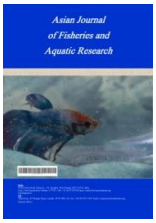

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Isolation of Probiotic Bacteria from *Macrobrachium rosenbergii* and Their Antagonistic Efficacy against Pathogenic Bacteria

Moni Krishno Mohanta[1*], Preonty Mallick[1], Md. Fazlul Haque[1], Md. Ariful Hasan[1] and Ananda Kumar Saha[1]

[1]Department of Zoology, Genetics and Molecular Biology Laboratory, University of Rajshahi, Rajshahi-6205, Bangladesh.

Authors' contributions

This work was carried out in collaboration among all authors. Authors MKM and PM designed the study, performed the statistical analysis and wrote the protocol and the first draft of the manuscript. Authors AKS and MAH managed analysis and the literature searches. Author MFH edited the manuscript and finalized it. All authors read and approved the final manuscript.

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ABSTRACT

The probiotic bacteria isolated from prawn, *Macrobrachium rosenbergii* was studied for their morphological and biochemical characteristics as well as their antagonistic efficacy against pathogenic bacteria. A total of three probiotic bacteria *viz.* White Colour Bacteria (WCB), Red Colour Bacteria (RCB) and Yellow Colour Bacteria (YCB) were isolated from intestine of healthy prawn while three pathogenic bacteria *viz.* PB1, PB2 and PB3 were isolated from infected antennae and muscles of moribund prawn. Depending on their physical and biochemical features, the probiotic isolates were gram-positive, rod shaped and motile bacteria belonging to *Bacillus* spp. and the pathogenic bacteria were also identified as gram-negative, cocci shaped and motile bacteria fit in *Enterococcus* spp., *Vibrio* spp. and *Micrococcus* spp. The optimum culture conditions of all isolates were at pH 7.0 and 37°C temperature. Results on the antibiogram profile of pathogenic bacteria revealed that majority of the isolates were sensitive (43.58%) or intermediate

**Corresponding author: Email: mkmohanta_zool@yahoo.com;*

(30.76%) against thirteen antibiotics. The probiotic bacterial antagonistic activities were tested against *Enterococcus*, *Vibrio* and *Micrococcus* spp.by cross-streak method. The results indicate that the strain of YCB showed inhibitory effects against *Enterococcus* spp. (5 mm), *Vibrio* spp. (5 mm) and *Micrococcus* spp. (3 mm). Similarly, WCB showed inhibitory effects against *Enterococcus* spp. (4 mm), *Vibrio* spp. (3 mm) and *Micrococcus* spp. (4 mm). RCB strains showed inhibition against *Micrococcus* spp. (3 mm) only, but not against *Enterococcus* spp. and *Vibrio* spp. Based on the results, it can be concluded that the isolated probiotic bacteria could be a good candidate to consider for further studies to control the pathogenic bacteria in prawn culture.

Keywords: Antibiotics; enterococcus; Macrobrachium rosenbergii; micrococcus; probiotic bacteria; Vibrio.

1. INTRODUCTION

Shrimp plays a vital role in delivering nutrition, earning foreign exchange and construction of rural employment for poverty improvement. Nowadays, shrimp plays a dominant role in the economy of Bangladesh. Every year it contributes 4.7% to GDP and about 8% to the total export earnings of the country [1].

However, in Bangladesh diseases caused by pathogens are very warning factors for the expansion of shrimp sector. Both freshwater prawn golda (*Macrobrachium rosenbergii*) and brackish water shrimp bagda (*Penaeus monodon*) farming in Bangladesh have been facing disease problem [2]. In shrimp and prawn farming, bacterial infection is one of the main problems all over the world. Member of the genus *Vibrio* including *V. cholera*, *V. harveyi* and *V. parahaemolyticus* have been identified as pathogenic species in shrimp which are the main cause of the larval deaths [3]. These pathogens which cause severe infections reduced production of prawn in ponds and hatchery [4].

