Iranian Journal of Fisheries Sciences 12(4),873- 886 2013

Screening and evaluation of indigenous bacteria from the Persian Gulf as a probiotic and biocontrol agent against *Vibrio harveyi* in *Litopenaeus vannamei* post larvae

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Received: June 2012 Accepted: December 2012

Abstract

Isolation of autochthonous bacteria from marine sources as a potential probiont in biocontroling against pathogenic *Vibrio* species in the shrimp culture industry was the aim of current research. A total of 198 bacterial strains were isolated from pond water, sediment, hepatopancreas and gut samples of shrimps after culturing the samples on Tryptic Soy Agar and incubated at 30 °C for 24-48 h. The isolates were tested for their antagonistic activity in contact with *Vibrio harveyi*. Two strains (IS02 and IS03) that isolated from the gut and pond sediment were showed antagonistic against *V. harveyi*. According to 16S ribosomal DNA gene sequence analysis, the strain IS02 was identified as *Bacillus subtilis* and IS03 as *B.vallismortis*. Further, the two bacterial species, *B. subtilis* and *B. vallismortis* were challenged separately for probiotic activity in the post larvae of *Litopenaeusvannamei* against pathogenic *V. harveyi*. The present study identified *B.subtilis* IS02 and *B.vallismortis* IS03 had biocontrol activity against *V. harveyiin vitro* and *in vivo* and they increase growth performance of *L.vannamei* in post larvae stage.

Keywords: Probiotic, *Litopenaeusvannamei,* Indiginous bacteria, *Vibrio harveyi*

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Introduction

In the recent two decades, aquaculture and mariculture rapidly developed. Aquaculture is one of the most important sources of animal protein (Lara-Flores et al., 2003). Currently, the aquaculture industry in Iran and other parts of the world has been facing serious problems due to microbial (Hosseini et al., 2004) and viral diseases (Afsharnasab et al., 2007).Vibriosis, especially luminous disease, has caused serious loss in shrimp hatcheries. Shrimp larvae are particularly susceptible to *Vibrio harveyi* and infection with this bacterium can lead to luminescent bacterial disease (Lavilla-Pitogo et al., 1990). Mass mortalities in shrimp hatcheries and culture ponds were caused by outbreaks of vibriosis have been recorded from many regions such as, Korea (Won and Park, 2008), Philippines (Lavilla-Pitogo et al., 1990; Lavilla-Pitogo et al., 1992; Lavilla-Pitogo and de la Pena, 1998) and Thailand (Ruangpan and Kitao, 1991) hatcheries. However, the abuse of antimicrobial drugs and disinfectants in prophylactic and treatment of diseases has led to the assay of resistant bacteria strains (Esiobu et al., 2002). Thus, the demand for environment-friendly sustainable aquaculture is increasing (Gatesoupe, 1999). Probiotics are non-pathogenic microorganisms that can be used instead of antibiotics as a biocontrol agent (Fuller, 1978; Gatesoupe, 1999; Mishra et al., 2001). One of the most recommended bacterial probiotics in shrimp culture belonged to Bacillaceae family (Ziaei-Nejad et al., 2006; Jiqiu et al., 2009; Liu et al., 2010).

Bacillus spp. are often show antagonistic activity against fish and shellfish pathogenic bacteria as well as other micro-organisms (Gatesoupe, 1999; Rengipipat et al., 2000). *Bacillus* spores have been utilized as bio control agents to cut down vibriosis in shrimp culture industry (Skjermo and Vadstein, 1999; Rengipipat et al., 2000).

The present study experimented to screen indigenous probionts from shrimp and habitat samples and effect of them versus pathogenic *V. harveyi* in laboratory conditions and small scale animal models. In addition, growth parameters were also investigated at post larvae stages with and without probiotic.

Materials and methods

A pathogenic strain of *Vibrio harveyi* was isolated from diseased shrimp from hatchery unit in Iran Shrimp Research Center, confirmed as *V. harveyi* by biochemical tests (Farmer, 2006) and partial 16S rDNA gene sequence analyses and have been deposited in Genbank under accession numbers (NCBI accession no GU974342.1). Shrimp pathogenic bacteria, *V. harveyi,* have been deposited in Iranian Research Organization for Science and Technology (IROST) under Persian Type Collection Center accession numbers PTCC 1755.

