randomly collected live prawns from each tank were measured for lengths and weights once every 3 weeks. Water quality was checked weekly for pH, dissolved oxygen and temperature. The amount of ammonium, total hardness and total alkalinity were also determined by test kits (HACH®) obtained from HACH Company, USA.

Statistical analysis: One-way analysis of variance (ANOVA) was used to determine any significant differences among the treatment groups. The comparison was done by using Randomized Complete Block Design (RCBD) test between the six treatments.

Challenge test of the probiotic treated giant freshwater prawns

Experimental conditions: After rearing shrimps in six feed treatments (prawn production section) until reach 120 days, 30 shrimps from each treatment (triplicate of 10 shrimps per tank) were transferred into a glass container (15"×18"×15" at 6" of water height) and challenge with *Aeromonas hydrophila* which was cultured and maintained using NB at 37°C, 200 rpm for 24 h. The bacterial suspension with a final concentration of 10^5-10^8 CFU mL^{-1} (300 mL) was added to each tank. The number of survival shrimps was recorded daily until 0% survival was reached in any treatment.

RESULTS

Low cost medium formulation for spore productions of *Bacillus* KKU02 and KKU03: Four abundant agricultural substrates which were sweet potato (*Impomoea batatasil*), cassava root (*Manihot esculenta*), rice (*O. sativa* Linn.) and sticky rice (*O. sativa* var. glutinosa), were added to the tested media as carbon sources in comparison to Nutrient Broth (NB). The results were shown in Fig. 1. It was found that all substrates could support the spore productions of

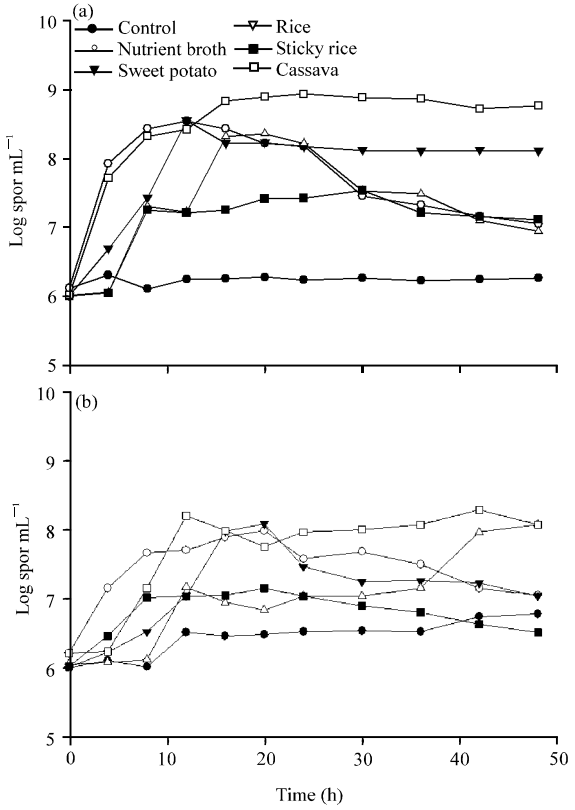

Fig. 1(a-b): Spore production of *Bacillus* (a) KKU02 and (b) KKU03 when using different agricultural substrates as carbon sources

Bacillus KKU02 and *Bacillus* KKU03 better than without adding any agricultural substrates (the control group). It could be seen that only cassava root and sweet potato supplementation gave an equal or better spore concentration than the nutrient broth. However, when consider the price of cassava and sweet potato, the cooked cassava root was the optimum carbon source for *Bacillus* KKU02 and *Bacillus* KKU03 spore productions which showed the highest concentration of *Bacillus* KKU02 and *Bacillus* KKU03 spores at 8.32 and 1.35×10^{8} spores mL^{-1}, respectively.

Supplementation of cooked cassava medium with mineral salts, 0.1 g L^{-1} $MgSO_4$ and 2.0 g L^{-1} $(NH_4)_2SO_4$, resulted in 1.75 times increase of spore productions in both *Bacillus* strains (Fig. 2). The highest concentrations of *Bacillus* KKU02 and *Bacillus* KKU03 spores were 1.62×10^{8} and 6.61×10^{7} spore mL^{-1}, respectively.

Various cassava concentrations (50, 100 and 200 g L^{-1}) for spore production were studied in order to obtain the optimum cassava concentration for spore production. The optimum cassava concentration for spore production of both *Bacillus* strains was 100 g L^{-1} which showed the highest spore concentrations of *Bacillus* KKU02 and *Bacillus* KKU03 at 1.78×10^{8} and 1.48×10^{8} spores mL^{-1}, respectively (Fig. 3).

