Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

Anti-*Aeromonas hydrophila* activity and characterisation of novel probiotic strains of *Bacillus subtilis* isolated from the gastrointestinal tract of giant freshwater prawns

Ananthaya Sansawat and Mongkol Thirabunyanon*

Biotechnology Section, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

* Corresponding author, e-mail: mthirabun@yahoo.co.th

Received: 17 March 2008 / Accepted: 20 May 2008 / Published: 23 February 2009

Abstract: The antagonistic activity of two *Bacillus* strains isolated from the gastrointestinal tract of giant freshwater prawns against *Aeromonas hydrophila* was evaluated in vitro. The characterisation of the novel probiotic strains of these bacilli was also performed. *Bacillus subtilis* P33 and 72 were found to have high inhibition activities against the growth of *A. hydrophila* by two assay methods: paper disc and well diffusion. Probiotic properties, namely acid and bile salt tolerance, autoaggregation, coaggregation, hydrophobicity and adhesion to Caco-2 cells, were further analysed. Survival rates in model gastrointestinal tract condition, viz. pH 2.5 for 3 h and 0.3% bile salt for 24 h, were shown to be more than 95% and 90% respectively. The ability of *B. subtilis* strains of P33 and P72 to adhere to epithelial cells of the host animal was measured by percentage autoaggregation (35.7 and 42.2%), coaggregation (11.1 and 11.6%), hydrophobicity in *n*-hexadencane (25.6 and 30.0%), xylene (32.2 and 36.1%), toluene (30.3 and 31.6%), and adhesion to Caco-2 cells (4.21 and 3.23 log cfu/ml respectively). These results indicate that both strains of *B. subtilis* P33 and P72 can be considered to be good novel probiotic candidates for use in the prawn aquaculture industry.

Keywords: probiotic, giant freshwater prawns, *Bacillus subtilis*, *Aeromonas hydrophila*, Caco-2 cells

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

Introduction

The giant freshwater prawn (*Macrobrachium rosenbergii*) is one of most commercially important food commodities in the world especially in many Asian countries such as Taiwan, Thailand, and Vietnam [1]. In the prawn aquaculture industry, *Aeromonas hydrophila* infection is considered a major cause of shell diseases and low rate of survival [2-3]. The use of antibiotics to prevent these diseases has normally been practiced in many cases although their indiscriminate use has led to increase in antibiotic resistance and residual level in the products [4-6], which has affected the shrimp export of Thailand [7]. Probiotics as microbial cells are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health [8]. The main purposes of using probiotics in aquaculture were shown to include competitive exclusion of pathogenic bacteria [9-10], as source of nutrients and enzymatic contribution to digestion [11] and enhancement of the immune response against pathogenic microorganisms [10],[12-14]. Although several investigations have reported the case of using potential probiotics in shrimp aquaculture, there seems to be no report of similar cases for the giant freshwater prawn. In this study, therefore, the characteristic activity and antagonistic ability of the novel probiotic strain of *Bacillus subtilis* isolated from the gastrointestinal tract of giant freshwater prawns were investigated.

Materials and Methods

Bacterial strains

The tested strains of *B. subtilis*, P33 and P72, were isolated from the intestines of giant freshwater prawns which were obtained from Chaopraya River, while the compared strain of *B. subtilis*, TISTR 08, and the pathogenic strain of *Aeromonas hydrophila*, TISTR 1321, were purchased from Thailand Institute of Scientific and Technological Research. The tested strains were fundamentally characterised by being gram positive, rod shape, spore former and catalase positive, thus indicating a general morphology or characteristic of *Bacillus* [15]. The strains were maintained at -80^{o} C in 20 % (v/v) glycerol (Merck, Germany) until further analysis.