Most of the hatchery operator used antibiotic drug and disinfectant to prevent bacterial diseases without proper scientific investigation into treatment regimes. Uncontrolled use of antibiotics and inappropriate selection of chemicals for use in shrimp hatcheries for lack of regulation are common in Bangladesh which in turn impose the risk of emergence of antibiotic resistant pathogens for human [5-7]. Some numerals of recent news, press issues and inquiries have risen up real community alarms about the care of antibiotic medication practice in aquaculture. There are number of probiotics which act together with the antagonism for chemicals or obtainable energy, the making of inhibitory composites, antagonism for blending sites, improvement of water quality and the enhancement of the immune response [8]. Many researchers proved probiotic organisms as a feed supplement to expand production rates [9] and protect free aquatic animals like fish from pathogenic bacteria [10].

The usage of probiotic microorganisms in aquaculture is a quite new concept and its play a key function in aquaculture industries [11]. Hence, the demand for the research on the use of probiotics bacteria in aquaculture is growing for environment-friendly approach [12]. Moreover, probiotic bacteria produce important enzymes and nutrients which are used for improving the growth of host organisms as well as fighting against pathogens [13,14]. Hence, the probiotics strains are used to treat disease, improve water quality and reduce pathogenic microbial population in shrimp rearing environment [15].

Probiotics are well-defined as microbes which are live when directed in tolerable volumes that deliberate a healthiness advantage to the host. Moreover, it visualizes that the probiotic bacteria have the capability to produce acids, enzymes, inhibitory compounds and other bio-molecules. From this point of views, the study was designed to isolate probiotic bacteria from healthy prawn and then to know the morphological and biochemical characteristics as well as antagonistic efficacy of probiotic isolates against pathogenic bacteria.

2. MATERIALS AND METHODS

2.1 Study Area and Duration

In this study, the healthy and moribund prawn samples were collected from Avaynagar Thana under Jessore District, Bangladesh and recycled as sources of inocula for the isolation of probiotic as well as pathogenic bacteria. The collection of prawn sample, isolation of bacteria and subsequent experiments were done from March to November 2019.

2.2 Collection of Prawn

The healthy and moribund giant freshwater prawn, *Macrobrachium rosenbergii* (40-50 g) were collected from Avaynagar Thana under Jessore District, Bangladesh. During transport to the laboratory, the samples were kept in ice and taken into sterile bags. They were then used as a source of inocula for the isolation of probiotic and pathogenic bacteria.

2.3 Isolation and Characterization of the Microbes from the Healthy and Moribund Prawn

Under sterile conditions, healthy prawn was dissected, the whole intestine carefully removed and suspended separately in1ml of physiological saline. Then suspended intestine was homogenized with Glass Pestle Tissue Grinders (PYREX™) by 50 strokes for each sample in sterile condition. One loopful of each suspension was separately inoculated into nutrient broth which was incubated for 24h at 37°C with shaking at 120rpm (revolution per minute) using shaker. Control flasks deprived of inoculums and also ready for incubated at 37°C with a shaker. The cultures that were found turbid after a period of 0 up to 2 days were used as inocula in further experiment.

For isolation of pathogenic bacteria, the affected prawn was dissected firstly. Secondly muscle and antenna were collected for pathogenic bacteria isolation [16]. Each sample was diluted from 10^{-1} to 10^{-7} and 0.1 ml of diluted sample was taken from 10^{-5}, 10^{-6}, 10^{-7} and spread on nutrient agar plate separately. One plate preserve as a control without spreading sample. At 37°C for 24 to 48 hours the plates were incubated.

2.4 Screening of Bacterial Isolates

One loopfull of primary enrichment culture was streaked on nutrient agar plates (Hi-media) and incubated for 24h at 37°C temperatures. The single colonies were found to grow on the medium. Four isolates were found from primary screening and further grown on nutrient agar plate. The single colony was selected and again streaking was done on nutrient agar plates for subculture under germ-free environments. Pure cultures of probiotic and pathogenic isolates which were obtained by repeated subculture of single colony were grown on slants by stab and streak method for storage purpose and subsequently for identification and biochemical characterization.