Giant freshwater prawn production using *Bacillus* KKU02 and *Bacillus* KKU03 in spore form as probiotic: Water quality during shrimp cultivation was also concerned in this study. The range of water quality parameters during experimental period in each prawn culture tank were shown in Table 1. All of the measured parameters which were pH, temperature, %DO, the amount of ammonium, hardness and alkalinity were in the ranges of 7.02-8.72, 23-30°C, 4.0-8.5%, 0-0.25, 120-250 and 80-180 ppm, respectively. These results were in the acceptable ranges suggested by Armstrong *et al.* (1976), New (1990) and Boyd and Zimmerman (2000).

The rearing prawns with the probiotic in six feed treatments for 90 days showed significant differences ($p\geq0.05$) of body weight and length gain between T1, T2,

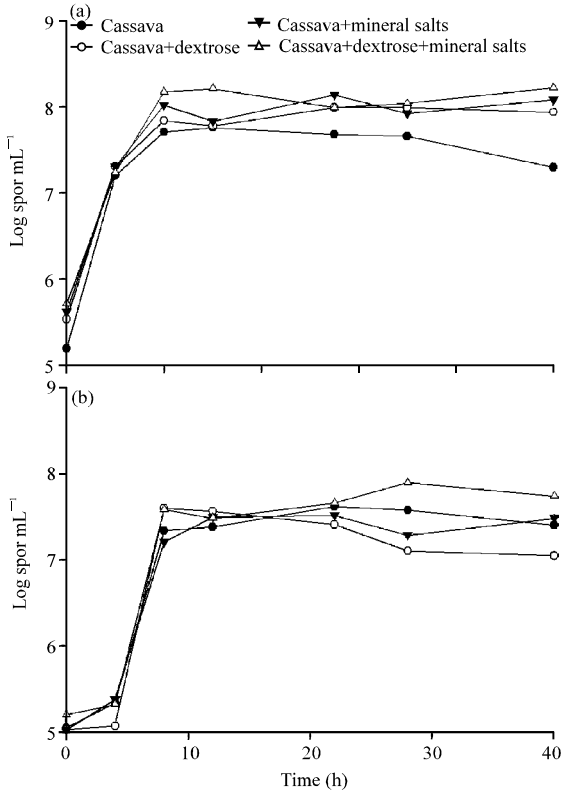

Fig. 2(a-b): Spore production of *Bacillus* (a) KKU02 and (b) KKU03) when supplementation with mineral salts in the cassava medium

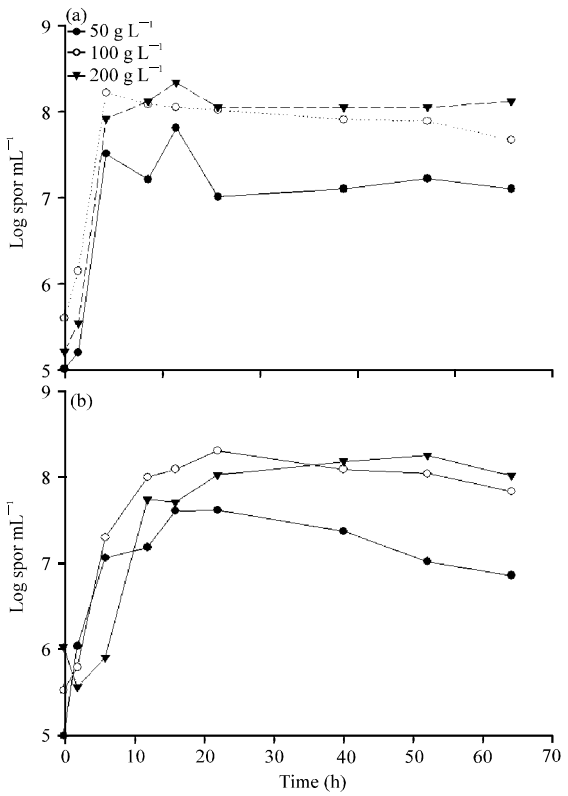

Fig. 3(a-b): Spore production of *Bacillus* (a) KKU02 and (b) KKU03 at various cassava concentrations

Table 1: Physical and chemical water quality parameters ranges during shrimp cultivation with 6 feed treatments of pure and mixed cultures of *Bacillus* KKU 02 and KKU03