Antagonistic activity of Bacillus strains against A. hydrophila

Two methods of agar diffusion assays were used in this study. The first was the paper disc diffusion assay, a modification of the paper disc (Durchmesser: 6 mm, Macherey-Nagel, Germany) diffusion method used as triplicate tests. Both groups of the bacterial strains (the tested strains including the compared strain and the pathogenic strain) were briefly grown in a nutrient broth (Merck, Germany), incubated at 37^{o} C for 18 h, and adjusted to an approximate concentration of 10^{8} cfu/ml. Each sterilised paper disc was impregnated with 20 µl of a diluted test bacterial isolate and placed on the surface of an agar plate which was previously inoculated with the indicator pathogen at a concentration of about 10^{7} cfu/ml. The plate was then incubated at 37^{o} C for 24 h and the inhibition zone around paper disc was recorded. The second method was the well diffusion assay in which the nutrient agar (Merck, Germany) plates were each overlaid with 10 ml of molten nutrient broth (Merck, Germany) containing 0.7% agar at 45° C and inoculated with the 18-h culture of the pathogenic strain above to obtain a final concentration of approximately 10^{6} cfu/ml. Upon solidification of both agar

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

layers, a sterile cork borer was applied to create wells of 8 mm in diameter. The cell-free supernatant (100 µl) from the broth containing the 18-h culture of the tested strains were transferred into each well and incubated at 37° C for 24 h under aerobic condition. *B. subtilis* TISTR 08 was used as the control. The inhibition of a clear zone around the well showing no growth of the indicator pathogen was recorded. Each sample was done in triplicate.

API 50 CHB assay

The profile of biochemical test of the isolates was evaluated with API 50 CHB (BioMérieux®, France) strips following the manufacturer's instructions. Briefly, bacteria were grown in nutrient broth at 37 °C for 18 h. After centrifugation at 5000 g for 15 min, cells were washed twice with sterile distilled water. The bacteria were adjusted with sterile distilled water to achieve an approximate concentration at 2 McFarland. The bacterial suspension was mixed with API 50 CHB medium and added into the wells of API 50 CHB strips. These suspensions were incubated at 37° C for 24 h. The ability of the bacterial strains to ferment 49 different carbohydrates was used to classify the strains.

Acid and bile salt tolerance assays

Acid tolerance was evaluated using a modified method of Conway et al. [16] and Pennacchai et al. [17]. Cultures were grown in nutrient broth (Merck, Germany) at 37°C for 18 h. One ml of bacterial suspension was transferred into 9 ml of sterile phosphate buffer saline solution adjusted to pH 2.5 with 5 N HCl (Merck, Germany). The initial bacterial concentration was almost 10^7 cfu/ml, which was then incubated at 37°C for 0 and 3 h. Viable bacteria were counted after incubation at 37° C for 24 h on nutrient agar. For bile salt tolerance, the method of Gilliland et al. [18] was performed. One ml of bacterial suspension was inoculated into 9 ml of sterile nutrient broth prepared with bile salt (Sigma, USA). About 0.3% of bile salt concentrate was applied. The suspension was incubated at 37° C on nutrient agar and viable bacteria were counted after exposure of 0 and 24 h.

Autoaggregation and coaggregation assays

Aggregation of bacterial isolates was evaluated by the method of Del Re et al. [19] as modified by Kos et al. [20]. For determination of autoaggregation, the tested bacteria were grown at 37° C for 24 h on a nutrient broth (Merck, Germany). After centrifugation at 5000 g for 15 min, cells were washed twice and resuspended in phosphate buffer saline to give a viable concentration of about 10^7-10^8 cfu/ml. Four ml of the cell suspension were mixed for 10 s to determine autoaggregation during 5 h of incubation at room temperature. The upper suspension was used in each hour by transferring 0.1 ml to another 3.9 ml of phosphate buffer solution, and the optical density at 600 nm was measured. Per cent autoaggregation was calculated by the formula: $1-(A_t/A_0) \times 100$, where A_t represents the absorbance at time t = 1, 2, 3, 4 or 5, and A_0 the absorbance at t = 0. For determination of coaggregation, the cell suspension was prepared similar to the autoaggregation assay. Two 2-ml aliquots of the cell suspensions were mixed together by vortexing for 10 s. About 4 ml of individual cell suspension was set aside as control group at the same time. The absorbance at 600 nm was measured after mixing and incubating at room temperature for 5 h. Coaggregation was calculated according to Handley et al. [21]: $(A_x + A_y)/2 - A_{(x+y)}/A_x + A_y/2 \times 100$, where A_x and A_y represented absorbance of each of the two strains.

