Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 10 (6): 230-245, 2015
ISSN 1816-4935 / DOI: 10.3923/jm.2015.230.245
© 2015 Academic Journals Inc.

Effect of Pond Fertilization with Vermicompost and Some Other Manures on the Pathogenic Bacterial Populations of Treated Waters

[1]Sunita Godara, [2]Ram C. Sihag and [1]Rajender K. Gupta
[1]Laboratory of Fish Biology and Aquaculture,
[2]Laboratory of Animal Behaviour and Simulated Ecology, Department of Zoology, CCS Haryana Agricultural University, Hisar, 125004, India

Corresponding Author: Sunita Godara, Laboratory of Fish Biology and Aquaculture, Department of Zoology, CCS Haryana Agricultural University, Hisar, 125004, India

ABSTRACT

Use of organic fertilizers has become very common in the modern piscicultural practices; vermicompost is a new addition to the list of such fertilizers. The efficacy of vermicompost was better than the other fertilizers both in keeping the hydro-biological parameters of treated waters in favourable ranges as well as in maximizing the fish growth. However, its effect on the pathogenic bacterial profile of treated waters is not known. To accomplish this objective, an experiment was performed in 5.54×6.15 m size ponds stocked with three species of Indian major carps viz. catla (*Catla catla* Ham.), rohu (*Labeo rohita* Ham.) and mrigal (*Cirrhinus mrigala* Ham.) at 30 fish per pond in 3:4:3 ratios, respectively. Six treatments viz. a control without any treatment (T_1), pig manure at 4,000 kg ha^{-1} $year^{-1}$ (T_2), poultry manure at 6,000 kg ha^{-1} $year^{-1}$ (T_3), cow dung at 10,000 kg ha^{-1} $year^{-1}$ (T_4), vermicompost at 10,000 kg ha^{-1} $year^{-1}$ (T_5) and vermicompost at 15,000 kg ha^{-1} $year^{-1}$ (T_6) were used to monitor their effect on the pathogenic bacterial populations in the treated pond waters. One fourth doses of fertilizers were applied 15 days prior to the fish stocking and the remaining doses were given at fortnightly intervals; the supplementary feed was given at 2% of the body weight of fishes. The pure culture of bacterial isolates segregated from the pond sediments were identified by primary, secondary and tertiary tests and confirmed for their pathogenicity through *in vitro* and *in vivo* tests. Overall, seven gram negative pathogenic bacterial strains (viz. *Aeromonas hydrophila, Escherichia coli*, *Enterobacter aerogens*, *Pseudomonas fluorescens*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Shigella* sp.) and three gram positive strains (viz. *Micrococcus luteus, Staphylococcus aureus* and *Staphylococcus* sp.) were isolated from the pond waters treated with different manures. The *in vitro* and *in vivo* tests confirmed the pathogenic nature of these bacteria. The abundances of the heterotrophic pathogenic bacteria were minimal in the pond waters treated with vermicompost at 10,000 kg ha^{-1} $year^{-1}$ followed by those treated with vermicompost at 15,000 kg ha^{-1} $year^{-1}$; the abundances of pathogenic bacteria were higher in waters treated with other fertilizers including under the control treatment. Under other than these two vermicompost treatments, the abundances of pathogenic bacteria were variable depending upon the bacterium-fertilizer type. On the basis of these results, vermicompost at 10,000 kg ha^{-1} $year^{-1}$ seemed to be the best among the five organic fertilizer treatments in controlling the abundance of pathogenic bacteria in the treated pond waters.

Key words: Organic manures, heterotrophic pathogenic bacteria, vermicompost, pond fertilization

INTRODUCTION

The fast increasing world population has presented complex challenges before the policy makers and the managers in the governments and the food producers in the fields; the most important challenge is the food shortage and malnutrition. More than half of the world population depends upon fish as a source of animal protein. Fish flesh is easily digestible and nutritionally better than the beef or mutton in quality. It contains all the essential amino acids and minerals i.e., iodine, phosphorus, potassium, iron, copper and vitamin A and D in desirable concentrations (Hussain, 1986; Abbas *et al*., 2010). Fish flesh has low carbohydrate and unsaturated fat contents. Thus, fish flesh serves as a valuable ingredient to a healthy diet. In fact, any diet that contains mainly cereals, starchy roots and sugar can be made nutritionally perfect for healthy growth by inclusion of fish in it (Choo and Williams, 2003; Sandhu, 2005; Yildirim, 2008). Due to an excellent source of omega-3 fatty acid, fish flesh is often recommended by the doctors to the heart patients. Beside this, fisheries sector provides vast and diverse employment opportunities to a large section of the society. Therefore, fisheries sector is very important not only as a main source of animal protein to ensure the food security (Sheikh and Sheikh, 2004) but also to improve the employment and income opportunities for poverty elimination, especially in the developing countries.