2.5 Microscopic Examinations and Identification of Bacterial Cells

For the identification of the bacteria, observations of morphological characters with a binocular light microscope (Labomed, USA), growth characteristics, antibiotic sensitivity test and biochemical tests *viz*. Triple Sugar Iron (TSI) test, Citrate utilization test, Sulfur Indole Motility Media (SIM) test, MacConkey agar test, Hydrogen Sulfide (H2S) Production Test, Potassium hydroxide (KOH) test, Catalase test, Oxidase test and Carbohydrate utilization tests were performed. The microorganisms were identified using Bergey's Manual of Systematic Bacteriology [17].

2.6 Determination of Optimum Growth Conditions

For the determination of optimum pH on bacterial growth, culture medium was adjusted to pH 5.0, 6.0, 7.0 and 8.0 by adding concentrated HCl or NaOH. Incubation temperature varied from 25°C to 42°C. Bacterial cell density of liquid cultures was determined at different time intervals by measuring optical density at 660nm with photoelectric colorimeter (AE-11M, Erma Inc., Tokyo) [18].

2.7 Antibiotic Sensitivity Test

Antibiotic sensitivity test of the isolates was performed as described by Saha and their colleagues [19]. Briefly, 1 ml of fresh broth culture of the isolate was spread uniformly on a nutrient agar plate with a sterile glass spreader. The plate was air-dried for few minutes and then antibiotic discs were placed on inoculated nutrient agar plates which were incubated at 37°C for 24 hours. After incubation, clear zones indicated inhibition of growth of the isolate.

2.8 Inhibitory Activity of Probiotic Bacteria on Pathogenic Bacteria (PB1, PB2 and PB3) by Cross-streak Method

Three strains of probiotic bacteria (RCB, WCB, YCB) and pathogenic bacteria (PB1, PB2, PB3) were cultured on nutrient agar and incubated at 37ºC for 24hours. Inhibitory activity tests were

done on nutrient agar plate by the cross-streak method [20]. Pathogenic bacteria *viz*. PB1, PB2, PB3 was streaked in a line separately and then probiotic bacterial strains *viz*. RCB, WCB, YCB were streaked separately in a perpendicular line. Every species pair was incubated at 37ºC for 48 hours and was cross-streaked in triplicate. After 48 hours of incubation the inhibitory activity was observed through linear zone of the plate and measured the zone with the helping of mm scale.

3. RESULTS

3.1 Isolation and Identification of the Bacterial Species

The isolates RCB, WCB and YCB were isolated from healthy prawn as well as the isolates PB1, PB2 and PB3 were isolated from moribund prawn. Results of morphological characteristics and microscopic analysis of bacterial cells are presented in Table 1 and Plate 1 while the biochemical and antibiotic sensitivity tests of the isolates are presented in Table 2a and 2b, respectively. This was accomplished simultaneously by gross colony morphology and a number of biochemical tests on the basis of presence (+) or absence (-) criteria.

3.2 Optimum Temperature and pH for Growth of Bacterial Isolates

To verify the effects of pH and temperature of growth medium on the growth rate of the isolates, a series of investigations were carried out which are presented in Figs. 1 and 2 respectively. The optimum pH for the growth of the isolates was 7.0 while extreme pH was 5.0 and 8.0 which restricted the bacterial growth. The optimum temperature for the growth of the isolates was found to be 37°C while the extreme temperatures were between 28° and 42°C which restricted the bacterial growth. At 37°C the rate of the best growth was found (OD 0.97) after 24hr of incubation.

Table 1. Colony morphology and microscopic observations of the isolated bacterial strains

Bacterial strain	Colony morphology				Microscopic observations		
	Color	Shape	Surface	Opacity	Gram staining	Shape	Motility
WCB	White	Irregular	Rough	Opaque	Gram +	Rod	Motile
RCB	Red	Circular	Smooth	Clear	Gram +	Rod	Motile
YCB	Yellow	Circular	Smooth	Clear	Gram +	Rod	Motile
PB1	White	Circular	Large	Smooth	Gram -	Cocci	Motile
PB2	White	Circular	Moderate	Smooth	Gram -	Rod	Motile
PB3	White	Circular	Small	Smooth	Gram -	Cocci	Motile

Table 2a. Biochemical test results for the isolated bacterial strains

Strain name	TSI	Citrate	SIM	MacConkey	H_2S production	KOH	Catalase test	Oxidase test	Maltose	Cellulose	Galactose	Lactose	Fructose	Glucose	Sucrose	Identification of bacteria
RCB	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	*Bacillus* sp.
WCB	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
YCB	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	*Bacillus* sp.
PB1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Enterococcus* sp.
PB2	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	*Vibrio* sp.
PB3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Micrococcus* sp.

