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Original article

Host associated mixed probiotic bacteria induced digestive enzymes in the gut of tiger shrimp *Penaeus monodon*

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ABSTRACT

The shrimp *Penaeus monodon* was used for the isolation of digestive enzyme producing host-associated probiotic bacteria. Gut was isolated from a healthy animal completely and morphologically different bacterial isolates were screened for the production of hydrolytic enzymes, such as, protease, amylase, lipase and cellulases. Based on their ability to produce enzymes, the potent probiotic bacteria were identified as *Bacillus subtilis* and *B. licheniformis* and these two were used for the preparation of probiotic diet for experimental trials. Probiotic diet was prepared by mixing the shrimp feed with 2 g probiotic/100 g artificial diet (F1), 4 g/100 g (F2), 6 g/100 g (F3), 8 g/100 g (F4) and 10 g/100 g (F5). Juvenile shrimp was fed with probiotic and control diet for a period of 7 weeks at 5 and 8% body weight for the first 3 and 7 weeks, respectively. After seven weeks, whole gut was dissected out and protease activity was estimated as 145 ± 12.3 U/g in control animal and increased as 710 ± 15.2 U/ g in F5 feed groups. Amylase activity was 139 ± 10.4 U/g in control and increased as 209 ± 13. 3 U/g in F5 group. Cellulase activities were 171 ± 9.3 in F5 groups and the control group showed only 102 ± 12.4 U/g. Lipase activity was 78 ± 3 U/g in F1 groups and it increased as 85 ± 5 U/g in F3 groups. These findings indicate the potential of host-associated bacteria to enhance the production of enzymes in the gut of juvenile *P. monodon*.

1. Introduction

Aquaculture is the one of the fastest growing sector throughout the world and Asian countries contributes more than 90% of total Global seafood production (FAO, 2012). Among these shrimp production contribute considerably in recent times. The rapid expansion and intensive culture of shrimp production have been affected by many diseases, including yellow head virus, white spot syndrome, early mortality syndrome and white faeces syndrome and problems related to water quality (Padmavathi et al., 2012). Among shrimps, *Penaeus monodon* is highly susceptible to these diseases (Tran et al., 2013). In shrimp farming, a range of disinfectants, antimicrobials and nutritional supplements are used to control or prevent shrimp diseases as well as maintaining water quality of farms. It was reported that, about 19% of the shrimp farms in Vietnam use enrofloxacin, ciprofloxacin and oxytetracycline to treat hepatopancreatic necrosis syndrome and early mortality syndrome. The use of probiotic bacteria increasing rapidly and effectively inhibit the growth of pathogens by colonizing shrimp gut and the production of various bioactive compounds show positive effect on shrimp pathogens (D'Arienzo et al., 2006). Also, probiotic bacteria incorporated into the feed critically enhance uptake of nutrients and enhance the growth in shrimp (Irianto and Austin, 2002; Van Doan et al., 2020). About 84% farmers mixed probiotic bacteria with the water to reduce environmental stress, whereas about 16% shrimp farmers preferred to mix

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probiotic organism with artificial pellet diet. The probiotic bacteria such as, *Lactobacillus acidophilius*, *Bacillus licheniformis*, *B. thuringiensis*, *B. subtilis* were frequently used in shrimp farms. In recent years, shrimp farmers used these probiotic feed for better yield and more than 90% of shrimp farmers use probiotics (Rico et al., 2013).