Bacterial strains were isolated from shrimp farming sites of Bushehr in Southwest of Iran and north coast of the Persian Gulf, from April to September 2010. Bacteria were obtained from three sources including sediment of shrimp ponds, water of shrimp ponds and gut and hepatopancreas of healthy shrimp. Samples were transferred to the laboratory in a container at 4°C. The shrimp gut content and hepatopancreas were aseptically removed from

a fresh healthy shrimp. All of the samples were in sterile 2.5% seawater by serial dilution method and cultured on non-selective media to enable isolation of as many strains as possible. Following (Buller, 2004), Tryptice Soy Agar (Merck,1.05458.0500, Germany) was made to 2.5% salinity with natural seawater (here after referred to as TSA-2.5% Sea) to provide a medium similar to that of the isolates' environment and incubated at 30º C (JSBI-250C, JSR Inc., Korea) for 24–48 h. Colonies were chosen on the basis of their dominance in cultures reflecting their dominance in the production system (Kesarcodi-Watson et al., 2009). Isolates were streaked for purity and stored at−70°C in skimmed milk (15% v/v) and sterile glycerol (20% v/v) (Day and Stacey, 2007). Before the experiments, bacteria were passaged in Tryptice Soy broth 2.5% (TSB-2.5% Sea) (Merck, 1.05459.0500, Germany), streaked and sub-cultured on TSA-2.5% Sea at 30°C to ensure purity.

The colonies from TSA-2.5%Sea were examined for their consistent antagonistic activity in a well diffusion agar against *V. harveyi* (Hjelm et al., 2004). All of the isolates were grown in 10 ml TSB-2. 5% sea for 48 h at 30°C in shaking incubator (200 rpm) (JSSI-200CL JSR Inc., Korea) and was centrifuged at 9500 rpm for 10 min in 4°C (3-16PK, Sigma Inc., Germany). The supernatant was sterilized by passage through a 0.45-µ Millipore filter (Millipore, MS®PES syringe filter, USA) and neutralized (pH 7.0) with 1 N NaOH(Balcazar and Rojas-Luna, 2007). Twenty ml of the Muller Hinton agar-2.5%Sea (Merck,1.05437.0500, Germany) were pipetted in 90 mm sterile Petri dishes and inoculated (1% v/v) with a *V. harveyi* suspension (OD=0.5 in 600nm wavelength). After drying for 30 min, four 6 mm wells were bored in each plate. Fifty µ1 of cell-free extracts of bacteria were pipetted into each well after diffusion of soup in Agra, the plates were incubated at 30°C and observed for clearing zones around the wells after 2, 3, and 7days. Neutralized TSB-2.5% Sea was used as controls to determine the possible inhibitory activity of the medium. The inhibition zones were measured by using a digital caliper. All experiments were carried out in triplicate to ensure feasibility and reproducibility.

Isolates from primary screening, according to the tolerance of isolates, stability and diameter of inhibition zone against *V. harveyi* were screened and dominant antagonistic isolates during the study were selected. Isolates which were identified as potential probiotics during the first screening, were repeated to confirm the reliability of the AWDA and secondary screening results (Fjellheim et al., 2010).

Overnight cultures of selected probiotics (18 h) were inoculated (5%) to 200ml TSB-2. 5% Sea and incubated at 30°C with aeration in shaking incubator at 150 rpm for 72 h. A sample was withdrawn after 2, 4, 6, 12, 24, 28, 48, 50 and 72 h for measurement of cell density in 600 nm, and antibacterial activity. The antibacterial activity of the cell-free neutralized (pH 7.0, 1M NaOH) supernatant was tested by agar well diffusion assay against *V. harveyi* and incubated for 24h, the best time of production of antibacterial metabolites and

the growth curve was obtained (Strompfova and Laukova, 2007; Guo et al., 2009).

