



## Probiotic effect of *Bacillus* species isolates on *Poecilia reticulata* Peters, 1859 (Guppy) and water quality

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### Abstract

The present work was carried out to isolate, identify, and characterize in *Bacillus* spp. native bacteria for probiotic activity from various soil samples collected from two townships of Dagon Myothit in Yangon Region. A total of 25 isolates of the genus *Bacillus* were obtained from soil samples collected sites. A total of 17 *Bacillus* isolates were identified down to four species, but the remaining eight isolates could only be identified to genus level. The four species identified were *Bacillus megaterium*, *Bacillus firmus*, *Bacillus pumilus* and *Bacillus larvae*. *Bacillus megaterium* (32%) and *Bacillus firmus* (24%) were most abundant in the studied soil samples. The remaining *Bacillus* spp. isolates (32%) could not be identified to species level. Two strains of isolates *Bacillus megaterium* and *Bacillus firmus* with high growth were used as probiotic strains and tested for probiotic effect on aquarium fish *Poecilia reticulata* (Guppy). After 21 days of fish culture with and without probiotics, dissolved oxygen (DO), ammonia nitrogen ( $\text{NH}_3\text{-N}$ ) were at improved levels in treated water with 100% survival of the guppies ( $n=25$ ). Nitrite nitrogen ( $\text{NO}_2\text{-N}$ ) increased in probiotic treated water. Viable cell count (CFU /mL) also increased in treated water might be due to growth of added probiotics. Hence, the studied two strains of *Bacillus megaterium* and *Bacillus firmus* could be suitable for probiotic treatment of water.

## Introduction

Bacteria are the most abundant microorganisms in the soil :  $6 \times 10^8$  cells per gram of soil is commonly observed. *Bacillus* species are the most common type of bacteria isolated from soil samples (Darbyshire and Greaves, 1973).

Most *Bacillus* spp. are effective degraders of biopolymers (i.e. proteins, starch, pectin etc). They play a significant role in the biological cycles of carbon and nitrogen. *Bacillus* also produce antibiotics against Gram-negative pathogens and fungi. In addition, some species such as *B. macerans* and *B. polymyxa* are nitrogen (N<sub>2</sub>) – fixers anaerobically and *B. firmus* and *B. circularis* are phosphate solubilizers providing nutrients to plants (Schippens, 1992, Hallmann *et al.*, 1998). Moreover, *B. thuringiensis* and *B. sphaericus* are reported to be effective bioinsecticides against insect pests of crop plants (Brown, 2001). Furthermore, many species including *B. subtilis*, are currently in use as probiotics to improve water quality and health of fish and prawns in aquaculture. (Motsara *et al.*, 1995)

Probiotics have been defined as microbial cell preparations, that when administered in adequate amounts, have beneficial effects on the health and well being of the host (Panigrahi and Azad, 2007). Probiotics are also reported to improve fish health by improving water quality (i.e dissolved oxygen, pH, nitrogen content etc.) and reducing pathogen load in aquaculture (Hong *et al.* 2005).

Seventy percent of protein, in the diet of people of Myanmar, is from freshwater fishes. Hence, aquaculture industry for fish and prawns is an important economic sector in our country, and the ever increasing demand for fish has led to search for means to improve yield (Sann Aung, 2009).

The present study was, therefore, conducted with the aim to search and test native soil bacteria with the following objectives: (i) to isolate and identify *Bacillus* spp. from different soil samples, (ii) to select from among the isolates and test them as probiotics for water quality and fish health improvement.

## Materials and Methods

**Study and sampling sites:** soil samples (500 g each) from 15 cm depth were collected from different sites in East and North Dagon Townships in Yangon Region.

**Study period:** research lasted from October, 2014 to March, 2015.

**Materials:** standard microbiological chemicals, and equipment, glassware were utilized. Nutrient agar (NA), Plate count agar (PCA), Bacillus agar, LB broth and nutrients broth were used as culture media. SIM, TSI, Indole, MR, VP, citrate as well as oxidase, catalase, urease, gelatinase tests were used for identification. All chemicals, reagents and media were purchased from Himedia, India through AMD Co. Ltd in Yangon.