Manipulation of intestinal biota using dietary supplements is an innovative approach to improve the growth performance and gut health of aquatic organisms (Han et al., 2015). For the past two decades, the practice of using probiotics in shrimp farming increasing due to their positive demand and alternate to chemotherapeutics (Dawood and Koshio, 2016). However, the selection of suitable probiotics is very important because of inappropriate bacteria may cause imbalance in the animal gut and negatively influence on immunomodulation, antagonistic activity against pathogens, contribution of the colonization resistance and digestibility of feed (Lazado et al., 2015). The typical probiotic microorganism should able to multiply and colonize in the host gut. In aquaculture production, the commercial probiotics used in terrestrial animals have ineffective and fail to colonize the gut of the host organism (Mohammadian et al., 2017). Many bacteria from the groups such as, pseudomonads, bacilli, lactic acid bacteria and *Saccharomyces cerevisiae* were reported as probiotics and these microbial flora enhanced the activity of digestive enzymes and manipulation of microbial flora in the gut of various fish species (Lobo et al., 2014; Suzer et al., 2008). However, the search of new probiotic organism continues. This is mainly because certain probiotic bacteria may colonize the gut of certain fishes and not other fish and a bacterium cannot be used to all fishes because of variation in the gut (Lazado et al., 2015).

The probiotic organisms are considered as an alternative to antibiotics in aquaculture practice to enhance immunity to the shrimps in aquaculture against various pathogenic bacteria (Ajitha et al., 2004). The probiotic bacteria have the ability to fight against various diseases caused by *Vibrio harveyi*, *V. alginolyticus*, *V. vulnificus* and *V. anguillarum* (Kongnum and Hongpattarakere, 2012). Also, lactic acid bacteria critically enhanced the growth of shrimp challenged with *V. alginolyticus* intra-muscularly (Ajitha et al., 2004) and *L. vannamei* was challenged with *V. harveyi* (Vieira et al., 2007). Moreover, the antagonistic property of lactic acid bacteria against *Vibrio* spp. is mainly dependent on the growth performance of probiotic bacteria in the gut of shrimps (Kongnum and Hongpattarakere, 2012). Generally, probiotic bacteria do not synthesize residues or drug resistance in aquatic organisms, probiotics as a alternate for chemical costly antibiotics have become a recent research topic of research in aquaculture biotechnology (Akhter et al., 2015). Many findings revealed that addition of probiotics with water or with artificial diet not only enhance the growth and survival of fishes, but also significantly reduce the outbreak of various diseases caused by bacteria, fungi and viruses by improving the immune system of shrimp (Kongnum and Hongpattarakere, 2012). These probiotics have been used in aquaculture to provide nutrition, control water quality, promote digestion and to control various diseases (Vieira et al., 2007; Hoseinifar et al., 2018). Selection of probiotic bacteria is critically important. It has been previously reported that the isolation of probiotic bacteria should be used to shrimp body, which promote the growth of the shrimp, enhance immunity and also reduce the outbreak of various diseases (Ajitha et al., 2004; Hoseinifar et al., 2018). Considering this fact, host-associated probiotic bacteria were isolated from the fish gut and the ability to induce digestive enzymes in juvenile shrimp was studied.

2. Materials and methods

2.1. Experimental animal

The shrimp, *Penaeus monodon* was collected for the isolation of probiotic bacteria. Intestinal samples were obtained from five animals (n = 5), pooled and used for the isolation of bacteria.

2.2. Enumeration and characterization of gut bacteria

Gut was removed totally from the shrimp using a sterile forceps, homogenized with phosphate buffer saline (pH 7.0 ± 0.2) using a glass homogenizer. It was further serially diluted using PBS up to 10^{-7} dilutions. 0.1 ml sample from three dilutions were selected and spread plated on nutrient agar (Himedia, Mumbai, India) for the isolation of aerobic bacteria. The plates were incubated for 24 h at 30 ± 2 °C and the colonies were counted. The results were expressed as colony forming units (cfu)/g of fish gut. The morphologically different colonies were purified and the microbiota population was estimated. The isolated bacteria were identified using Gram staining, motility test, Kovac's oxidase test, catalase test and fermentation with various sugars.

