International Journal of Pure and Applied Zoology ISSN (Print) : 2320-9577
Volume 1, Issue 4, pp: 326-333, 2013 ISSN (Online): 2320-9585

http://www.ijpaz.com

Research Article

EFFECTIVE ROLE OF PROBIOTIC ISOLATE *BACILLUS SP. ON THE GROWTH OF LABEO ROHITA*

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Article History: Received 22nd September 2013; Accepted 14th November 2013; Published online 19th November 2013

ABSTRACT

The present investigation was aimed to isolate and assess the bacterial samples from the rhizosphere soil. Out of twenty strains, four strains were assessed for their probiotic activity towards the growth of fish in the particular habitat. The experiments were conducted by admixing the strain with water and fortified with the commercial feed. Results pertaining to the present investigation clearly revealed that only one strain (MA4 -Antagonistic effect) was showed better activity. It was identified as *Bacillus sp*. Identified strain was examined for its effect on fish growth in the form of probiotics. Furthermore, the bacterial strain mixed in water enhanced the water qualities and also significantly increased the length and weight of freshwater fish, *Labeo rohita.*

Key words: *Bacillus sp.* probiotic isolate, growth, *Labeo rohita.*

INTRODUCTION

The word "probiotic" is 'organisms and substances which contribute to intestinal microbial balance' (Parker, 1974). It can be also defined as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989). This revised definition has put forward the importance of live cells as the essential component of a potential probiotic and it clears the confusion created by the use of the term '*substances*'. However, "an effect on intestinal microbial balance" has been defined and demonstrated only in a few cases. Later, Tannock (1997) proposed the definition as "living microbial cells administered as dietary supplements with the aim of improving health". The concept of microfloral manipulation was first appreciated by Metchnikoff (1907) who examined the consumption of yoghurt and found an effect on the longevity of Bulgarian peasants.

A beneficial effect by application of certain beneficial bacteria in human, pig, cattle and poultry nutrition has been well documented (Jong, 1993). On the other hand, the use of such probiotics in aquaculture is a relatively a new concept (Kozasa, 1986). There is a paucity of information available with regard to probiotics in aquaculture, even though, several reports deal with enhancement of growth of oyster larva with addition of certain bacterial mixture (Douillet and Langdon, 1994). This necessitate the demand of search for microorganisms (Symbionts) that may contribute to beneficial gut fauna in fish, because it has become clear that the microfauna in the gut plays an important role with respect to the well-being and health of fish. In spite of the limited information available on the effect of beneficial bacteria on the growth and health of

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fish, some results indicate the applicability of probiotics in intensive farming as well. According to Austin *et al*. (1995) the bacteria *Vibrio alginolyticus* was found to reduce diseases in Atlantic salmon (*Salmo salar*) caused by infection of common pathogenic strains (*Aeromonas salmonicida* and *V. anguillarum*) and thus to have a probiotic effect of bacterial strains associated with and skin mucus of adult marine turbot (*Scophthalmus maximus*) and (*Limanda limanda*), and found them to suppress the growth of the fish pathogen *V. anguillarum*. Over time, man and microbe have reached an intricate state of co-existence through mutual adaptation. All warm blooded animals are profoundly dependent on the microbial world. Despite the inclination to regard microorganisms as the enemy, the majority of these tiny life forms favor cohabitation and cooperation not conflict. While some microorganisms are villains, others, termed probiotics, can and do play a very beneficial role in maintaining health. Such probiotic microorganisms mainly consist of lactobacilli, *Enterococcus*, *Lactococci*, and *Bifidobacteria*. Nowdays, a number of preparations are commercially available and introduced to fish and shrimps, molluscans farming as feed. While decaying organic waste matter known as sludge and fuel gas such as ammonia, nitrate and sulphide create serious health problem of fish and reduce the promotion of growth algae. This process reduce the foul odours and increase the dissolved oxygen content of water and reduce the algal blooms and make healthy problem for pond (Sivakumat *et al*., 2009). With these backgrounds, the present study was aimed to investigate the power of probiotics (*Bacillus sp.*) on the growth of *Labeo rohita.*

MATERIALS AND METHODS

Collection and processing of the Sample

The soil samples were collected from in and around Yercaud Geographic coordinate: Latitude: 11° 45’ N / Longitude: 78°.10’ E. Site elevation: 1464.0 M amsl), Salem District, Tamil Nadu, India.