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

Hydrophobicity assay

Determination of cell surface hydrophobicity was evaluated according to the ability of the microorganisms to partition into hydrocarbon from phosphate buffer solution using the method of Savage [22]. Bacterial isolates were grown in nutrient broth (Merck, Germany) at 37°C for 24 h. After being centrifuged at 5000 g for 15 min, the pellets (bacterial precipitates) were washed twice with phosphate buffer solution and optical density of the bacteria at 450 nm adjusted to 0.5 A. About 1 ml of bacterial suspension was added with 60 µl of a hydrocarbon, viz. *n*-hexadecane (Fluka, Germany), xylene (Fisher, England), or toluene (Merck, Germany), and vortexed for 1 min followed by determination of optical density of the water phase. Hydrophobicity was calculated according to the equation: $[(OD_{450}$ before $- OD_{450}$ after$)/OD_{450}$ before$] \times 100 =$ % hydrophobicity.

Adhesion assay on Caco-2 cells

The method of Gagnon et al. [23] was performed with a little modification. Caco-2 cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% (v/v) fetal calf serum (Hyclone, USA) inactivated at 56°C for 30 min, 1% (v/v) non-essential amino acids (Hyclone, USA), and 1% (v/v) penicillin-streptomycin (10,000 IU/ml and 10,000 µg/ml; Hyclone, USA). Cells were incubated at 37° C in 5% CO_2 in air. For the bacterial adhesion assay, Caco-2 cells were seeded with 1 ml of culture medium containing 10^5 viable cells/well in 24-well tissue culture plates. The culture medium was changed every 48 h while Caco-2 cells were used at post-confluence after 15 days to become fully differentiated. The medium of non-supplemented DMEM was replaced at least 1 h before adhesion assay. Tested bacteria from the 18-h cultures in nutrient broth (Merck, Germany) were harvested and washed twice with phosphate buffer saline. Cells were resuspended in non-supplemented DMEM to achieve a concentration of 10^8 cfu/ml. After washing the Caco-2 twice with phosphate buffer saline, 0.5 ml of bacterial suspension was added to each well and incubated at 37° C for 1 h in 5% CO_2 in air. Removing unattached bacteria was performed by washing with the sterile phosphate buffer saline 3 times. Caco-2 cells were lysed with 0.1% Triton X-100 (Merck, Germany) for 5 min. The concentration of adhered bacterial cells were enumerated by plate counting in triplicate on nutrient agar and then incubating at 37° C for 24 h. The adhesion of bacterial strains to Caco-2 cells was expressed as log cfu/ml by comparing the initial and viable bacteria in the DMEM suspension. Each adhesion assay was performed in triplicate.

Results and Discussion

The diversity of microbial population in the gastrointestinal tract of prawns had been found in many different prawn species in research findings (data not shown) which showed that the *Bacillus* strains have characteristics of being Gram positive and spore formers having rod shape and the ability to produce catalase enzyme [15]. Both isolates of *B. subtilis* P33 and P72 were found to be catalase positive, an indicator that anaerobic spore-forming *Clostridium* spp. were absent. Barbosa et al. [24] reported that catalase positive property is a characteristic of *Bacillus*, thus separating it distinctly from *Clostridium* spp.