**Methods:** firstly, soil sample (1 g each) was dissolved in distilled water (1 ml) and centrifuged (3000 rpm, 1 min). Supernatant (1 ml) was diluted to  $10^{-3}$  level and each dilution (25  $\mu$ l) was spread on nutrient agar (NA) and plate count agar (PCA) and incubated at 35°C overnight. Total viable counts were made on PCA for CFU/g. Subculture were made from colonies on NA and studied for colony and cell morphology as well as for biochemical characteristics for species identification of genus *Bacillus*.

Two isolates of *Bacillus* spp. were selected as probiotics and tested on water quality and guppy fish (*Poecilia reticulata*) in aquarium tanks for one month duration. Fish survival and water quality of pH, DO (dissolved oxygen), NH<sub>3</sub>-N (nitrate nitrogen) were monitored in treated tank and compared to control tank.

## Results

A total of 25 isolates of *Bacillus* spp. were obtained and were found to be Gram-positive rods having creamy white colonies. Biochemical tests identified four isolates as *Bacillus megaterium*, *Bacillus larvae*, *Bacillus firmus* and *Bacillus pumilus*. Total viable cell counts ranged from  $0.15 \times 10^2$  CFU/mL to  $10.02 \times 10^5$  CFU/mL (Tables 1 and 2).

Two identified species of *Bacillus megaterium* (isolate code SIb), and *Bacillus larvae* (isolate code S VIII a) were selected as probiotics for their high growth at  $10^5$  CFU/mL levels on *Bacillus* agar at 35°C (Plate 1).

*B. megaterium* and *B. larvae* at 1:1 ratio were mixed in nutrient broth cultures and inoculated at  $10^5$  CFU/mL dosage in a total volume of 10 ml on day 7 and day 14 of guppy culture (n=25) in aquarium Tank II. Tank I was control without probiotic treatment but had guppy (n=25) in the same volume of 64 litres water. Fish survival, water parameters and cell counts were monitored during the experiments. The results are shown in Table 3 and Figs. 1 and 2).

Table 1. Biochemical characteristics of identified isolates

Sr. No.	Species (Isolate Tests code)		<i>Bacillus megaterium</i>	<i>Bacillus pumilus</i>	<i>Bacillus firmus</i>	<i>Bacillus larvae</i>
			S I b	S II b	S II d	S VIII a
1.	Gram reaction		+	+	+	+
2.	Indole test		-	-	-	-
3.	Oxidase test		-	-	-	-
4.	Catalase test		+	+	+	-
5.	Gelatin liquefaction		-	-	-	-
6.	Methyl Red test		+	+	+	-
7.	Voges-Proskauer		-	-	-	-
8.	Utilization of citrate		-	-	-	-
9.	Urease test		+	+	-	+
10.	Nutrient agar (growth)		+	+	+	+
11.	<i>Bacillus</i> agar (growth)		+	+	+	+
12.	TSI	Slant/bult	A/A	K/A	K/A	A/A
		H <sub>2</sub> S gas	-	-	-	-
		Gas production	-	-	-	-

Table 2. Growth of identified isolates on Bacillus differentiation agar at 35° C overnight