(+ = microbial growth, - = no growth)

Table 2b. Antibiotic sensitivity tests of the isolates

Antibiotics	Zone of inhibition (mm)					
	RCB	WCB	YCB	PB1	PB2	PB3
Neomycin	16 (S)	15 (I)	9 (R)	18(S)	19(S)	8(R)
Cefradine	17(S)	16 (S)	17 (S)	15(I)	12(I)	15(I)
Tetracycline	20 (S)	16 (S)	17 (S)	15(I)	11(I)	16(S)
Cephradine	7 (R)	17 (S)	8 (R)	6(R)	17(S)	8(R)
Erythromycin	22 (S)	20 (S)	12 (I)	20(S)	20(S)	22(S)
Ciprofloxacin	16 (S)	25 (S)	16 (S)	25(S)	20(S)	22(S)
Gentamicin	18 (S)	17 (S)	18 (S)	11(I)	17(S)	18(S)
Amoxycillin	7 (R)	9 (R)	8 (R)	8(R)	6(R)	7(R)
Doxycycline	20 (S)	19 (S)	18 (S)	20(S)	17(S)	11(I)
Kanamycin	7 (R)	9 (R)	5 (R)	6(R)	6(R)	15(I)
Ceftozidine	9 (R)	9 (R)	8 (R)	11(I)	8(R)	9(R)
Rifampicin	15 (I)	15 (I)	16 (S)	17(S)	20(S)	20(S)
Ampicillin	17 (S)	15 (I)	15 (I)	11(I)	15(I)	11(I)

***(5-10 mm) = Resistance to antibiotics (R), (15-20 mm) = Sensitive to antibiotics (S), (10-15 mm) = Intermediate resistance (I)*