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

Based on the recently proposed use of probiotic bacteria to prevent shrimp diseases [25], this study was aimed to identify the novel probiotic strains in order to apply them as a disease control in giant freshwater prawn aquaculture. The characteristics of a successful probiotic consist of antimicrobial activity against intestinal pathogens, acid and bile tolerance, and the ability to adhere to and colonise the intestinal tract [26-27]. The determination of the antimicrobial activity of *B. subtilis* P33 and P72 which were isolated from the gastrointestinal tract of giant freshwater prawns against *A. hydrophila* was performed by paper disc and well diffusion assay. The antagonistic effect of these isolates on the growth of indicator pathogen could be determined by the appearance of clear inhibition zones around the paper disc or well (Figure 1 and Table 1). Previous studies showed that *Bacillus* species could produce a large number of antimicrobials [28]. In addition, the cell-free extracts of *B. subtilis* BT23 showed greater inhibitory effects against the growth of *V. harveyi* which was isolated from the black gill disease of *Penaeus monodon* [29]. The *B. subtilis* UTM 126 possessed an antimicrobial activity against pathogenic *Vibrio* strains that included *V. alginolyticus*, *V. parahaemolyticus* and *V. harveyi* [25]. All these suggest that the antimicrobial-producing strains of *B. subtilis* P33 and P72 may play an important role in suppressing the growth of harmful *A. hydrophila*.

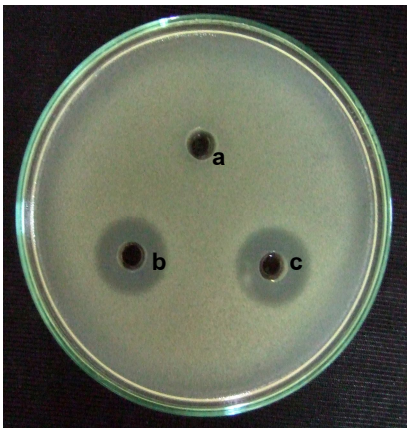

Figure 1. Agar well diffusion assay showing antagonistic activity of *Bacillus* strains against *A.hydrophila* TISTR 1321: (a) *B. subtilis* TISTR 08, (b) *B. subtilis* P33, and (c) *B. subtilis* P72

Table 1. Antagonistic activity of *Bacillus* strains against *A. hydrophila* TISTR 1321

Bacteria	Inhibition zone (mm.)	
	Paper disc diffusion	Well diffusion
B. subtilis TISTR 08	-	-
B. subtilis P33	14.5±0.5	18.3±0.6
B. subtilis P72	13.7±1.3	19.0±1.0

Note: - = no inhibition

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

The method using biochemical technique was applied in identifying the type of *B. subtilis* P33 and P72. These isolates were subjected to sugar fermentation pattern analysis by API 50 CHB test strip. Exhibiting a rate of 96.6%, both P33 and P72 isolates were identified as belonging to the species of *Bacillus subtilis*.

In order to survive in the gastrointestinal tract, a probiotic candidate must be resistant to the salivary enzyme, gastric acid and bile, and able to establish itself in the intestinal microbiota. The tolerance of both *B. subtilis* P33 and P72 strains to acid (pH 2.5) and bile salt (0.3%) were reported as % survival rate (Figure 2). The low pH tolerance of both strains was shown to be more than 95%. The growth of these strains in nutrient broth containing 0.3% bile salt after 24-h incubation indicated a high rate of tolerance of more than 90% for both strains. Previously, probiotic strains of *Bacillus* species and *B. subtilis* MA 139 were shown to exhibit resistance to bile salts and simulated gastric conditions [30], and in fact, some *Bacillus* species were frequently found in the intestinal tract [24,31]. These findings suggest that both of these probiotic candidates could survive transit through the gastrointestinal tract and establish themselves in the intestinal environment in which they may cause effective action.

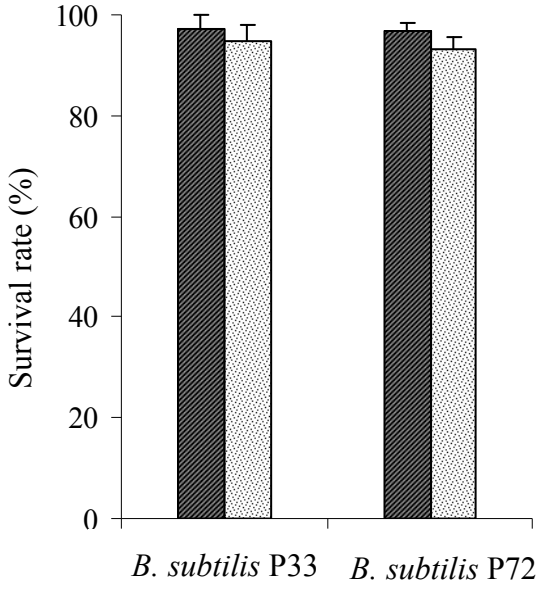

Figure 2. Tolerance of *B. subtilis* P33 and P72 strains to ▨ acid (pH 2.5) and ▩ bile salt (0.3%)