2.3. Determination of digestive enzyme activity of probiotic bacteria

The probiotic bacterial strains were screened for the production of extracellular protease, amylase, lipase and cellulase using plate screening method. Protease production of the selected bacterial strains was performed by inoculating the bacteria on nutrient agar plates with 1% (w/v) casein with bromocresol green (Vijayaraghavan and Vincent, 2013). A clear transparent zone was observed after 24 h. Amylase activity was screened using soluble starch (1%, w/v) as a substrate (Gopinath et al., 2017). Enzyme activity was observed after the plate was flood with 1% Gram's Iodine solution. For cellulase screening, the isolates were grown on nutrient agar plates containing 1% (w/v) carboxy methyl cellulose. After 24 h incubation, the plate was flooded with Congo red (1%) prepared in double distilled water (Vijayaraghavan and Vincent, 2012). To screen lipase activity, the selected probiotic bacteria were grown on nutrient agar containing 1% tributyrin. After 48 h incubation, a clearance zone was observed around the colonies. Enzyme production was expressed as mean zone of diameter (mm) (Samad et al., 1989).

2.4. Potential probiotic bacteria

The digestive enzyme producing non-pathogenic bacteria were used to enhance the digestibility of juvenile shrimp. The bacterial strains were cultured individually in nutrient broth medium (pH 7.0) and incubated for 48 h. After 48 h, the bacterial cells were centrifuged at 5000 rpm for 10 min and the pellet was washed twice with phosphate buffered saline (PBS, pH 7.2). The washed pellet was lyophilized and used for the preparation of probiotic diet.

2.5. Identification of probiotic probiotic strains

The potent probiotic bacterium was subjected to morphological, biochemical analysis and 16S rDNA analysis. Genomic DNA was extracted by chloroform methanol method.PCR amplification was performed using a forward (5′-GGTTACCTTGTTACGACTT-3′) and a reverse (5′-AGAG TTTGATCCTGGCTCAG-3′) primer. The amplified PCR product was purified by agarose gel electrophoresis and sequenced.

2.6. Experimental diet formulation

The experimental diet was prepared in aseptic conditions. The basal diet contains crude protein (51.2%), crude lipid (10.34%), ash (11.02%), moisture (10.1%) and energy (5.74 kcal g^{-1}). The shrimp diet was prepared by mixing shrimp (120 g), squid meal (20 g), wheat (50 g), squid liver (40 g), rice (100 g), wheat flour (200 g), soy bean meal (100 g), vitamin mixture (10 g), mineral mix (40 g), fish oil (15 g) and gelatine (10 g). The artificial diet was spread in a tray and the probiotic bacteria (*Bacillus subtilis* and *B. licheniformis*) were mixed with experimental diet at various concentrations (2, 4, 6, 8 and 10 g probiotic/100 g of feed). Six different experimental diets with probiotic bacterium were prepared with concentration of 1×10^4 (F1), 1×10^5 (F2), 1×10^6 (F3), 1×10^7 (F4), 1×10^8 (F5) CFU g^{-1} of feed. The prepared artificial diet was spread in the sterile trays and the absorption of probiotics was achieved by spraying the suspended probiotic bacteria with different experimental feeds. After spraying, the feed was air dried in a vent hood at the room temperature (30 ± 2°) for overnight, and the unique moisture content as well as bacterial concentration in the feed was maintained. Finally the probiotic supplemented feed was stored in vacuumed heavy-duty plastic containers at 4 °C. To the control diet probiotic bacteria were not incorporated (Wouters et al., 2001).

2.7. Experimental setup

Experimental *P. monodon* juvenvile was divided into six groups. Each experimental group consists of 10 shrimps (n = 10). These were fed with experimental and control diet twice a day (morning and evening) for a period of 7 weeks at 5 and 8% body weight for the first 3 weeks and 7 weeks, respectively. After experimental period, growth rate and enzyme activities were calculated. To determine the growth rate three animals were randomly selected from each group and growth performance was analyzed.