After isolation and purification, Gram staining was done on selected bacteria. The DNA of two probionts (IS02 and IS03), were extracted by IBRC Gram Positive Bacterial Genomic DNA Extraction Kit, and the 16S ribosomal DNA gene was amplified by using eubacterial universal primers, forward primer: 5'-TTGGAGAGTTTGATCCTGGCTC–3' and reverse primer: 5'-AGGAGGTGATCCAACCGCA– 3' (Sigma). PCR was carried out with 3 µl DNA as the template in a 50µl reaction mixture containing primers (0.5µl from each of them), $MgCl_2$ 50mM (1.5µl) (Cinnagen), deoxynucleoside triphosphate 10mM (0.8 µl) (Cinnagen) and Taq DNA polymerase 5U/µl (0.5 µl) (Cinnagen) along with PCR buffer 10X (5 µl) (Cinnagen). After the initial denaturation for 5 min at 94°C, 36 cycles consisting of denaturation at 94°C for 1 min, annealing at 62°C for 40 sec and 80 sec extension at 72°C, followed by a final extension at 72°C for 10 min and cooling to 4°C. The confirmation of 16S rDNA amplification was performed by electrophoresis of final product in agarose gel (1%). Amplified DNA fragments (1500bp) were cloned into the pGEM vector following the directions provided (Promega). Recombinant bacteria were discovered by blue-white screening and confirmed by PCR. Plasmids containing the insert were purified and utilized as a template for DNA sequencing. The amplified 16S rDNA of isolates was sequenced by Sanger sequencing method, and more comparison was made with available sequences in ez-taxon (Chun et al., 2007) and NCBI (National Center for Biotechnology Information) BLAST program.

Probiotic strains were inoculated in TSB-2.5% Sea and incubated in shaking incubator at 30°C overnight. The cells were centrifuged (2000g) and sediments were washed with sterile sea water twice and resuspended in the same buffer. A suspension containing 10^7–10^8 CFU/ml of bacteria was prepared and the absorbance was adjusted to 0.25±0.05 at 600 nm for each probiotic. In addition to verifying the relationship between absorbance at 600 nm and CFU per milliliter of probiotic bacteria dilution plating method was used.

The basal diet for the supplementation of probiotics was commercial shrimp feed (Havoorash Co., Iran). Bacterial suspensions were surface coated on the feed by spraying (10^5 CFU/g feed). Estimating the probiotic concentration in the prepared feed was determined by plate counting on TSA-2.5% Sea.

Litopenaeus vannamei post- larvae were obtained from a commercial shrimp hatchery in the Province of Bushehr, Iran. The shrimps were maintained in a water bath thermostatically controlled at 26±1°C. The shrimp had not been exposed to shrimp diseases and were deemed pathogen-free by standard microbiological techniques. Shrimp were acclimatized for 5 days before use in order to ensure adequate health. After the acclimation period, the average weight of the shrimp was 0.3±0.15g and 180 shrimp were introduced into three sets of troughs in triplicates (20 shrimp in each trough), and filled with filtered seawater, salinity maintained at 35±2 ppt, pH at 7.8 ± 2.0 and

temperature at 26 ±1°C. First triplicate was treated with feed supplemented with 10^5 CFU/g of *B. subtilis* IS02 (T1) and the others were treated with feed supplemented with 10^5 CFU/g of *B. vallismortis* IS03 (T2) for 28 days; the third group served as the control and was fed with a regular diet during the entire trial period. Shrimps in all groups were fed twice daily. The water temperature was held at 26 ± 1°C during the whole trial. The weight and the general health of the shrimp were recorded. After 28 days of probiotic supplementation, the experimental infection was carried out by the immersion method.

V. harveyi IS01 was grown for 18h at 30°C in TSB-2.5% Sea. After incubation cells harvested by centrifugation at 5000rpm for 10 min, washed and suspended in sterile sea water in order to adjust the number of bacteria at 10^4–10^5 CFU/ml). Shrimps in all nine of the tanks were exposed to *V. harveyi* IS01 (10^4–10^5 CFU/ml) for 24h. After infection, the shrimps were kept under the initial experimental conditions. The accumulated mortality of the shrimp was recorded for 15 days (Vaseeharan and Ramasamy, 2003; Balcazar and Rojas-Luna, 2007).

The weights of all shrimps were determined at the start (Initial Weight) and at the end (Final Weight) of the 28 day experiment. The Daily Weight Gain (DWG; g d−1) was calculated as (Vijayagopal et al., 2008) :

$$\frac{\text{Final Weight(g)} - \text{Initial weight (g)}}{28d}$$

The Relative Gain Rate (RGR %) was calculated as:

$$\frac{\text{Final weight(g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100\%$$

The Specific Growth Rate (SGR %) was calculated as:

$$\frac{\text{Ln[Final Weight(g)]} - \text{Ln[Initial weight(g)]}}{28d} \times 100\%$$

The results were analyzed by the One-Way ANOVA test and Games-Howell and Duncan Post-Hoc test to determine differences ($P<$ 0.05) between testing groups. All statistics were performed with PASW, version 18 (IBM® SPSS®, USA).