The autoaggregation percentage of the tested isolates was determined during a period of 5 h (Table 2). In the beginning, the percentage of autoaggregation ranged between 10.5–14.2%, and then continually increased every hour. In the final 5th hour, the autoaggregation registered a high percentage of 35.7–42.2. Coaggregation of these isolates with *A. hydrophila* was expressed as per cent reduction in the absorbance of a mixed suspension after 5 h. The rates of both isolates were 11.1 and 11.6% for *B. subtilis* P33 and P72 respectively. Previous studies showed that the property of aggregation is related to cell adherence and interacts closely with undesirable bacteria [19,32]. The strains with the highest autoaggregation or coaggregation were selected for further tests in probiotic screening steps [33]. The method of coaggregation with gut pathogens may be useful for screening to identify potential probiotic

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

strains [20,34]. Both of the tested strains in this study exhibited high autoaggregation and moderate coaggregation. A similar result was observed in which a probiotic strain of *L. acidophilus* M92 showed a high score in autoaggregation but lower score in coaggregation with pathogens [19]. The inhibitor producing lactic acid bacteria which coaggregate with pathogens may constitute an important host protective mechanism against infection in the urogenital tract [35], as well as in the gastrointestinal tract [36].

The use of *n*-hexadencane, xylene, and toluene to evaluate the hydrophobic cell surface properties of the tested *Bacillus* isolates showed a rather consistent result. The hydrophobicity of *B. subtilis* P33 and P72 strains was 25.6–30.0 % in *n*-hexadecane, 32.2–36.1 % in xylene, and 30.3-31.6% in toluene (Table 2). Surface hydrophobicity was determined in order to test for possible correlation between this physico-chemical property and the ability to adhere to the intestinal mucus as suggested [37].

Table 2. Adhesion property of *B. subtilis* P33 and P72 by different testing methods: hydrophobicity, autoaggregation and coaggregation

Bacteria	Hydrophobicity (%)			Aggregation (%)					
				Auto- (h)					Co-
	Hexadec-ane	Toluene	Xylene	1	2	3	4	5	
B. subtilis P33	25.6±1.6	30.3±9.4	32.2±5.6	14.3±0.0	21.4±10.1	35.7±10.1	35.7±10.1	35.7±10.1	11.1±0.0
B. subtilis P72	30.0±2.6	31.1±3.7	36.1±2.0	10.6±0.8	36.7±4.7	47.2±3.9	41.7±11.8	42.2±3.1	11.7±3.7

Adhesion to the intestinal epithelium and mucus is found to be associated with stimulation of the immune system [38-39], and adhesion to the intestinal mucosa is also crucial for transient colonisation [39], an important prerequisite for probiotics to control the balance of the intestinal microbiota [40]. The ability to adhere to the intestinal mucosa is therefore an important criterion for in vitro probiotic selection [41], hence the use of Caco-2 cells in this study. The result indicated that both of the two tested strains could adhere to Caco-2 cells. The adhesion to Caco-2 cells was 4.21 and 3.23 log cfu/ml for *B. subtilis* P33 and P72 respectively (Figure 3). Similar results were found using several other species such as *Lactobacillus* sp. [42], *L. casei rhamnosus* [43], *L. rhamnosus* DR20 and *Bifidobacterium lactis* DR10 [44], *L. fermentum* [45], and *L. plantarum* [46]. These findings suggest that both *B. subtilis* P33 and P72 strains have the ability to adhere to the epithelial cells of the host animals.