2.8. Analysis of digestive enzymes from the shrimp gut

Juvenile shrimp gut was dissected out carefully and ground with phosphate buffered saline (pH 7.0). The sample was centrifuged and the supernatant was used for enzyme assay. Amylase activity of the sample was assayed using 3,5-dinitrosalicylic acid (DNS) method. 0.1 ml sample was mixed with 1% soluble starch prepared in sodium phosphate buffer. The reaction was performed for 10 min at 30 ± 2 °C and DNS reagent (1 ml) was added and kept on a boiling water bath for about 10 min. It was cooled and 10 ml double distiller was added. The absorbance of the sample was read at 540 nm against reagent blank (Worthington, 1993). Maltose was used for the preparation of standard curve for the determination of amylase. To determine cellulase activity carboxy methyl cellulose was used as the substrate. The remaining steps were same like amylase activity determination (Ghose, 1987). Protease activity was determined from the gut sample using casein (1%, w/v) as a substrate. The sample (0.2 ml) was incubated with substrate (2.5 ml) for 30 min and the reaction was terminated using trichloro acetic acid (10%, w/v). It was centrifuged and the clear supernatant was used for analysis. L-tyrosine was used as the standard (Lowry et al., 1951). Lipase activity of the sample was also determined as suggested by Snell and Snell (1971).

2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) to find the significance of variation between variables (treatment and control group) and the p value <0.05 was considered as significant.

3. Results and discussion

P. monodon was used to analyze the diversity of microbial flora in the gut. The total viable count (TVC) of the sample ranged between 0.93×10^8 and 1.58×10^8 cfu/g in the gut of *P. monodon*. These were pure cultured and a total of 35 bacteria were selected for characterization studies based on morphological differences. *Bacillus* sp., *Micrococcus* sp., *Corynebacterium*, and *Staphylococcus* were the dominant species in the gut of *P. monodon*. Gram-positive bacteria population was high than Gram-negative bacteria. In our study, four major groups were identified, including, *Proteobacteria, Bacteroides, Fusobacteria* and *Firmicutes*. From these major phyla, *Protobacteria* consists of more than 80% bacteria and *Firmicutes* consists of 17% total bacteria. *Fusobacteria* and, *Bacteroides* represent only 2%, 1%, respectively (Fig. 1). However, Lactobacillus sp. was not detected from the gut sample of *P. monodon*, which was reported previously in the gut of *P. monodon*. The isolated bacteria were subjected to enzyme screening (protease, amylase, lipase and cellulase) and potent enzyme producers were further selected. Distribution of hydrolytic enzyme producing bacterial isolates from the gut of *P. monodon* is presented in Fig. 2. Here more than 90% of the isolated bacteria were protease producing capacity whereas cellulase producers were very less.

The present study shows that supplementing probiotic mixture incorporated at various concentrations in the artificial feed improves the performance of growth and digestive enzyme in the fish gut. Moreover, enzyme activity significantly increased with increased concentrations of probiotics. Protease activity was found to be high in F4 experimental group, which was found to be high than other groups. These findings suggested induced effect of probiotics up to 8% feed and this dose strongly recommended to induce enzyme synthesizing ability (Figs. 3–6). The stimulatory effect of probiotic mixture has been reported previously in tiger shrimp *Penaeus monodon* (Rengpipat et al., 1998), *Homarus gammarus* L. (Daniels et al., 2010), and *Penaeus vannamei* by *Bacillus* sp. and photosynthetic bacteria mixture (Wang, 2007). Previously, Wang and Xu (2006) used mixed probiotic organisms and reported induced effect than individual probiotics in common carp. In shrimps, the growth performance is mainly due to the activity of digestive enzymes (Hong et al., 2005). In *Fenneropenaeus indicus*, supplementation of probiotics enhanced amylase and lipase activity in the digestive tract after fed with *Bacillus* sp. (Ziaei-Nejad et al., 2006). Liu et al. (2009) found that the probiotic *Bacillus* sp. enhanced proteolytic activity in *Litopenaeus vannamei* (Moriarty, 1996).