Treatment	pH	Temperature (°C)	Disolved oxygen (%)	Ammonia (mg L^{-1})	Hardness (mg L^{-1})	Alkalinity (m g L^{-1})
1	7.5-8.22	23-30	4.50-8.0	0-0.25	120-250	80-180
2	7.25-8.5	24-30	4.25-7.5	0-0.25	120-250	80-180
3	7.7-8.72	24-30	4.50-8.5	0-0.25	120-250	80-120
4	7.4-8.45	23-29	4.25-7.5	0-0.25	120-250	80-120
5	7.02-8.2	24-29	4.00-7.25	0-0.25	120-275	80-120
6	7.24-8.47	23-30	4.25-7.5	0-0.25	120-250	80-120

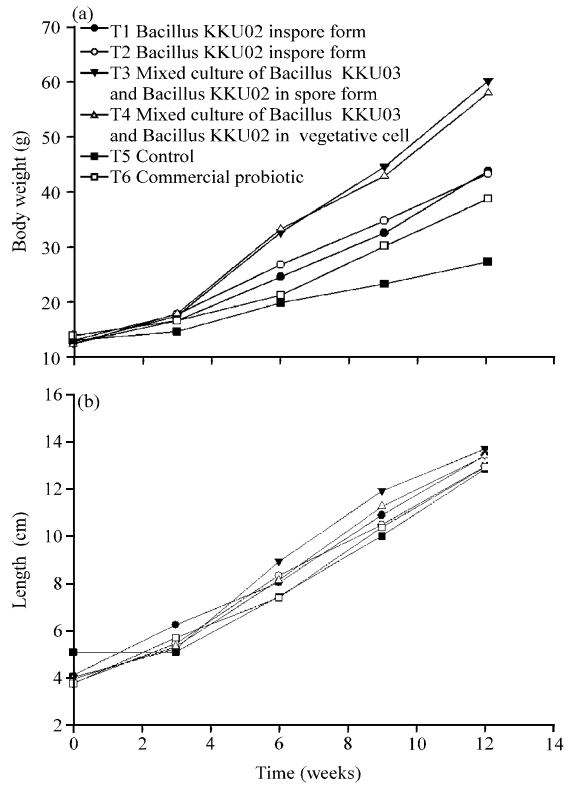

Fig. 4(a-b): (a) Body weight and (b) Length of giant freshwater prawn after rearing for 90 days in six feed treatments

T3, T4, T6 and the control group (T5) during probiotic feeding, as shown in Fig. 4. The prawns fed with mixed culture of spore form (T3) exhibited the highest body weight and length at 59.75 g and 13.50 cm, respectively. On the other hand, the body weight and length of the probiotic treatment groups T1, T2, T4 and T6 were not significantly different from each other ($p \geq 0.05$).

Challenge test of the treated giant freshwater prawn: After 3 days of post challenge with *A. hydrophila*, 50% of shrimps in the control group (T5 without any probiotic supplementation) were dead while more than 70% of shrimps in all probiotic treatment groups still survive, as

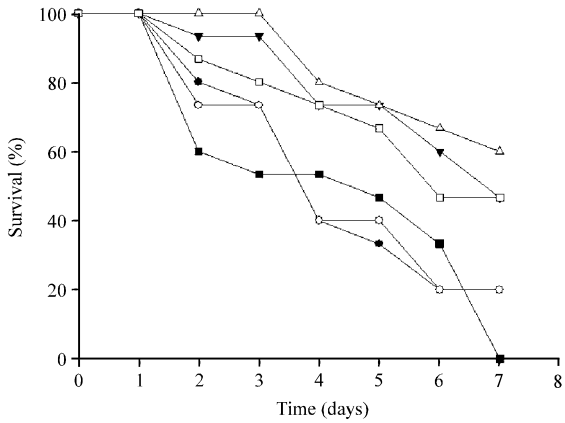

Fig. 5: Percentage of survival in challenged prawn after challenging prawns with *A. hydrophila* for 7 days compared to the control group

shown in Fig. 5. All shrimps in the control treatment (T5) could not survive after 7 days of the challenge. In contrast, the survival rate of prawn in mixed culture treatments both spore and vegetative cell, could survived more than 45% at day 7 of the challenge which were comparable or even better than the commercial probiotic. The lower percentages of shrimp survival in T1 and T2 after 3 days of the challenge were because some shrimps were dead during molting.