Results

The number of bacteria strains was isolated from seawater, sediment; hepatopancereas and gut content of healthy shrimp were 198 strains. They were included Gram negative and Gram positive bacilli, Gram positive and Gram negative cocci (Fig. 1). The primary screening of 198 strains revealed that 14 strains exhibited antagonistic activity against *V. harveyi*. Among them 64.3% was Gram positive bacilli and 35.7% were Gram negative bacilli. 14 antagonistic isolates were chosen for further evaluation of probiotic properties, however, as some isolates did not tolerate the storage conditions, and according to largest inhibition zone the numbers were reduced to 2 dominant antagonistic isolates during the study. Properties such as stability, dominance and stable inhibition zone against *V. harveyi* were emphasized when selecting the isolates. The maximum inhibition activity against the *V. harveyi* belonged to strain IS02 and strain IS03 and they were assessed for their in vivo activity.

The 16S rDNA sequences of selected bacterial isolates were analyzed and identified the probiont IS02 as *Bacillus subtilis* and IS03 as *Bacillus vallismortis*.

Culture supernatants of probiont bacteria were sampled at various times during growth cycle and tested for, growth kinetic and anti *V. harveyi* activity. The highest antibacterial activity of IS02 was reached at the end of stationary growth phase, then a decrease in the bactericidal activity of culture supernatants occurred (Fig. 2). IS03 has the maximum antibacterial activity in the middle of stationary growth phase and activity of its decrease after 72 h of culture (Fig. 3).

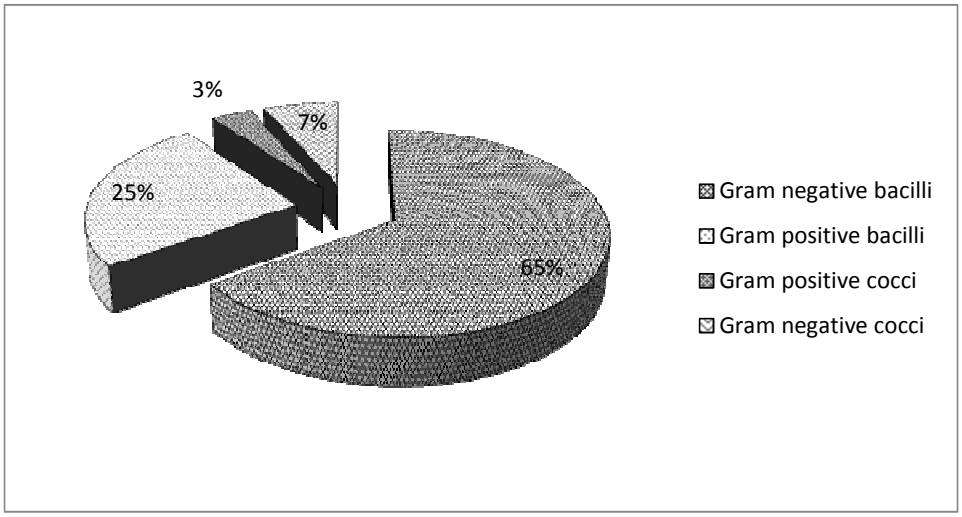

Figure 1: Morphologic diversity of bacterial strains isolated from water, sediment and shrimp in percent