The increased growth of shrimp in this study might be due to increased enzyme activity induced by the incorporation of

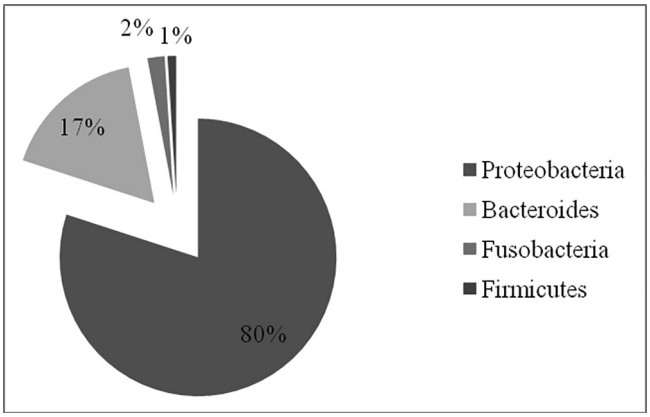

Fig. 1. Distribution of microbial genera isolated from the gut of *P. monodon*.

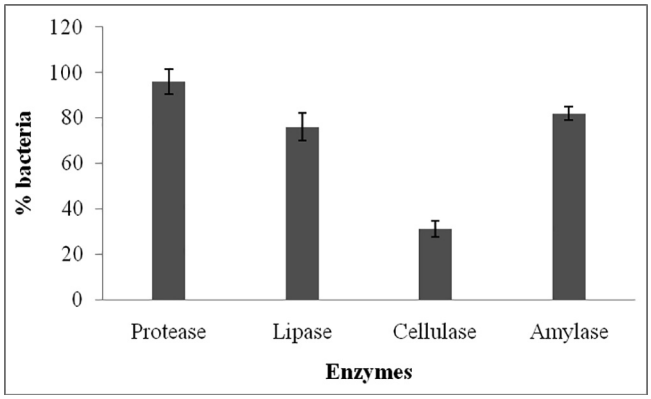

Fig. 2. Distribution of hydrolytic enzyme producing bacterial isolates from the gut of *P. monodon*.

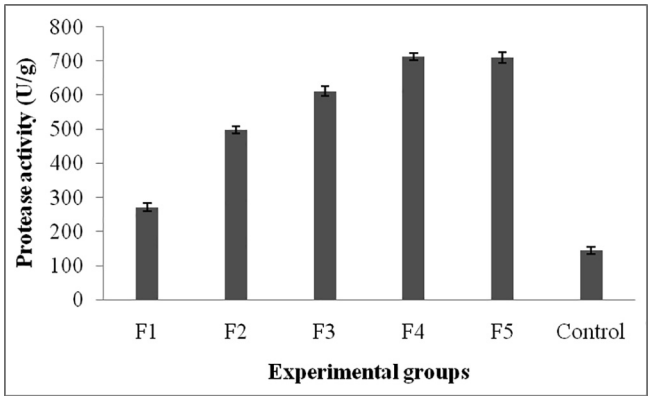

Fig. 3. Protease activity (U/g) of control and experimental juvenile shrimp fed with five experimental diets (F1-F5). Gut was completely dissected out and enzyme activity was expressed as U/g after 7 weeks culture. *Error bar ± standard deviation.*

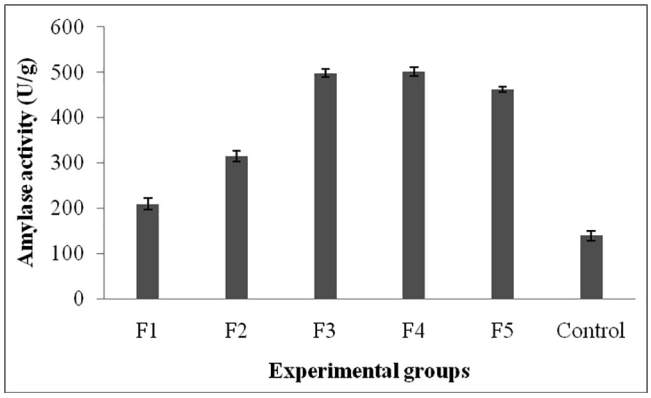

Fig. 4. Amylase activity (U/g) of control and experimental shrimp fed with five experimental diets (F1-F5). Gut was completely dissected out and enzyme activity was expressed as U/g after 7 weeks culture. *Error bar ± standard deviation.*