Isolation of Bacterial species

The sterilized plate with nutrient agar medium was prepared and marked with respective dilutions. With the help of sterile pipettes, 0.1 ml of the diluted sample was dispensed on Petri plate rotator and with the help of sterilized L-rod, the sample was evenly spread. The plates were incubated at 37^{o}C for 24 hrs.

Identification of probiotic bacteria by morphological characterization

Gram’s staining

The bacterial isolates were Gram stained according to the procedure Cappuccino and Sherman, (1996).

Biochemical test (KB0002 Hi assorted™ Biochemical kit)

The following biochemical tests were carried out according to the methods described by Cappuccino and Sherman (1991). The biochemical tests were carried out for Citrate utilization, Lysine decarboxylase, Ornithine decarboxylase, Urease, Phenyl alanine deamination, Nitrate reduction, H_2S production, Glucose, Adonitol, Lactose utilization, Arabinose and Sorbitol utilization according to company instruction (Himedia, Mumbai). The isolated organism 50 µl was inoculated into each well in the test kit by surface inoculation method. The kit was incubated at 37^{o}C for 24 hours. The results were observed based on the principle of pH change after substrate utilization. On incubation, organisms undergo metabolic changes which are indicated by a color change in the media that can be either interpreted visually or after the addition of reagent (KB0002 Hi assorted ™ Biochemical kit).

Carbohydrate utilization kit

The carbohydrate tests are based on the principle of pH change and substrate utilization. On incubation, the bacteria undergo metabolic changes which are indicated by a spontaneous color change in the medium. The organisms were analyzed for the utilization of carbon sources like Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Mellibiose, Sucrose, L-Arabinose, Mannose, Inulin, Sodium gluconate, Glycerol, Salicin, Glucosamine, Dulicitol, Inositol, Sorbitol, Mannitol, Adonitol, α-Methyl D-Glucoside, Ribose, Rhamnose, Cellobiose, Melezitose, α-Methyl D-mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malonate and Sorbose (KB009 Hicarbohydrate kit).

The sample (50 µl) was inoculated into the well as provided in the kit and incubation was carried out at 37°C for 24 hrs.

Collection and Acclimatization of test animals

The freshwater fish *L. rohita* weighting about an average of 1.54 ±0.4 g were collected from local fish farm in Mettur reservoir, Salem, Tamil Nadu.

Water quality parameters

Temperature, pH, Dissolved oxygen, Nitrate, Ammonia from fish lank water samples were analyzed once in week.

Examination of fish growth

Total length was measured using steel graduated scale. Fresh weight was taken by weighing the animal in live condition in an electronic balance once in a week. From the data collected, food consumption, mean weight, growth rate, average growth and relative growth were calculated.