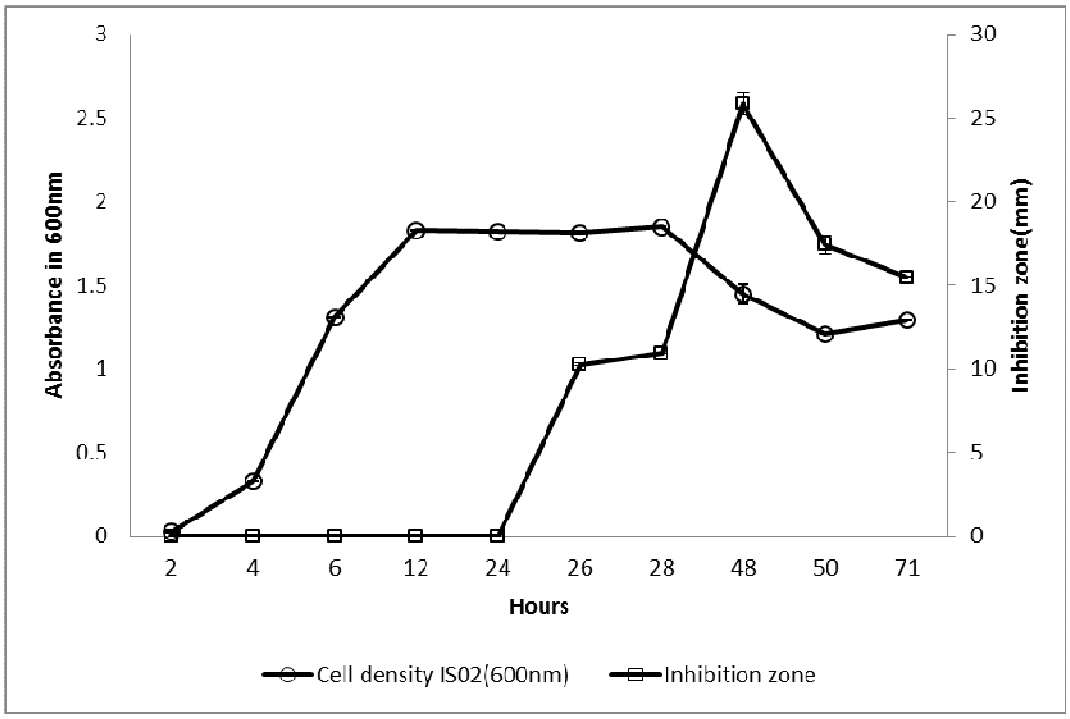

Figure 2: Growth curve of *B. subtilis* IS02 and inhibition zone of bacteria soup on *V. harveyi* in different time

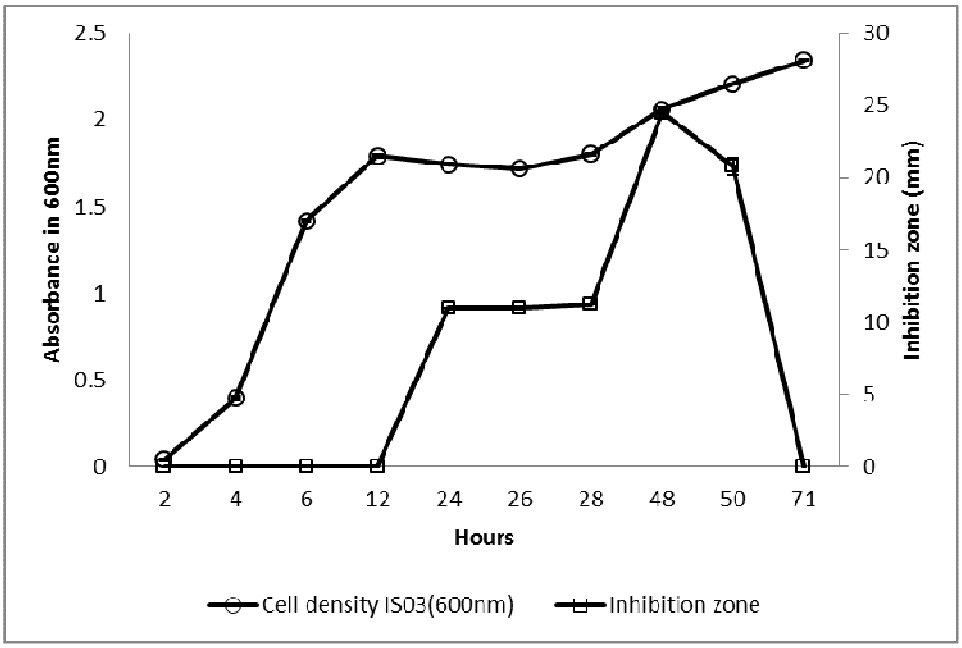

Figure 3: Growth curve of *B. vallismortis* IS03 and inhibition zone of bacteria soup on *V. harveyi* in different time

According to obtained results the *B. subtilis* IS02 and *B. vallismortis* IS03 48h culture soup caused a clearing zone with a diameter of 25.86±0.6mm and 24.49±0.04mm against *V. harveyi* IS01.