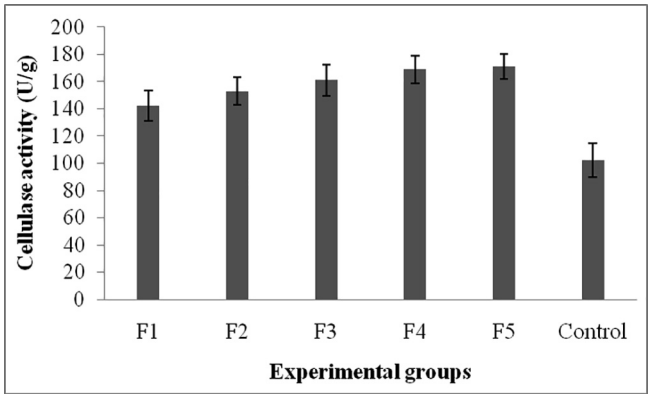

Fig. 5. Cellulase activity (U/g) of control and experimental shrimp fed with five experimental diets (F1-F5). Gut was completely dissected out and enzyme activity was expressed as U/g after 7 weeks culture. *Error bar ± standard deviation.*

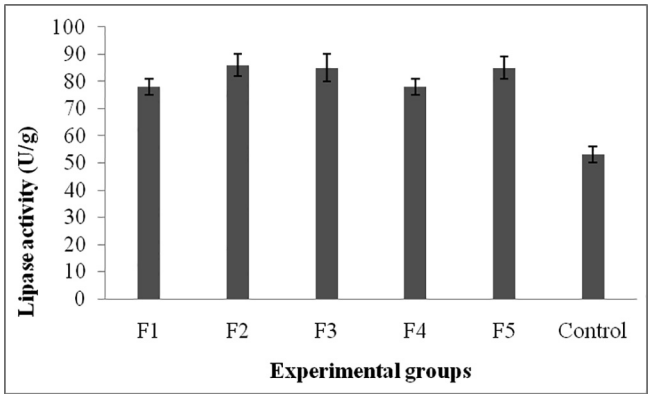

Fig. 6. Lipase activity (U/g) of control and experimental shrimp fed with five experimental diets (F1-F5). Gut was completely dissected out and enzyme activity was expressed as U/g after 7 weeks culture. *Error bar ± standard deviation.*

probiotics with the feed. Generally, digestive system of shrimp is induced in the early post-larval (PL) and larval stages, where the probiotic bacteria critically induced the activity of digestive enzymes. Also, the bacterial species, mainly bacteria from the genus *Bacillus* produce various exoenzymes in the gut (Moriarty, 1997). However, it is often difficult to distinguish between activity due to enzymes synthesized by the probiotic bacteria and that due to enzymes synthesized by the shrimp. Moreover, the extracellular enzymes synthesized by the probiotic bacteria would represent good ratio than enzyme activity of the gut (Ziaei-Nejad et al., 2006), and the presence of probiotic bacteria in the gut might critically stimulate the production of various endogenous enzymes. The supplementation of probiotics in aquaculture system instead of antibiotics in aquaculture has attracted much more attention by various workers recently (Mohammadian et al., 2017). The findings of this present experiment clearly showed that amylase, lipase and protease activity in the gut of experimental group (T4 and T5) were increased significantly ($p < 0.05$) and it may due to the stimulation of probiotics or digestive enzyme secretion by these groups of probiotics. In fishes, intestine has complex microbial system and there are various kinds of bacterial communities with complex structures in the gut (Villasenor et al., 2013). Generally, the growth of the aquatic organisms, including fishes depends on the absorption of food materials, transformation of multiple nutrients in the digestive system after digestion of feed (Nejad et al., 2006). Also, enzyme activity in the digestive system can be mainly used to measure the digestive level of fishes, and used to assess the growth of aquatic animals (Wang et al., 2018).