RESULTS

The selected bacterium was identified as *bacillus* sp. performed by the following cultural and biochemical characters (Table 1, 2, and 3). The water quality parameters pH ammonia, nitrite (mg/ml), nitrate and dissolved oxygen were analysed (Table 4). The mean pH of 7.2 was noticed in water treated with control feed. CF+MA1 increased the pH value slightly (0.2). In the same way, increased pH of 7.6 was noticed in the remaining treatment groups such as CF+MA2; CF+MA3 and CF+MA3 as evidenced from the table 4. Similarly, ammonia level in the commercial feed was found to be 138 mg/ml. The treatment of CF+MA4 greatly declined the level of dissolved ammonia followed by CF+MA2, CF+MA3 and CF+MA1 were recorded as 80, 106 and 140 mg/ml. It was interesting to note that the increase in the ammonia level in CF+MA1 to 2 g, and the result is on par with the commercial feed. Furthermore, the nitrite level was found to be decreased in CF+MA4 (12.01 mg/ml). The nitrate levels of 30.13 in CF+MA4, 32.8 in CF+MA2, 32.98 in CF+MA1 and 33.08 in CF+MA3. Whereas, the dissolved oxygen level was found to be increased in CF+MA3 and CF+MA4 i.e 5.8 mg/ml and 5.68 mg/ml respectively (Table 4). The fish growth was measured every seven days. The length and weight was assessed. The fish growth was more than control. However, the linear growth of fish was higher in probiotic treated than control fish. The application of water probiotic significantly reduced the levels of ammonia, nitrate and nitrite (Table 5)

Table 1. Cultural characteristics, morphological and biochemical characteristics of *Bacillus sp.*

S. No.	Tests	Observation
	Morphology	
1	Colony property	On nutrient agar, colonies are circular, smooth round, waxy, slight yellow to white and mucoid producers no pigment.
2	Spores staining	Ellipsoidal and cylindrical, central sub terminal, swelling the sporangium.
3	Gram's staining	Gram positive rod.
4	Motility	Motile.
	Biochemical characters	
1	Indole production test	Positive
2	Methyl red test	Positive
3	Voges Proskauer test	Positive
4	Catalase	Positive
5	Gelatin Liquefaction	Positive
6	Casinase	Positive
7	Starch hydrolysis	Positive

Table 2. Biochemical results for *Bacillus sp.b*y KBOO2 Hi Assorted ™ Biochemical Kit.

S. No.	Biochemical Tests	MA1 strain
1	Citrate Utilization	-
2	Lysine decarboxylase	+
3	Ornithine	+
4	Decarboxylase	-
5	Urease	-
6	Phenyl alanine	+
7	Deamination	-
8	Nitrate reduction	+
9	H_2S production	-
10	Glucose	-
11	Adonitol	-
12	Lactose utilization	-
13	Arabinose utilization	-
14	Sorbitol	-

+ (Positive), - (Negative)

Table 3. Carbohydrate fermentation tests for *Bacillus sp.* by KBOO9 Hi Carbohydrate ™ Kit**.**

S.No.	Carbohydrate	MA4 strain
1	Lactose	-
2	Xylose	-
3	Maltose	+
4	Fructose	-
5	Dextrose	+
6	Galactose	-
7	Raffinose	-
8	Trehalose	+
9	Mellibiose	-
10	Sucrose	-
11	L-Arabinose	-
12	Mannose	-
13	Inulin	-
14	Sodium Gluconate	-
15	Glycerol	+
16	Salicin	-
17	Glucosamine	+
18	Dalicitol	-
19	Inositol	-
20	Sorbitol	-
21	Manitol	-
22	Adonitol	-
23	α Methyl D-Glucoside	-
24	Ribose	+

25	Cellobiose	-
26	Melzitose	+
27	α Methyl D-mannoside	-
28	Xylitol	+
29	ONPG	-
30	Esculin	-
31	D-Arabinose	-
32	Citrate	-
33	Malanate	-
34	Sorbose	-

Table 4. Water quality parameters in culture tanks treated with bacterial strains.