The mean final weight of shrimps after 28 days were 1.08±0.3g in the groups supplemented with *B. subtilis* IS02, 1.01±0.3g in the groups supplemented with *B. vallismortis* IS03 and 0.77±0.2g in control groups (table 1). The statistical analysis showed significant difference ($P< 0.05$) in the final weight between treatment and control.

The mean values of Daily Weight Gain (DWG), Relative Gain Rate (RGR) and Specific Gain Rate (SGR) after 28 days in all groups treated with probiotics were significantly higher than those of the Control ($P<0.05$). These results show that probiotics food composed of probiont bacteria increased growth performance in shrimps. There was meet did not observe mortalities during 28 days in probiotic treatment groups.

Table 1: The growth performance of shrimp fed diets supplemented with probiotic strains for 28 days

Mean values[1]	C	T1	T2
Initial Weight (g/shrimp)	0.298 ± 0.15[a]	0.320±0.15[a]	0.294±0.15[a]
Final Weight (g/shrimp)	0.770±0.20[a]	1.077±0.30[b]	1.010±0.30[b]
DG (gd^{-1})	0.017±0.008[a]	0.027±0.01[b]	0.025±0.01[b]
RGR (%)	157.67±72.6[a]	236.35±88.5[b]	237±98.8[b]
SGR (%)	3.23±0.00[a]	4.185±0.96[b]	4.21±1.087[b]

[1]: Values are mean ± SD for each row. Means with the same superscript are not significantly different ($P< 0.05$). C—control (no probiotic provided); T1—received the *B. subtilis* IS02 as probiotic; T2—received the *B. vallismortis* IS03 as probiotic.

To investigate whether selected bacteria are able to protect shrimp against vibriosis infection, shrimps were infected with *V. harveyi* by the immersion method. The cumulative mortality of shrimp was reduced by *B. subtilis* IS02 and *B. vallismortis* IS03 whereas final mortality of infected shrimp not treated by probiotic strains was 95% in 15^{th} day after infection but with treats by *B. subtilis*IS02 and *B. vallismortis* IS03, it was 22.5% and 17.5% respectively (Fig. 4). Statistical analysis demonstrated significant differences ($P< 0.05$) in mortality between treatment and control groups. Mortality in control groups started from first week but in probiotic treatment groups, it began from the second week.

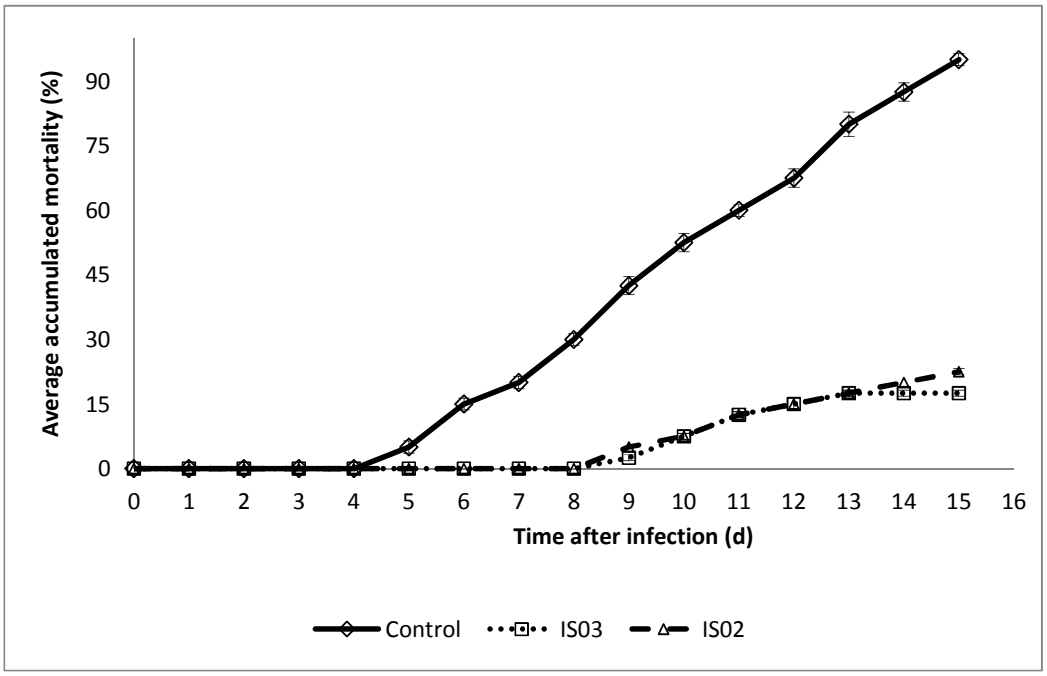

Figure 4: Cumulative mortality of *Litopenaeus vannamei* infected with *V. harveyi* with and without probiotic treatment of *B. subtilis* IS02 and *B. vallismortis* IS03. vs, Control.