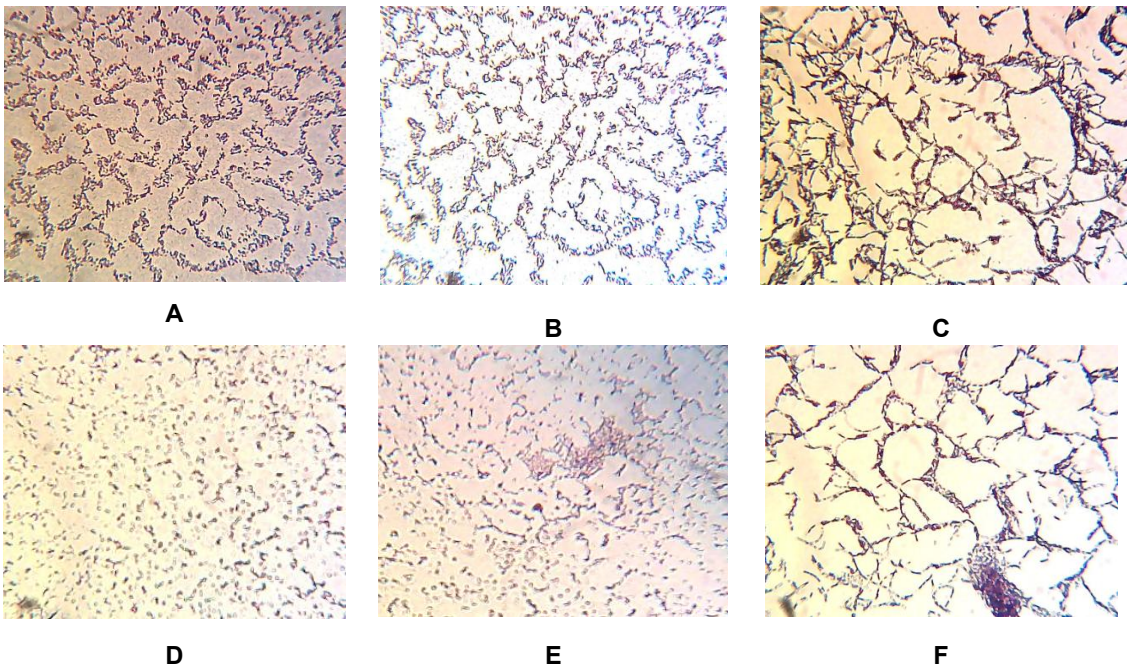

Plate 1. Gram staining tests for the isolates A (RCB), B (WCB), C (YCB), D (PB1), E (PB2) and F(PB3) respectively. The bacterial cells taken from the pure culture of the isolates were Gram stained and then microphotograph were taken with DSLR camera connected light microscope at 400X magnification

3.3 Inhibitory Activity of Probiotic Bacteria (YCB, RCB, WCB) against *Enterococcus*, *Vibrio* and *Micrococcus* spp. by Cross-streak Method

Inhibitory activity of probiotic bacteria *viz.* YCB, RCB and WCB against *Enterococcus*, *Vibrio* and *Micrococcus* spp. were evaluated by cross-streak methods (Plate 2). The results indicate that the strain of YCB showed inhibitory effects against *Enterococcus* spp. (5 mm), *Vibrio* spp. (5 mm) and *Micrococcus* spp. (3 mm) respectively. WCB showed inhibitory effects against *Enterococcus* spp. (4 mm), *Vibrio* spp. (3 mm) and *Micrococcus* spp. (4 mm) respectively. RCB strains only

showed inhibition against *Micrococcus* spp. (3 mm) but did not show any inhibition against *Enterococcus* spp. and *Vibrio* spp. (Plate 2).

4. DISCUSSION

Microbial diseases occur at all stages of commercially important culture of prawn and are responsible for huge economic loss. Bacterial infections cause more disease problems over all than any other infection. The use of antibiotics in aquaculture for controlling pathogens is increasing rapidly day by day without any concerns. Without decreasing the shrimp production the fundamental purposes in aquaculture is to reduce utilization of antibiotics so that avoid the typical drawbacks from the practice of antibiotics mostly increase of negative environmental effects and their resistance [21].