S.No	Parameters	CF	Average value			
			CF+MA1	CF+MA2	CF+MA3	CF+MA4
1	pH	7.2	7.4	7.6	7.6	7.6
2	Ammonia (mg/ml)	138	140	80	106	76
3	Nitrite (mg/ml)	12.34	12.66	13.14	12.013	12.01
4	Nitrate (mg/ml)	31.67	32.98	32.8	33.08	30.13
5	Dissolved oxygen (mg/ml)	4.5	4.7	5.6	5.8	5.68

Table 5. Average length and wet weight gain of *Labeo rohita.*

Treatment		Average value					
		Initial	7th day	14th day	21st day	28th day	35th day
Control	Length(cm)	2.5±0.031	2.9±0.01	3.6±0.37	4.1±0.40	4.5±0.42	4.70±0.43
	Weight(g)	15±0.27	17±0.32	18±0.34	18.5±0.36	18.88±0.36	19.01±0.26
CF	Length(cm)	2.5±0.31	3.3±0.36	4.5±0.42	5.5±0.46	5.7±0.47	6.41±0.50
	Weight(g)	15.17±0.27	16.56±0.31	17.75±0.34	19.76±0.38	21.87±0.43	22.60±0.45
CF+MA1	Length(cm)	2.5±0.31	3.5±0.37	3.8±0.38	4.5±0.42	5.1±0.45	5.75±0.47
	Weight(g)	15.78±0.29	16.98±0.32	17.54±0.33	20±0.39	21.98±0.43	22.98±0.45
CF+MA2	Length(cm)	2.5±0.31	3.8±0.38	4.1±0.40	4.56±0.42	5.3±0.46	5.80±0.48
	Weight(g)	15.24±0.28	17±0.32	17.85±0.34	19.87±0.39	20±0.39	21±0.41
CF+MA3	Length(cm)	2.5±0.31	4±0.4	4.5±0.42	4.98±0.44	5.7±0.47	5.9±0.48
	Weight(g)	15.25±0.28	17.68±0.34	17.99±0.34	19±0.37	20.66±0.4	21.76±0.43
CF+MA4	Length(cm)	2.5±0.31	4.56±0.4	4.78±0.19	5±0.44	5.7±0.47	5.9±0.48
	Weight(g)	16±0.30	18±0.34	18±0.34	18.56±0.36	21±0.470.41	21.23±0.42

Values are average of minimum 25 fishes; CF-Commercial feed MA –represent *Bacillus* sp.

DISCUSSION

The present study reveals that the strain MA4 *Bacillus* sp, improving water quality parameters as well as fish growth by bacterial pathogen which is comparable to commercial probiotic strain Lactobacilli, Streptococci, etc. and control also, in addition to that the water probiotics gave good results than feed probiotics. Likewise, Sivakumar *et al*. (2009) and Sharma (1999) stated that the application of water probiotic significantly reduced the levels of ammonia, nitrate, nitrite and dissolve oxygen than feed probiotic application, while bacterial culture incorporated into pellet feed to the fish gave better results not than water probiotic. The strain was Gram positive, rod shaped, endospore forming, and motile bacteria. According to Bergey's manual of Determinative Bacteriology, the selected bacterium were identified and confirmed as *Bacillus cereus*.

In recent years there has been considerable increase in the probiotic in aquaculture. The probiotics were defined as live microbial feed supplement that improve the health of man and

terrestrial livestock (Ghosh *et al*., 2002). In order to overcome fish diseases, scientists have selected certain beneficial microbes, to be used as feed additives. Hjelm *et al*. (2004) coined the term probiotics and defined the term as "organisms and substances which contribute to intestinal microbial balance". Probiotics can also be considered as microbes to improve the nutritive value of an animal feed (Ibrahim *et al*., 2004). Until recently, one of the most frequent procedures used to avoid the establishment of undesirable bacteria in a target organism was the administration of antibiotics in the water. In the present study, bacteria isolated from medicinal plants showed higher seed germination efficiency and also had antagonistic activity against fish pathogenic bacteria. Most efficient bacteria were identified as *Bacillus* sp. *Bacillus* is known to improve seed germination and plant growth in several crop plants (Irianto and Austin, 2003). In addition, *Bacillus* sp. is also producing siderosphores, growth hormones, enzymes and organic acids that enhance the growth of agricultural crop plants.