DISCUSSION

Low cost medium formulation for spore productions of *Bacillus* KKU02 and KKU03: *Bacillus* spores have been reported as probiotic uses both in human and animal from a lot of scientists (Hoa *et al*., 2001; La Ragione *et al*., 2001; Casula and Cutting, 2002; La Ragione and Woodward, 2003; Hong *et al*., 2005; D'Arienzo *et al*., 2006; Hong *et al*., 2008; Cutting, 2011). *Bacillus* KKU02 and KKU03 were reported earlier in using as probiotic in giant freshwater shrimp (Deeseenthum *et al*., 2007). In order to extend the shelf life of these 2 *Bacillus* isolates, their spore forms were investigated. In order to use spores as probiotic for feeding shrimp, mass production of spore must be considered, including cheap medium formulation

for spore production. Thailand has a lot of cheap agricultural products which could be used for bacteria cultivation. Four abundant agricultural products which were sweet potato, cassava root, rice and sticky rice, were tested in order to obtain the best substrate for spore productions of *Bacillus* KKU02 and KKU03. Fortunately, cassava which was the cheapest agricultural substrate, was the optimum substrate for our *Bacillus* spore productions. This could be seen from the obtained spore concentrations which were comparable to those obtained from the Nutrient broth. The control group which contained only dextrose, showed the lowest spore number, possibly because of the limitation of carbon source.

Some minerals, such as calcium magnesium and manganese, have been reported in enhancing spore formulation of some *Bacillus* sp. (Amaha *et al*., 1956; Curran, 1958, Kolodziej and Slepecky, 1964; Cote and Gherna, 1999; Monteiro *et al*., 2005; Dechmahitkul *et al*., 2007; Omer, 2010). In our previous studies, $MgSO_4$ and $(NH_4)_2SO_4$ were used as mineral and nitrogen sources in culturing *Bacillus* KKU 02 and KKU 03 (Deeseenthum *et al*., 2007). Thus, these 2 compounds were tested by adding in the cassava medium for spore production. The results showed that the spore concentration was increased 1.7 times, compared to the medium without supplementation.

The concentration of carbon source was also important in spore production. Kang *et al.* (1992) reported that when glucose higher than 200 g L^{-1}, *Bacillus thuringienesis* could not produce its spore. The concentration of cassava in the spore production medium was, thus, examined. The results showed that reducing the cassava to 100 g L^{-1} could still get the spore concentration equivalent to when 200 g L^{-1} were used.

Thus the optimum conditions for spore production of *Bacillus* KKU02 and KKU03 were cassava 100 g L^{-1}, dextrose 20 g L^{-1}, $MgSO_4$ 0.1 g L^{-1} and $(NH_4)_2SO_4$ 2.0 g L^{-1}.

Giant freshwater prawn production using spore of *Bacillus* KKU02 and *Bacillus* KKU03 as probiotic: Water qualities, such as pH, %DO, the amount of ammonium, hardness and alkalinity, had some effects on shrimp growth (Armstrong *et al.,* 1976; New, 1990; Boyd and Zimmerman, 2000). Water in the culture tanks was changed once a week in order to make sure that water had no effect on the death and could be used for culturing shrimps. The results showed that water quality during shrimp cultivation was in the acceptable range. Thus, growth performance of the cultured shrimps was affected by the studied *Bacillus* probiotic.

After feeding shrimps with probiotic, both in vegetative cells and spore forms of *Bacillus* KKU02 and KKU03 it was found that the weight of the shrimps was significantly higher than feeding only the commercial feed, as shown in Fig. 4, although the shrimp length in all treatments, except in T3, was not significantly different. In addition, the mixed cultures, both live cells and spore forms, exhibited the better results than using pure spore cultures and the commercial probiotic. These results were similar to our previous report when live cells were used (Deeseenthum *et al*., 2007). This was possibly because the mixed probiotic *Bacillus* sp. enhanced nutrients utilization in shrimps, as these 2 isolates of *Bacillus* could produce amylase and protease (Deeseenthum *et al*., 2007). Moreover, these results also indicated that the spore forms of these two isolates of *Bacillus* gave the same results when the vegetative cells were used. Thus, the spore of these 2 *Bacillus* isolates could also be used as probitics. The pure *Bacillus* spore of both stains, in addition, showed the equivalent obtaining weight to the commercial probiotic treatment. These results indicated that our *Bacillus* stains had a high potential for commercialization.