In shrimps, there are various studies demonstrated the positive impact of probiotics on the growth; however the mechanism of action of probiotics is not clearly explored. It was previously hypothesized the induction of digestive enzymes such as, protease, lipase and amylase, stimulate the production of enzyme activities in the host fish species (Wang, 2007). In aquaculture *Lactobacillus* spp. have also been frequently used for the beneficial effect of shrimps and reported range of enhanced digestive enzymes,

including amylase and proteases (Suzer et al., 2008). Also, Zokaeifar et al. (2012) observed enhanced digestive enzyme activity in *L. vannamei* treated with probiotic bacterium, *Bacillus subtilis*. Another possible mechanism was also proposed. Epithelial cell microvilli of intestine provide great surface area for absorption, the increase in the density of enterocytes and increase in the height of enterocytes can enhance nutrient adsorption ability (Zhang et al., 2012).

The supplemented probiotics at all concentrations resulted improved growth and enhanced enzyme activity due to the activity of mixed probiotics. This kind of result was reported previously by Swain et al. (1996) in Indian carp, *Labeo rohita*, and also reported by Ghosh et al. (2003) in the rohu fingerlings. Noh et al. (1994) used yeast culture and probiotic organism to study the growth performance of Israeli carp and achieved enhanced growth. Bogut et al. (1998) used a commercial probiotic, *Streptococcus faecium* to enhance the growth and the diversity of gut microbial flora in carp *Cyprinus carpio*. The positive influence of probiotics in *Fenneropenaeus indicus* was also reported by Ziaei-Nejad et al. (2006). The increased digestive enzyme activity obtained with formulated diet containing mixed probiotics improved the digestion of starch, cellulose, fat and protein, which improved better growth with probiotic added feed. Similar findings were reported previously by various workers. Lara-Flores et al. (2003) used mixed probiotics (*Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae*) to enhance the growth of Nile tilapia (*Oreochromis niloticus*). Tovar-Ramírez et al. (2004) used live yeast to enhance the larvae development in European sea bass (*Dicentrarchus labrax*). Ziaei-Nejad et al. (2006) used *Bacillus* spp. as a probiotic bacterium to enhance the ability of digestive enzymes in the Indian white shrimp *Fenneropenaeus indicus*.

The commercially available probiotics are mainly from the genus *Bacillus*, *Lactobacillus*, *Nitrosomonas* and yeast. Bacteria from these genera have been frequently used in aquaculture practice (Wang 2007). In our study, we isolated various bacteria from the genus *Bacillus* showed enhanced enzyme activity. It was reported that the bacterial species such as, *B. thuringiensis*, *B. pumilus*, *B. cereus*, *B. licheniformis* and *B. clausii* have been frequently used for the formumation of probiotic feed (Hong et al., 2005). These bacterial species produce various antimicrobial metabolites and these stimulate immune system and also showed inhibitory effect on various pathogens (D'Arienzo et al., 2006). Among probiotic bacteria, *Bacillus* species has various advantages because of the ability to produce spores and these spores are stable at room temperature and can be stored at room temperature in long periods (Hong et al., 2005).

4. Conclusions

In conclusion, the present finding indicated that supplementation of probiotic bacteria isolated from the host species promoted the production of digestive enzymes in *P. monodon* juvenile. The mixed probiotic bacteria stimulated the enzyme activity in the gut in dose dependent manner. These probiotics promote gut bacterial flora and increase digestibility of feed. *Bacillus subtilis* and *B. licheniformis* enhanced protease, amylase, lipase and cellulase activity in the shrimp gut. Host-associated bacterial species could enhance the digestive enzyme activity in shrimps than other sources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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