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

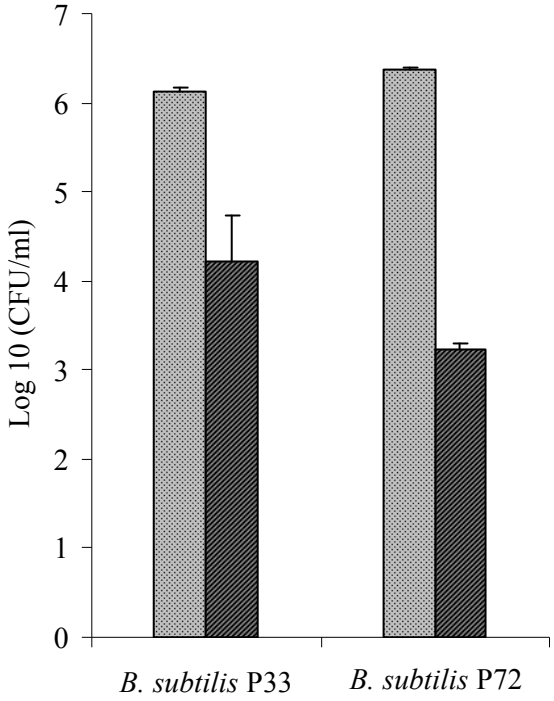

Figure 3. Adhesion of *B. subtilis* P33 and P72 to Caco-2 cells for initial bacterial (▩) and adhered bacterial (▨) concentration

Conclusions

The *B. subtilis* strains of P33 and 72 which originally came from the gastrointestinal tract of the giant freshwater prawn, were found to show inhibiting activities against the growth of *A. hydrophila*. The probiotic property of both strains could survive in acidic medium (pH 2.5) and 0.3 % bile salt solution. The two strains also exhibited ability to adhere to epithelial cells as shown by aggregation, hydrophobicity and adhesion to Caco-2 cells, thus indicating that they could be considered as good novel probiotic candidates for use in the prawn aquaculture industry.

Acknowledgement

This work was supported by the Master Research Grant from TRF (Thailand Research Fund), Thailand.

References

1. M. B. New, "Freshwater prawn farming: Global status, recent research and glance at the future", *Aquac. Res.*, **2005**, *36*, 210-230.
2. D. V. Lightner and R. M. Redman, "Shrimp diseases and current diagnostic methods", *Aquaculture*, **1998**, *164*, 201-220.
3. B. Sung, S. F. Hwang, and F. M. Tasi, "Responses of giant freshwater prawn (*Macrobrachium rosenbergii*) to challenge by two strains of *Aeromonas* spp.", *J. Invert. Path.,* **2000***, 76,* 278-284.