The selection of probiotic bacteria was usually based on their antagonistic activity against pathogens. Our potential bacterial stain was antagonistic to *Vibrio harvei* that was isolated from fish intestine (Jack *et al.,* 1994). A total of nine isolates were tested against *V. herveyi*, among these only one strain inhibited the growth of pathogen at higher level than other strains. Jones and Hoffer (2002) and her co-workers found that *Bacillus* produces bacteriocins, siderophores, lysozymes, proteases, hydrogen perioxides that are inhibiting the pathogenic microbes. In aquaculture practice, water quality deteriorates mainly due to accumulation of metabolic wastes. In this work *Bacillus* sp improved the water quality parameters and also reduced pathogenic bacteria load at significant level than the commercial strain *Lactobacillus* sp. This could be due to the degradation of organic matter facilitates nutrients recycling and competes with other pathogenic bacteria (Sanders *et al*., 2003). This is true in our study also. But, compared to Bacillus, *Lactobacillus* improved the health of fishes to some extent but water quality parameters were much improved than control.

In present study the water quality reduces higher in water probiotic than feed probiotic. Because water probiotic are directly applied to the tank culture, those reducing organic matters load. *Bacillus* sp. of bacteria is reported to more efficiently improve water quality. *Bacillus* also reduced the quantity of ammonia, nitrite in the water (Skjermo and Vadstein, 1999). In the present study, application of water probiotic significantly reduced the levels of ammonia, nitrite, and nitrate than feed probiotic application.

Bacterial culture incorporated into pellet feed gave good result but not comparatively better than water probiotic. Because, probiotic bacteria such as Lactic acid bacteria, *Streptococcus*, *Saccharomyces* showed enhanced survival, growth and immunity of fish (Sugita *et al*., 1996). *Bacillus* sp improved water quality parameters as well as fish growth by reducing bacterial pathogens, which is comparable to commercial strain of *Lactobacillus* and control. In addition, application of water probiotics gave good result than feed probiotics. The best performance of fish in terms of growth performance and feed utilization efficiency was recorded at the bacterial supplementation of 2×105 CFU/g in diet. Probiotic diet supplementation resulted in better growth performance and feed utilization than in controls.

The nutritive value of *Daphnia* meal as an alternate protein source has been demonstrated. Similar results were reported by Ghosh *et al.* (2003) and Bairagi *et al.* (2004) about Indian carp. Yanbo and Zirong (2006) suggested that the addition of probiotic reduced the culture cost of fishes in cultivation systems which were confirmed by our results. Probiotics induce useful microflora into larval intestine and cause high growth performance. *Daphnia* meal was supplemented with probiotic to increase its efficiency Similar results were reported by Bairagi *et al.* (2002), using intestinal bacterial strains (*Bacillus* spp.) for fermentation of duckweed (*Lemna polyrhiza*) leaf meal for feeding of *L. rohita*. In their study, the growth and feed utilization efficiency of rohu were higher than the control group and similar results were observed regarding *Daphnia* meal in our study. Our results indicate the suitable concentration of probiotic in this experiment, for bacterial supplementation of diet was 2×105 CFU/g. Similarly, Ghosh *et al.* (2003) indicated that *Bacillus circulans* supplementation in diets of rohu had the best growth performance at about 2×105 cells per 100 g of feed. Using *Bacillus subtilis* and *Bacillus circulans* in formulated

diets for rohu fingerlings led to an increase in PER (Protein Energy Ratio) and LER, and a decrease in FCR in experimental treatments (Bairagi *et al*., 2004).

CONCLUSION

In conclusion, different levels of probiotics *Bacillus* resulted in different performance. The experiments showed that the probiotic *Bacillus* highly increase the growth performances and feeding efficiency selected freshwater fish fingerlings of *L. rohita.*

ACKNOWLEDGEMENT

The authors are thankful to the head of the institution, Government Arts College (Autonomous), Salem for providing the necessary facilities.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this article.

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