Investigation of effect of probiotic treatment on vibriosis of shrimp brought out that shrimp mortality was decreased by both probiotic strains under in vivo conditions.

Discussion

Shrimp aquaculture is a major industry throughout many countries in the Asia-Pacific region and Latin America, but FAO has reported disease outbreaks as a major inhibitor factor for development of aquaculture industry worldwide (Alexandra, 1991; Lavilla-Pitogo et al., 1998; Subasinghe et al., 2001; Martin et al., 2004). Moreover, use antibiotics in a prophylactic way, has resulted in the development of (multiple) antibiotic resistance (Cabello, 2006), the continuous development of antibiotic-resistant bacteria is reducing the efficacy of current treatment and is a threat to public health (Karunasagar et al., 1994). With this growing need for an alternative to antibiotics, the concept of sustaining, rather than fighting, the microbial community on the farm has arose. A good pool of bacterial isolates is urgently needed in the search for probiotic candidates. There is increasing evidence in a diverse range of species that promoting a healthy gut microbial community and essential for growth and disease resistance of animals (Fuller, 1989; Moriarty, 1999; Verschuere et al., 2000; Collado et al., 2007; Decamp et al., 2008).

There is no clear suggestion that probiotic candidates isolated from the host perform better than isolates that are from a different habitat

(Verschuere et al., 2000), but logically one would assume that the best place to look for beneficial bacteria is among the host's own flora. In the present study in first screening process Gram positive bacilli had the most frequency percent among isolates after second screening process, two Gram positive bacilli bacteria were selected from the normal mid-gut microbiota and sediment of ponds of *L. vannamei* culture. These bacteria have a significant and stable antagonist effect on *V. harveyi*. Molecular identification indicated that the organisms were belonged to *Bacillaceae* family and consequently the potential for its application on farms became greater. *Bacillaceae* produce a range of antimicrobial activities throughout their life cycle, and probiotic candidates that produce inhibitory substances has identified good probiotics in aquaculture (Abriouel et al., 2011), however this approach will not detect other modes of probiotic action (e.g. immunostimulation, production of digestive enzymes, competition for attachment sites or pathogen inhibition). Probiotics decrease the likelihood of antibiotic resistant genes being transferred from the probiotic to the pathogen (Temmerman et al., 2003; Huys et al., 2006). In this research according to 16SrDNA analyzing the isolates was identified as *B. subtilis* strain IS02 and *B. vallismortis* strain IS03.

The daily addition of *B. subtilis* IS02 and *B. vallismortis* IS03 at a concentration of 10^5 CFU/g feed in shrimp larvae culture system can increase the SGR and RGR of shrimp. This is the first report that *B. subtilis* IS02 and *B. vallismortis* IS03 were used as probiotic for prevention of the vibriosis in post larvae of *L. vannamei* and growth enhancement. Shrimp mortality was followed by *V. harveyi* alone treatment, when the larvae are pretreated with probiont for 28 days and then exposed to *V. harveyi*, their mortality is significant ($P< 0.05$) reduced. Mortality in control groups started earlier than probiotic treatment groups and it may be referred to the immune stimulant effect of selected probiotic.