Sr . No	Isolate		Total count CFU/ mL	Log value
	Isolate code	Scientific name		
1.	SI a	<i>Bacillus megaterium</i>	0.88 x 10 <sup>5</sup>	4.94
2.	SI b	<i>Bacillus megaterium</i> *	10.02 x 10 <sup>5</sup>	6.00
3.	SI c	<i>Bacillus</i> sp.	0.95 x 10 <sup>5</sup>	4.95
4.	SII a	<i>Bacillus firmus</i>	0.18 x 10 <sup>2</sup>	1.25
5.	SII b	<i>Bacillus pumilus</i>	0.26 x 10 <sup>2</sup>	1.41
6.	SII c	<i>Bacillus megaterium</i>	0.24 x 10 <sup>2</sup>	1.38
7.	SII d	<i>Bacillus firmus</i> *	0.92 x 10 <sup>2</sup>	4.96
8.	S III a	<i>Bacillus megaterium</i>	0.79 x 10 <sup>5</sup>	4.87
9.	S III b	<i>Bacillus</i> sp.	0.21 x 10 <sup>5</sup>	4.32
10.	S III c	<i>Bacillus larvae</i>	0.11 x 10 <sup>5</sup>	4.04
11.	S III d	<i>Bacillus firmus</i>	0.48 x 10 <sup>5</sup>	4.68
12.	SIII e	<i>Bacillus</i> sp.	0.19 x 10 <sup>5</sup>	4.27
13.	SIII f	<i>Bacillus megaterium</i>	0.45 x 10 <sup>5</sup>	4.65
14.	SIV a	<i>Bacillus</i> sp.	0.91 x 10 <sup>2</sup>	1.95
15.	SIV b	<i>Bacillus</i> sp.	1.10 x 10 <sup>2</sup>	2.04
16.	SIV c	<i>Bacillus megaterium</i>	0.89 x 10 <sup>2</sup>	1.94
17.	SIV d	<i>Bacillus firmus</i>	0.15 x 10 <sup>2</sup>	1.17
18.	SIV e	<i>Bacillus</i> sp.	0.47 x 10 <sup>5</sup>	4.67
19.	S V a	<i>Bacillus</i> sp.	0.59 x 10 <sup>5</sup>	4.77
20.	S V b	<i>Bacillus megaterium</i>	0.48 x 10 <sup>5</sup>	4.68
21.	S V c	<i>Bacillus firmus</i>	0.19 x 10 <sup>4</sup>	3.27
22.	SVI a	<i>Bacillus</i> sp.	0.18 x 10 <sup>4</sup>	3.25
23.	SVI b	<i>Bacillus megaterium</i>	0.19 x 10 <sup>4</sup>	3.27
24.	SVII a	<i>Bacillus firmus</i>	0.12 x 10 <sup>4</sup>	3.07
25.	SVIII a	<i>Bacillus larvae</i>	0.54 x 10 <sup>5</sup>	4.73

Table 3. Recorded water parameters and number of fish in Tank I and Tank II during the test of probiotics.

Waters Parameters (mg/L)	Day 1		Day 7		Day 14		Day 21		Remarks
	T1	T2	T1	T2	T1	T2	T1	T2	
DO	4.0	4.0	4.0	4.5	5.5	5.5	5.5	4.5	Lower in Tank 2
NH <sub>3</sub> -N	0.06	0.06	0.02	0.06	0.02	0.03	0.09	0.04	Much lower in Tank 2
NO <sub>2</sub> -N	-	-	0.13	0.19	0.01	0.01	0.01	0.02	Increase in Tank 2
NO <sub>3</sub> -N	-	-	-	-	-	-	-	-	Not detected
pH	8.0	8.0	8.5	8.0	8.0	8.0	8.0	8.0	slight change
Temperature (C°)	24	25	26	26	25	25	26	26	Similar
No. of fish surviving	25	25	25	25	25	25	20	25	100 % survival in Tank 2

-, not detected

T<sub>1</sub>, Tank I (Control) T<sub>2</sub>, Tank II (Treated)



A . Colonies of *Bacillus megaterium*



B. *Bacillus megaterium* cells  
(1000 ×)



C. Colonies of *Bacillus larvae*



D. *Bacillus larvaecells*  
(1000 ×)

Plate 1 Colonial and cell morphology of selected probiotic *Bacillus* isolates on *Bacillus* differentiation agar (35°C)