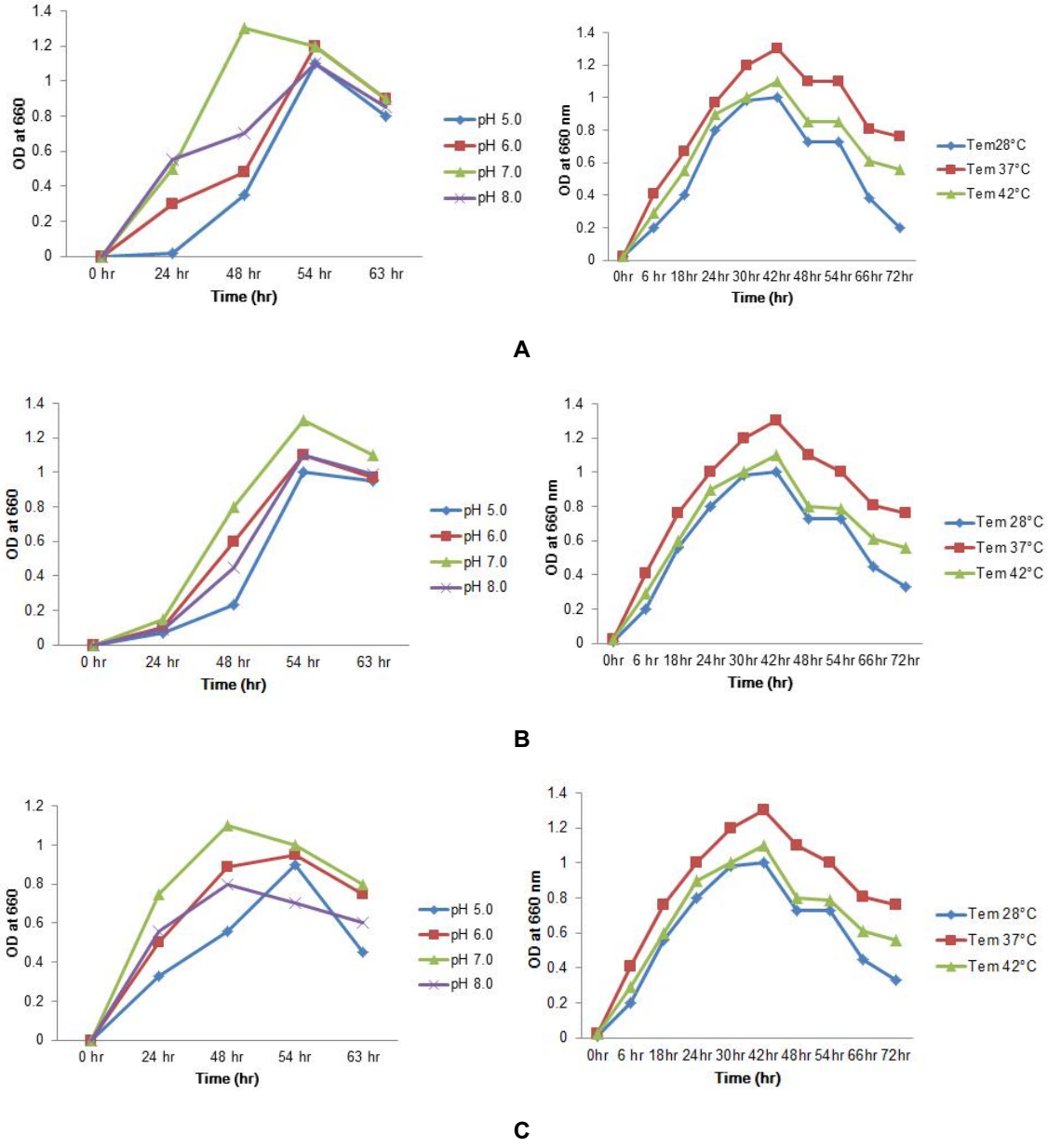

Fig. 1. Optimum pH and temperatures for growth of bacterial isolates A (WCB), B (RCB) and C (YCB)