4. J. A. Plumb, "Health Maintenance and Principal Microbial Diseases of Cultured Fishes." Iowa State University Press, Ames, **1999.**
5. S. Vasudevan, "Probiotics and their role in shrimp hatcheries", *Fish. Chim.*, **2000**, *19*, 57-59.
6. H. Sorum and M. Sunde, "Resistance to antibiotics in the normal flora of animal", *Vet. Res.*, **2001**, *32*, 227-241.
7. S. Rengpipat, W. Phianphak, S. Piyatiratitivorakul, and P. Menasveta, "Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth", *Aquaculture*, **1998**, *167*, 301-313.
8. F. J. Gatesoupe, "The use of probiotics in aquaculture", *Aquaculture*, **1999**, *180*, 147-165.
9. B. Gomez-Gil, A. Roque, and J. F. Turnbull, "The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms", *Aquaculture*, **2000**, *191*, 259-270.
10. J. L. Balcázar, "Evaluation of probiotic bacterial strains in *Litopenaeus vanamei*", Final Report, National Center for Marine and Aquaculture Research, Guayaquil, Ecuador, **2003**.
11. S. Ziaei-Nejad, M. H. Rezaei, G. A. Takami, D. L. Lovett, A. M. Mirvaghefi, and M. Shakouri, "The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*", *Aquaculture*, **2006**, *252*, 516-524.
12. S. Rengpipat, S. Rukpratanporn, S. Piyatiratitivorakul, and P. Menasveta, "Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11)", *Aquaculture*, **2000**, *191*, 271-288.
13. M. Gullian, T. Fabiano, and R. Jenny, "Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*", *Aquaculture*, **2004**, *233*, 1-14.
14. S. V. Alavandi, K. K. Vijayan, T. C. Santiago, M. Poornima, K. P. Jithendran, S. A. Ali, and J. J. S. Rajan, "Evaluation of *Pseudomonas* sp.PM11 and *Vibrio fluvialis* PM17 on immune indices of tiger shrimp *Penaeus monodon*", *Fish Shellfish Immunol.*, **2004**, *17*, 115-120.
15. J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams, "Bergey's Manual of Determinative Bacteriology" , 9th Edn., Williams and Wilkins, Baltimore, **1994**.
16. P. L. Conway, S. Gorbach, and B. R. Goldin, "Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cell", *J. Dairy Sci.*, **1987**, *70*, 1-12.
17. C. Pennacchia, D. Ercolini, G. Blaiotta, O. Pepe, G. Mauriello, and F. Villani. "Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics", *Meat Sci.*, **2004,** *67*, 309-317.
18. S. E. Gilliland, T. E. Staley, and L. J. Bush, "Importance of bile tolerance of *Lact. acidophilus* strains for use as dietary adjunct", *Int. J. Food Microbiol.*, **1984**, *29*, 105-109.
19. B. D. Re, B. Sgorbati, M. Miglioli, and D. Palenzona, "Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*", *Lett. Microbiol.*, **2000**, *31*, 438-442.
20. B. Kos, J. Šuškovic, S. Vukovic, M. Šimpraga, J. Frece, and S. Matošić, "Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92", *J. Appl. Microbiol.*, **2003**, *94*, 981-987.
21. P. S. Handley, D. W. S. Harty, J. F. Wyatt, C. R. Brown, J. P. Doran, and A. C. C. Gibbs, "A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrilar and fimbriate strains of *Streptococcus salivarius*", *J. Gen. Microbiol.*, **1987**, *133*, 3207-3217.

22. D. C. Savage, "Growth phase, cellular hydrophobicity, and adhesion in vitro of lactobacilli colonizing the keratinizing gastric epithelium in the mouse", *Appl. Environ. Microbiol.*, **1992**, *58*, 1992-1995.
23. M. Gagnon, E. Kheadr, G. Le Blay, and I. Fliss, "In vitro inhibition of *Escherichia coli* O157:H7 by *Bifidobacterial* strains of human origin", *Int. J. Food Microbiol.*, **2004**, *92*, 69-78.
24. T. M. Barbosa, C. R. Serra, R. M. La-Ragione, M. J. Woodward, and A. O. Henriques, "Screening for *Bacillus* isolates in the broiler gastrointestinal tract", *Appl. Environ. Microbiol*., **2005**, *71*, 968-978.
25. J. L. Balcázar T. Rojas-Luna, and D. P. Cunningham, "Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*", *J. Invert. Path.*, **2007**, *96*, 147-150.
26. S. Salminen, A. von Wright, L. Morelli, P. Marteuau, D. Brassart, W. M. de Vos, R. Fonden, M. Saxelin, K. Collins, G. Mogensen, S. E. Birkeland, and T. Mattila-Sandholm, "Demonstration of safety of probiotics – a review", *Int. J. Food Microbiol.,* **1998**, *44*, 93-106.
27. M. E. Sanders, L. Morelli, and T. A. Tompkins, "Spore formers as human probiotics: *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*", *Comprehensive Rev. Food Sci. Food Safety*, **2003**, *2*, 101-110.
28. M. C. Urdaci and I. Pinchuk, "Antimicrobail activity of *Bacillus* probiotics", in "Bacteria Spore Formers: Probiotics and Emerging Applications" (Ed. E. Ricca, A. O. Henriques, and S. M. Cutting), Horizon Bioscience Press, Norfolk, **2004**, pp. 171-182.
29. B. Vaseeharan and P. Ramasamy, "Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*", *Lett. Appl. Microbiol.*, **2003**, *36*, 7-83.
30. L .H. Duc, H. A. Hong, T. M. Barbosa, A. O. Henriques, and S. M. Cutting, "Characterization of *Bacillus* probiotics available for human use", *Appl. Environ. Microbiol.*, **2004**, *70*, 2161-2171.
31. G. Casula and S. M. Cutting, "*Bacillus* probiotics: Spore germination in the gastrointestinal tract", *Appl. Environ. Microbiol.*, **2002**, *68*, 2344-2352.
32. C. Gusils, S. González, and G. Oliver, "Some probiotic properties of chicken lactobacilli", *Can. J. Microbiol.*, **1999**, *45*, 981-987.
33. M. A. Ehrmann, P. Kurzak, J. Bauer, and R. F. Vogel, "Characterization of lactobacilli towards their use as probiotic adjucts in poultry", *J. Appl. Microbiol.*, **2002**, *92*, 966-975.
34. M. C. Collado, J. Meriluoto, and S. Salminen, "Measurement of aggregation properties between probiotics and pathogens: In vitro evaluation of different methods", *J. Microbiol. Meth.,* **2007**, *71*, 71-74.
35. G. Reid, J. A. McGroarty, R. Angotti, and R. L. Cook, "*Lactobacillus* inhibitor production against *Escherichia coli* coaggregation ability with uropathogens", *Can. J. Microbiol.*, **1988**, *34*, 344-351.
36. R. J. Spencer and A. Chesson, "The effect of *Lactobacillus* spp. on the attachment of enterotoxigenic *Escherichia coli* to isolated porcine enterocytes", *J. Appl. Bacterial*., **1994**, *77*, 215-220.
37. T. Wadstrom, K. Andersson, M. Sydow, L. Axelsson, S. Lindgren, and B. Gullmar, "Surface properties of lactobacilli isolated from the small intestine of pigs", *J. Bacterial.*, **1987**, *62*, 513-520.