Challenge test of the treated giant freshwater prawn after raring for 90 days: The survival of probiotic fed shrimps was enhanced after challenging with *A. hydrophila*, especially in the mixed culture, confirming the advantages of probiotic use. This was possibly because of the immune stimulation or pathogen growth inhibition by the probiotic *Bacillus* sp. which was reported by a number of investigators (Sakai *et al*., 1995; Itami *et al*., 1998; Moriarty, 1998; Rengpipat *et al*., 2000; Bachere, 2003; Vaseeharan and Ramasamy, 2003; Balcazar *et al*., 2006; Pandiyan *et al*., 2013). The low survival of shrimps in the pure spore cultures of *Bacillus* KKU02 (T1) and KKU03 (T2) after 3 days of challenge was possibly because the shrimps accidentally molted during the challenge test. The molting shrimps were easily attacked by the healthy shrimps and easily to be infected by the pathogen. The mixed culture, both live (T3) and spore (T4) forms, showed the highest percentage of survival which was better than the commercial probiotic (T6). These results confirmed that our mixed *Bacillus* culture had a high potential in commercial use. However, more aspects in using these two isolates of *Bacillus*, such as the safety and production cost, have to be studied further.

ACKNOWLEDGMENTS

The authors would like to thank the Office of the Higher Education Commission, Thailand for supporting

by grant fund under the program "Strategic Scholarships for Frontier Research Network for the Join Ph.D. Program Thai Doctoral degree" for this research, Graduate School, Khon Kaen University and Agricultural Biotechnology Research Center for Sustainable Economy, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand, for the financial support of this research. Thank you to the Department of Biotechnology, Faculty of Technology, Khon Kaen University and the Fermentation Research Center for Value Added Agricultural Products, Khon Kaen University, Khon Kaen, Thailand and the Department of Environmental Science, Faculty of Science, Udon Thani Rajabhaj University, Udon Thani, Thailand for providing facilities for this research.

REFERENCES

Amaha, M., Z.J. Ordal and A. Touba, 1956. Sporulation requirements of *Bacillus coagulans* var. *Thermoacidurans* in complex media. J. Bacteriol., 72: 34-41.

Armstrong, D.A., M.J. Stephenson and A.W. Knight, 1976. Acute toxicity of nitrite to larvae of the giant Malaysian prawn, *Macrobrachium rosenbergii*. Aquaculture, 9: 39-46.

Austin, B., L.F. Stuckey, P.A.W. Robertson, I. Effendi and D.R.W. Griffith, 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii*. J. Fish Dis., 18: 93-96.

Bachere, E., 2003. Anti-infectious immune effectors in marine invertebrates: Potential tools for disease control in larviculture. Aquaculture, 227: 427-438.

Balcazar, J.L., I. de Blas, I. Ruiz-Zarzuela, D. Cunningham, D. Vendrell and J.L. Muzquiz, 2006. The role of probiotics in aquaculture. Vet. Microbiol., 114: 173-186.

Bly, J.E., S.M.A. Quiniou, L.A. Lawson and L.W. Clem, 1997. Inhibition of saprolegnia pathogenic for fish by *Pseudomonas fluorescens*. J. Fish Dis., 20: 35-40.

Boyd, C. and S. Zimmerman, 2000. Grow-Out Systems-Water Quality and Soil Management. In: Freshwater Prawn Culture-The Farming of *Macrobrachium rosenbergii*, New, M.B. and W.C. Valenti (Ed.). Blackwell Science, Oxford, UK., pp: 221-238.

Cartman, S.T. and R.M. La Ragione, 2004. Spore Probiotics as Animal Feed Supplements. In: Bacterial Spore Formers: Probiotics and Emerging Applications, Ricca, E., A.O. Henriques and S.M. Cutting (Eds.). Horizon Bioscience, Norfolk, UK., ISBN: 9781904933021, pp. 155-161.

Casula, G. and S.M. Cutting, 2002. Bacillus probiotics: Spore germination in the gastrointestinal tract. Applied Environ. Microbiol., 68: 2344-2352.

Cote, R. and R.L. Gherna, 1999. Medium Formulation and Design, *E. Coli* and *Bacillus* Spp. In: Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation, Flickinger, M.C. and S.W. Drew (Eds.). John Wiley and Sons, New York, USA., ISBN: 9780471138228, pp: 1676-1683.

Curran, H.R., 1958. The Mineral Requirements for Sporulation. In: Spores, Halverson, H.O. (Ed.). American Institute of Biological Sciences, Washington, DC., USA., pp: 1-9.

Cutting, S.M., 2011. Bacillus probiotics. Food Microbiol., 28: 214-220.