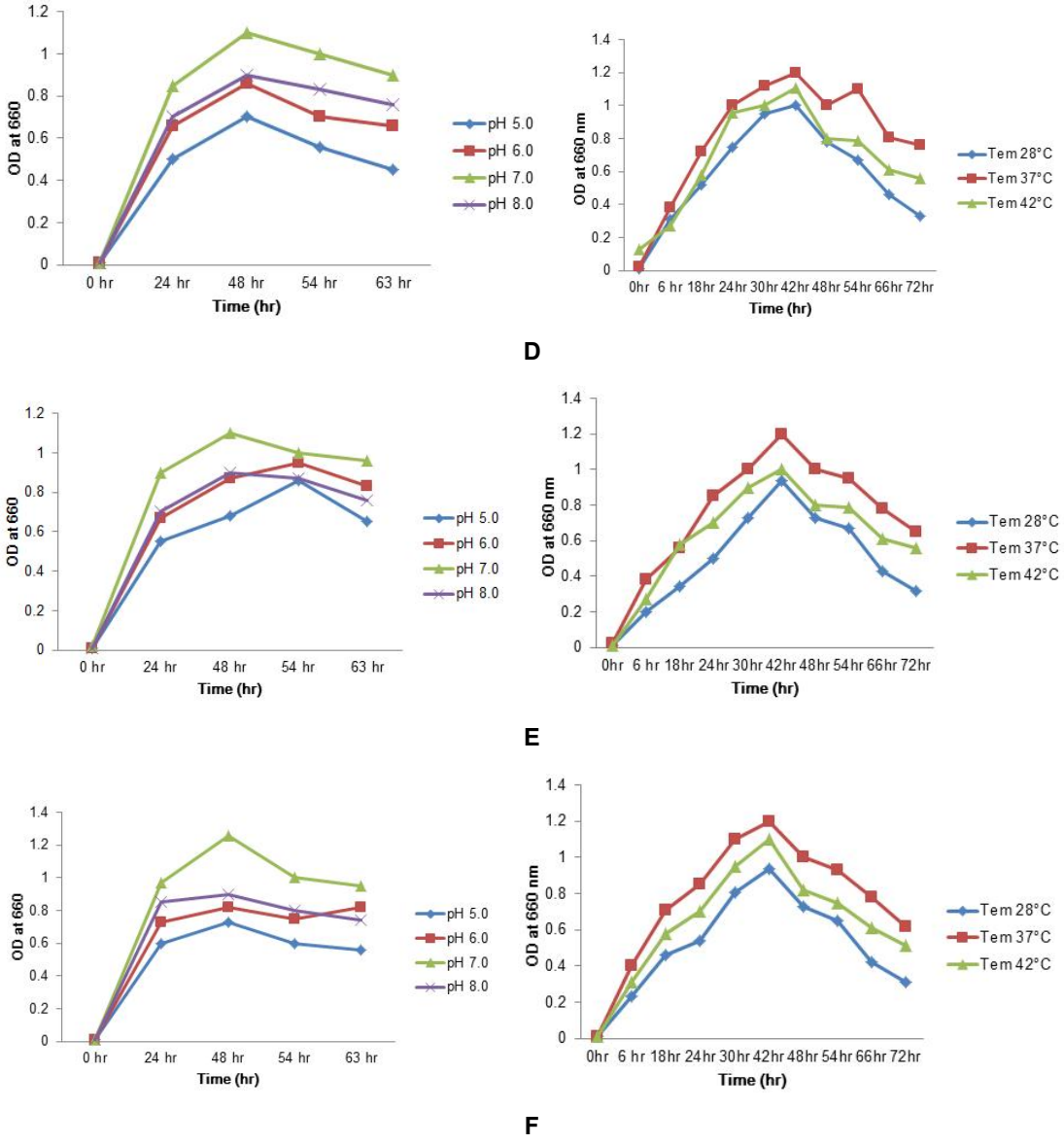

Fig. 2. Optimum pH and temperatures for growth of bacterial isolates D (PB1), E (PB2) and F (PB3)

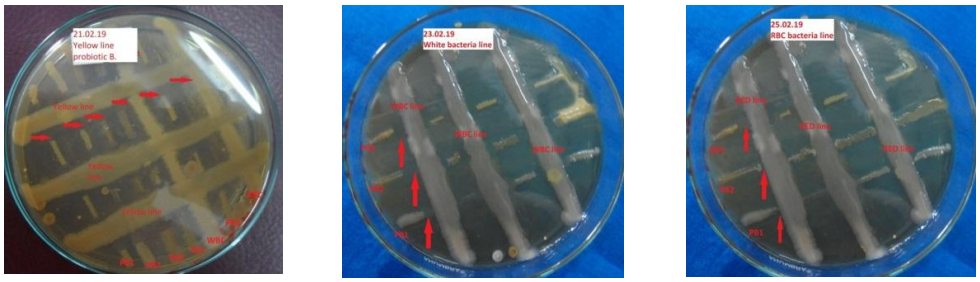

Plate 2. Inhibitory activity of probiotic bacteria A (YCB), B (WCB) and C (RCB)

Use of probiotic bacteria in shrimp aquaculture help in the biological control of disease through improved shrimp immunity, shrimp growth performance and pathogen inhibition [22]. Many studies have shown that in aquaculture systems compounds produced by bacteria that could be used to inhibit bacterial pathogens [23,24]. To manage the health of shrimps the use of such compounds to control shrimp pathogens is now gaining importance in shrimp farming as it can be a better alternate and cost effective than applying antibiotics.