Maejo Int. J. Sci. Technol. **2009**, *3*(01), 77-87

38. E. H. Beachey, "Bacterial adherence: Adhesion – receptor interactions mediating the attachment of bacteria to mucosal surfaces", *J. Infect. Dis.*, **1981**, *43*, 325-345.
39. M. Juntunen, P. V. Kirjavainen, A. C. Ouwehand, S. J. Salminen, and E. Isolauri, "Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection", *Clin. Diagn. Lab. Immunol.*, **2001**, *8*, 293-296.
40. M. Collado, J. Meriluoto, and S. Salminen, "In vitro analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus", *Food Res. Int.*, **2007**, *40*, 629-639.
41. L. Morelli, "In vitro selection of probiotic lactobacilli: A critical appraisal", *Curr. Issues Intest. Microbiol.*, **2000**, *1*, 59-67.
42. E. M. Tuomola and S. J. Salminen, "Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures", *Int. J. Food Microbiol.*, **1998**, *41*, 45-51.
43. C. Forestier, C. De Champs, C. Vatoux, and V. Joly, "Probiotic activities of *Lactobacillus casei rhamnousus*: In vitro adherence to intestinal cells and antimicrobial properties", *Res. Microbiol.*, **2001**, *152*, 167-173.
44. P. K. Gopal, J. Prasad, J. Smart, and H. S. Gill, "In vitro adherence properties of *Lactobacillus shamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*", *Int. J. Food Microbiol.*, **2001**, *67*, 207-216.
45. L. Baccigalupi, A. D. Donato, M. Parlato, D. Luongo, V. Carbone, M. Rossi, E. Ricca, and M .D. Felice, "Small surface-associated factors mediate adhesion of a food-isolated strain of *Lactobacillus fermentum* to Caco-2 cells", *Res. Microbiol.*, **2005**, *156*, 830-836.
46. P. A. Maragkoudakis, G. Zoumpopoulou, C. Miaris, G. Kalantxopoulos, B. Pot, and E. Tsakalidou, "Probiotic potential of *Lactobacillus* strains isolated from dairy products", *Int. Dairy J.*, **2006**, *16*, 189-199.