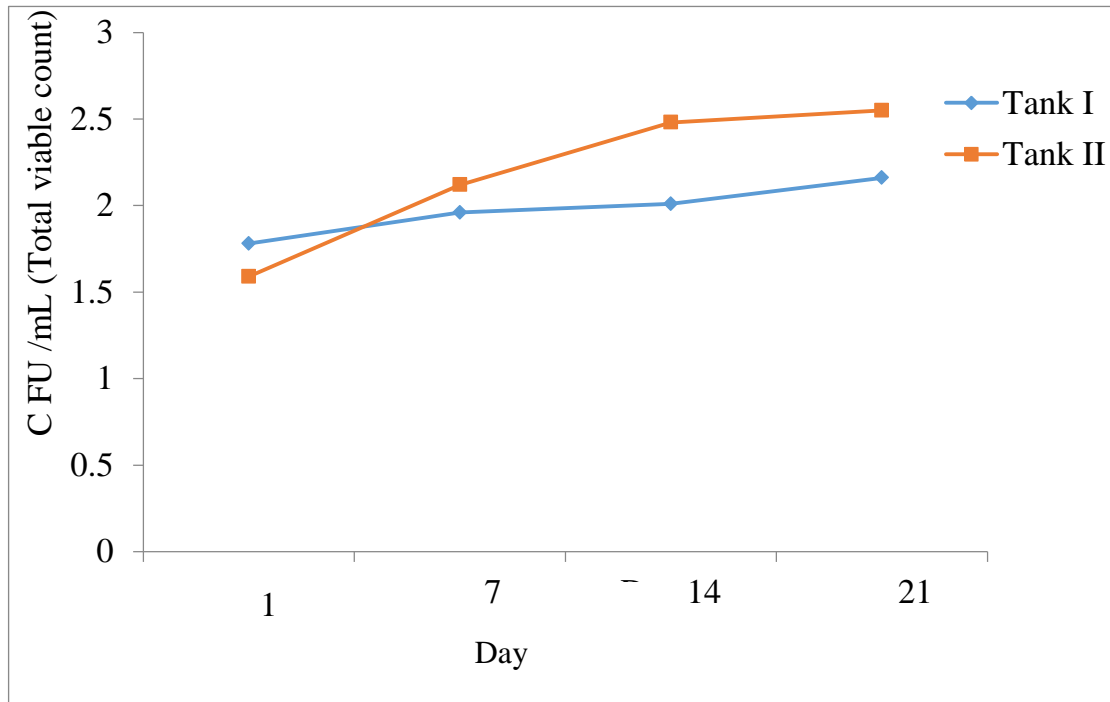
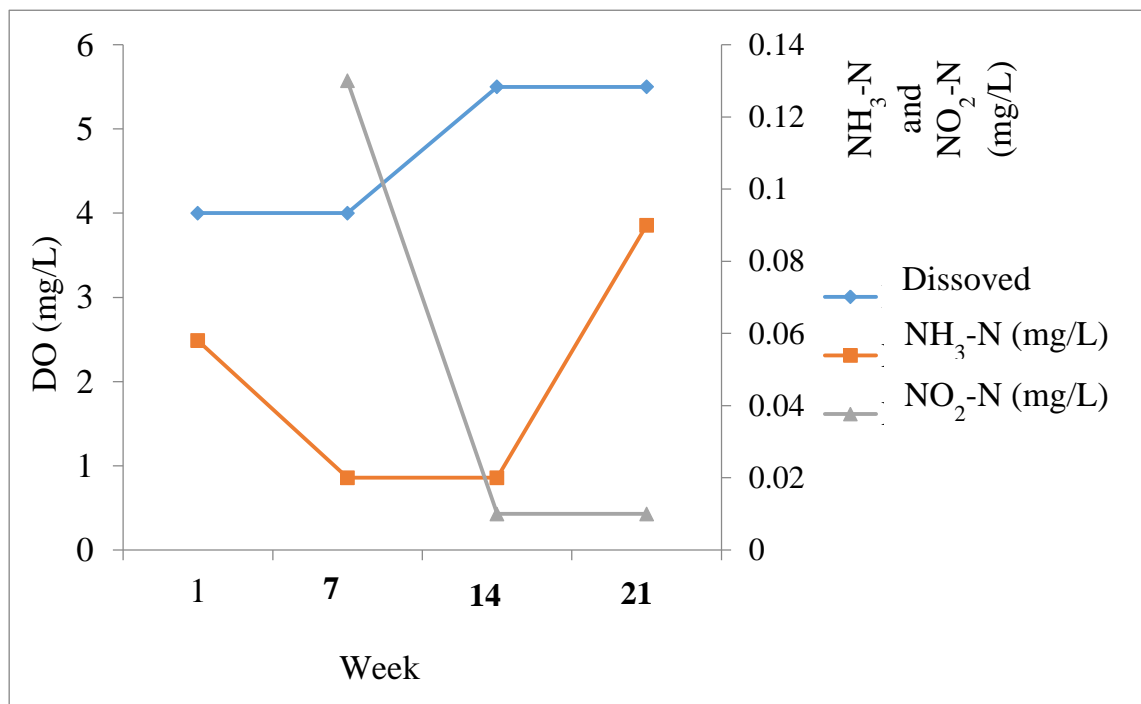
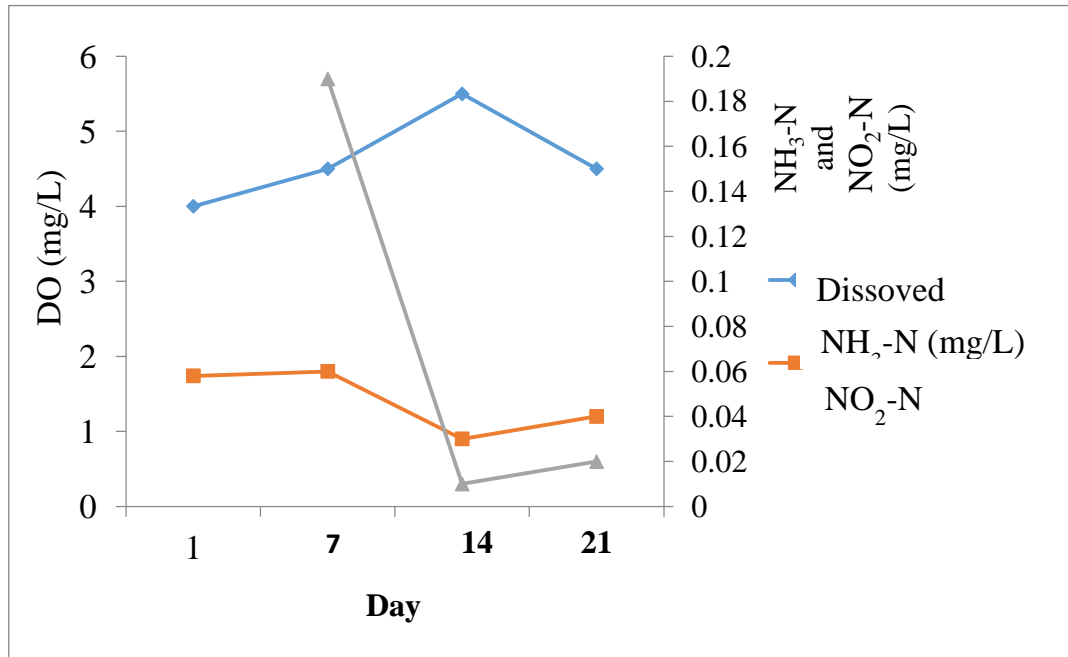