D'Arienzo, R., F. Maurano, G. Mazzarella, D. Luongo, R. Stefanile, E. Ricca and M. Rossi, 2006. *Bacillus subtilis* spores reduce susceptibility to *Citrobacter rodentium*-mediated enteropathy in a mouse model. Res. Microbiol., 157: 891-897.

Dechmahitkul, W., C. Youkong, K. Poomputra, S. Akeprathumchai and P. Mekvijitsaeng, 2007. Study on media formulation and production process of *Bacillus subtilis* spores for animal probiotics. KMUTT Res. Dev. J., 30: 251-259.

Deeseenthum, S., V. Leelavatcharamas and J.D. Brooks, 2007. Effect of feeding *Bacillus* sp. as probiotic bacteria on growth of giant freshwater prawn (*Macrobrachium rosenbergii* de Man). Pak. J. Biol. Sci., 10: 1481-1485.

Gatesoupe, F.J., 1994. Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic vibrio. Aquat. Living Resour., 7: 277-282.

Gram, L., J. Melchiorsen, B. Spanggaard, I. Huber and T.F. Nielsen, 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Applied Environ. Microbiol., 65: 969-973.

Hoa, T.T., L.H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P.H. Van and S.M. Cutting, 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Applied Environ. Microbiol., 67: 3819-3823.

Hong, H.A., L.H. Duc and S.M. Cutting, 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev., 29: 813-835.

Hong, H.A., J.M. Huang, R. Khaneja, L.V. Hiep, M.C. Urdaci and S.M. Cutting, 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. J. Appl. Microbiol., 105: 510-520.

Itami, T., M. Asano, K. Tokushige, K. Kubano and A. Nakawaga *et al*., 1998. Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. Aquaculture, 164: 277-288.

Kang, B.C., S.Y. Lee and H.N. Chang, 1992. Enhanced spore production of *Bacillus thuringiensis* by fed-batch culture. Biotechnol. Lett., 14: 721-726.

Kolodziej, B.J. and R.A. Slepecky, 1964. Trace metal requirements for sporulation of *Bacillus megaterium*. J. Bacteriol., 88: 821-830.

La Ragione, R.M. and M.J. Woodward, 2003. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. Vet. Microbiol., 94: 245-256.

La Ragione, R.M., G. Casula, S.M. Cutting and M.J. Woodward, 2001. *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. Vet. Microbiol., 79: 133-142.

Monteiro, S.M., J.J. Clemente, A.O. Henriques, R.J. Gomes, M.J. Carrondo and A.E. Cunha, 2005. A procedure for high-yield spore production by *Bacillus subtilis*. Biotechnol. Prog., 21: 1026-1031.

Moriarty, D.J.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture pond. Aquaculture, 164: 351-358.

New, M.B., 1990. Freshwater prawn culture: A review. Aquaculture, 88: 99-143.

Omer, A.M., 2010. Bioformulations of bacillus spores for using as biofertilizer. Life Sci. J., 7: 124-131.

Pandiyan, P., D. Balaraman, R. Thirunavukkarasu, E.G.J. George, K. Subaramaniyan, S. Manikkam and B. Sadayappan, 2013. Probiotics in aquaculture. Drug Invet. Today, 5: 55-59.

Rengpipat, S., S. Rukpratanporn, S. Piyatiratitivorakul and P. Menasaveta, 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture, 191: 271-288.

Sakai, M., T. Otubo, S. Atsuta and M. Kobayashi, 1995. Enhancement of resistance to vibriosis in rainbowtrout, *Oncorhynchus mykiss* (Walbaum), by oral administration of bovine lactoferrin. J. Fish Dis., 18:187-190.

Sanders, M.E., L. Morelli and T.A. Tompkins, 2003. Sporeformers as human probiotics: *Bacillus*, *Sporolactobacillus* and *Brevibacillus*. Compre. Rev. Food Sci. Food Saf., 2: 101-110.

Smith, P. and S. Davey, 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. J. Fish Dis., 16: 521-524.

Vaseeharan, B. and D. Ramasamy, 2003. Control of pathogenic *Vibrio* spp. By *Bacillus subtilis* BT 23, a possible probiotic treatment for black tiger shrimp *Penaeus mondon*. Lett. Applied Microbiol., 36: 83-87.

Westerdahl, A., J.C. Olsson, S. Kjelleberg, P.L. Conway, 1991. Isolation and characterization of turbot (*Scophtalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied Environ. Microbiol., 57: 2223-2228.