When morphological and bio-chemical tests were complete the microorganism was found that gram-positive, rod-shaped and motile bacteria belong to the genus of *Bacillus* sp. The isolates were showed hazy appearance in the motility media and also were positive for TSI, SIM, H2S production, catalase, citrate utilization, and oxidase tests. This result is supported by the findings of Purivirojkul [25]. A study revealed that spore-forming bacteria like *Bacillus* spp.can be used as probiotics [26]. *Bacillus* spp. may display many kinds of different enzymes that destroy biofilms and slime which allow *Bacillus* spp. over other bacteria and their antibiotics to enter slime layers from place to place in Gram-negative bacteria. Also *Bacillus* spp. are not likely to use genes for antibiotic resistance or virulence from Gram-negative bacteria [27]. Earlier many researcher reported that many species of *Bacillus* such as *B. licheniformis, B. subtilis* and *B. toyoi*, were used as probiotics in aquaculture [13].

Three pathogenic bacteria *viz*. *Enterococcus* sp., *Micrococcus* sp. and *Vibrio* sp. were isolated and identified from muscle and antenna of moribund prawn. When physiological and biochemical tests were done, the microorganisms were found that gram-negative, cocci-shaped, rod-shaped and motile bacteria. The isolates showed positive result for TSI, SIM, H_2S production, catalase, citrate utilization, and oxidase tests. This result is supported by the findings of Narasimhan et al. [28]. It was also reported eleven different bacterial strains such as *Aureobacterium faciens, Bacillus subtilis, Micrococcus luteus, Escherichia coli, Aeromicrobium erythreum, Vibrio cholarae, Pseudomonas putida, Enterobacter aerogens, Pseudomonas aeruginosa, Enterococcus* and *pseudo avium* isolated from Omsakthi Aquafarmat Sedhubhavasatramin Thanjavur District, Tamil Nadu, India [28]. Purivirojkul [25] reported that from infected fairy shrimp showing black disease symptoms the pathogenic bacterium *Aeromonas hydrophila* WSI was isolated. Six species of *Vibrio* were isolated from shrimp farms in north coastal Andhra Pradesh, India [29]. Besides, many workers have reported about pathogenic bacteria isolated from diseased prawn *viz*. *Shigella* spp., *Aeromonas* spp., *Klebsiella* spp., *Pseudomonas* spp., and *Staphylococcus* spp. [30]. The effects of our current study show that the isolated pathogenic bacteria are most important pathogens in prawn culture ponds causing financial losses and severe mortalities which agreed with previous reports by Dalmin et al. [31]. Development of the prawn culture practice and control the pathogenic bacteria are very vital. By consequently, an improved description of probiotics for aqua-flora has been proposed [32].

The result of the cross-streak methods revealed that the probiotic bacteria namely YCB, RCB and WCB showed the antagonistic effect against pathogenic bacteria *viz. Enterococcus*, *Vibrio* and *Micrococcus* spp. This result is supported by the findings of Purivirojkul [25]. Purivirojkul [25] also noted that the putative probiotic displayed the highest inhibition in the cross-streak experiments was identified as *B.vallismortis* by16S rDNA sequencing. It was also reported that many strains of *Bacillus* spp. such as *B.thuringiensis* NEB17 cerein 8A and *B. licheniformis* bacillocin 490 release chemical substances with bactericidal effect on other microbial populations [8,33-36]. However, *B. vallismortis* can be used for biocontrol of fungal, bacterial and viral diseases of plant, but use of this *Bacillus* strain in aquaculture has been reported in many studies [37-39].

5. CONCLUSION

In the present study, it was found that the probiotic bacteria which were isolated from giant fresh water prawn were three different species of *Bacillus.*The isolated probiotic bacteria showed remarkable antagonistic efficacy against three pathogenic bacteria *viz. Enterococcus*, *Vibrio* and *Micrococcus* spp. which were isolated from moribund prawn. Both probiotic and pathogenic isolates showed the best growth at pH 7 and 37°C temperature. All studied probiotic and pathogenic isolates were resistant or intermediate to Amoxicillin, Kanamycin and Ceftozidine while they were sensitive or intermediate to all other tested antibiotics. Altogether, it can be concluded that the probiotic isolates were good inhibitor of

pathogenic isolates indicating that the probiotic isolates might be a potential candidate for controlling the pathogenic bacteria in aquaculture. Hence, the future study should be focused on the exploration of optimal parameters for using these isolates as probiotics in prawn culture.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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