Fig. 1 Bacteria population changes without probiotics in Tank I and with probiotics in Tank II



A. Water quality of tank I (control)



B. Water quality of tank II (treated)

Fig 2 Variation in some water quality parameters in control and treated tanks during guppy culture

## Discussion

The present work was an effort to cultivate local or native bacteria of genus *Bacillus*, from soil of various sites, to be tested as probiotics for treatment of aquarium fish health and water quality.

Four species of *Bacillus*, namely *B. megaterium*, *B. larvae*, *B. fimus* and *B. pumilus* were isolated and identified from among the 25 isolates obtained in the present study.

Growth studies on *Bacillus* differentiation agar at 35°C revealed that *B. megaterium* isolate and *B. larvae* isolate had high growth at  $10^5$  CFU/mL levels. Hence, the latter two species were selected as probiotics and used in treating guppy fish (*Poecilia reticulata*) culture and its water quality in the present study.

Probiotic inoculant in water were reported to enter fish by mouth into their intestines (Planas *et al.*, 2004). Probiotics may serve as dietary supplements to improve the intestinal microbial balance of fish (Irianto and Austin, 2002). Probiotic actions include competitive exclusion of pathogenic bacteria, direct uptake of dissolved organic waste of fish, and digestion of other organic material by extracellular enzymes in water (Balcazar *et al.*, 2006).

In the present study, there was a reduction of ammonia nitrogen ( $\text{NH}_3\text{-N}$ ) in probiotic treated tank 2, whereas  $\text{NH}_3\text{-N}$  rose considerably in control tank 1. The  $\text{NH}_3\text{-N}$  is product of fish faeces .

On the other hand DO (dissolved oxygen) was slightly decreased in treated tank 2 but it was still at good level. similarly, pH and nitrite nitrogen did not changed much in treated tank 2. And guppy survival was 100% (n=25) after 21 days of treatment, but it was only 80% in control tank.

Overall results indicated that the probiotic strains tested in the present work could be suitable for application in freshwater fish cultures.

### **Conclusion**

Overall results indicated that the two soil isolates of *Bacillus megaterium* and *B. larvae* are promising strains as probiotics that have beneficial effects on fish survival and reducing ammonia nitrogen from fish faecal waste in the aquarium water.

### **Suggestions for future work**

The present work regarding the two probiotics species of *B. megaterium* and *B. larvae* should be studied further for the presence of genes with beneficial effects on water quality, and fish survival.

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