This result finds support of other workers. Rengipipat et al. (1998) showed that inoculation of Bacillus S11 to *P. monodon* post-larvae that were challenged by pathogenic luminescent bacteria led to the significant survival of them(Rengpipat et al., 1998).Vaseeharan and Ramasamy (2003) have reported treatment of *P. monodon* adults by the probiont *Bacillus subtilis* BT23 reduced the mortality of them against *V. harveyi* (Vaseeharan and Ramasamy, 2003). Marine bacterium *Alteromonas* spp. (10^6 CFU ml) has a protective effect and reduced mortality of *P. monodon* larvae against *V. harveyi* (Abraham et al., 2004). Balcazar et al. (2007) recorded that feed conversion ratio in shrimp nourished by probiotic diets was higher than control groups, and these shrimps showed lower mortality than the control group after challenging by *V. parahaemolyticus.*(Balcazar et al., 2007). Hill et al. (2009) reported *Bacillus pumilus* isolated from the mid-gut of *P.monodon*, strongly inhibited *V. harveyi* and *V. mimicus* (Hill et al., 2009). Guo et al. (2009) suggests that survival and metamorphosis of shrimp improved with the addition of probiotic *B. fusiformis* (Guo et al., 2009). The mechanism of these bacteria was not well known previously but today we know several eukaryotes (including plants and fungi) and prokaryotes

(e.g. Bacillaceae), that can produce bioactive metabolites. The bioactive metabolites interfere with quorum sensing pathways in several gram negative species (Dong et al., 2002; Teasdale et al., 2009; Defoirdt et al., 2011) and stopped their pathogenicity.

In present study, neutralized culture soup of probionts, *Bacillus subtilis* IS02 and *B. vallismortis* IS03 exhibit zone of clearance against *V. harveyi*. Consequently, the antagonistic activity of probionts can be related to the production of bioactive metabolites by them and inhibits the growth of *V. harveyi* or quorum sensing pathways. We measure the growth kinetics and determined the time in which candidate probiotic produce maximum antibacterial compound in soup of culture medium for determining the best time of harvesting probiotic bacteria. It is mentioned that *Bacillus* species produce a large number of bioactive metabolites against bacteria, fungi, protozoa and viruses. Antibiotics and bacteriocins are examples of bioactive metabolites produce by *Bacillus* strains. Most of the peptide antibiotics produce by them are active against gram-positive bacteria; however compounds such as Polymyxin, Colistin, and Circulin exhibit activity almost exclusively upon gram-negative bacteria, whereas Bacillomycin, Mycobacillin, and Fungistatin are effective against molds and yeasts (Mannanov and Sattarova, 2001). Advance research is required to clarify the mechanism of the beneficial effects of probiotic bacteria and purification of bioactive compound for further purposes in industry especially aquaculture.

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غربالگری و ارزیابی باکتری های بومی خلیج فارس به عنوان پروبیوتیک در کنترل زیستی باکتری ویبریو هاروی (*Vibrio harveyi*) پست لارو میگوی سفید غربی (*Litopenaeus vannamei*)

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تاریخ دریافت: خرداد ۱۳۹۱ تاریخ پذیرش:آذر ۱۳۹۱

چکیده

هدف پژوهش حاضر جداسازی باکتری های بومی دریایی به عنوان پروبیوتیک در کنترل زیستی باکتری های پاتوژن سویه های ویبریو (*Vibrio spp.*) در صنعت پرورش میگو بود. در مجموع ۱۹۸ سویه باکتریایی از آب، رسوب، هپاتوپانکراس و روده میگوها پس از کشت در محیط تریپتیک سوی آگار و گرمخانه گذاری در دمای ۳۰ درجه سانتیگراد به مدت ۴۸-۲۴ ساعت جداسازی شد. این جدایه ها از نظر فعالیت آنتاگونیستی علیه باکتری ویبریو هاروی (*Vibrio harveyi*) مورد آزمون قرار گرفتند. دو سویه (IS02 و IS03) جداسازی شده از روده و رسوبات استخر بیشترین ویژگی بازدارندگی از رشد را بر روی باکتری *V.harveyi* داشتند. بر اساس نتایج توالی یابی ژن 16SrDNA، باکتری IS02 به عنوان *Bacillus subtilis* و باکتری IS03،*B. vallismortis* شناسایی شد. سپس اثر پروبیوتیکی هر دو باکتری به صورت جداگانه در کنترل باکتری *V. harveyi* پاتوژن در میگوهای سفید غربی مورد ارزیابی قرار گرفت. بر اساس نتایج این پژوهش هر دو باکتری IS02 و IS03 توانایی کنترل زیستی باکتری *V. harveyi* را در شرایط درون تن و برون تن داشته و سبب افزایش شاخص‌های رشد پست لارو میگوی سفید غربی می گردند.

کلمات کلیدی:پروبیوتیک، میگوی سفید غربی، باکتری های بومی، ویبریو هاروی

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