In the recent years, utilization of animal wastes for pond fertilization has become a very common practice in the modern aquaculture systems (Dhawan and Kaur, 2002a, b; Hossain *et al*., 2003; Jha *et al*., 2004). However, this practice has caused noticeable harm to the environment by proliferating growth of pathogenic bacteria like *Aeromonas* sp. and *Pseudomonas* sp. in the water bodies (Hojovec, 1977; Sugita *et al*., 1985; Jinyi *et al*., 1987; Quines, 1988). Freshwater fish in Indian ponds and other water bodies across the globe commonly suffer from bacterial diseases such as various kinds of skin ulcerations, albinoderma, erythroderma, furunculosis and verticle-scale disease, primarily caused by *Aeromonas* sp. and *Pseudomonas* sp. (Das, 2004; Sihag and Sharma, 2012). Among the various practices, depending upon the variable inputs, semi-intensive carp culture practices in rural aquaculture involve utilization of various organic manures for plankton (natural food) production. These manures are either directly utilized by the fish or they enrich the aquatic ecosystem comprising of autotrophic (plankton) and heterotrophic microbial communities (Schroeder, 1987; Muendo *et al*., 2006). Nearly half of the fish currently consumed as food worldwide are raised in fish ponds rather than caught in the wild. In most of the situations, cultured fish remain healthy even in the continuous presence of pathogens. However, when environmental stresses occur and the balance shifts in favor of the disease, the characteristic pathogens flourish. Due to the outbreak of disease in aquaculture industry, use of antibiotic has led to the development of drug-resistant strains resulting in reduction of natural defense mechanism in the aquacultural animals (Sihag and Sharma, 2012).

Many organic fertilizers are being used for pond fertilization (Dhawan and Kaur, 2002a, b; Hossain *et al*., 2003; Jha *et al*., 2004); vermicompost is the new addition to this pool which not only promotes the fast growth of fishes (Godara *et al*., 2015a) but also keeps the aquatic environment (hydro-biological parameters) congenial for their growth (Godara *et al*., 2015b). Vermicompost is a product of vermi-biotechnology that is frequently used in agro-ecosystems as organic fertilizer (Saini *et al*., 2008a, b, 2010). The advantage of use of vermicompost as organic fertilizer is the quick availability of nutrients in 'ready-to-uptake' forms (Nath and Lannan, 1992). In our earlier studies, we investigated the effect of vermicompost as pond fertilizer on fish growth (Godara *et al*., 2015a) and hydro-biological parameters of the treated waters (Godara *et al*., 2015b). However, what will be the effect of vermicompost vis-à-vis other animal based fertilizers on the heterotrophic

pathogenic bacterial profiles of the treated waters is not known. The present study is an attempt at finding a solution to this problem of paramount importance.

MATERIALS AND METHODS

Experimental design: This study was carried out at the Fish Farm and Fisheries Biology Laboratory of the Department of Zoology in Chaudhary Charan Singh Haryana Agricultural University, Hisar, India. The experiment was performed in fish ponds each with a size of 5.54×6.15 m from September, 2011 to August, 2012 (Godara *et al*., 2015a). The experimental ponds, after cleaning and application of lime at 200 kg ha^{-1} $year^{-1}$, were filled with inland ground water obtained from the deep tube wells and were allowed to stabilize for about 15 days. Three fish species of Indian major carps viz. catla (*Catla catla* Ham.)- a surface feeder, rohu (*Labeo rohita* Ham.)- a column feeder and mrigal (*Cirrhinus mrigala* Ham.)- a bottom feeder, commonly used for composite fish culture in India, were selected for this experiment. The water level throughout the experiment was maintained at 1,54 m as described by Godara *et al*. (2015a).

Treatments and pond fertilization: In this experiment, there were six different treatments each with four replications; the pond devoid of any fertilizer acted as the control (T_1). To fertilize the ponds, other five treatments included semi dried pig manure applied at 4,000 kg ha^{-1} $year^{-1}$ (T_2), poultry manure at 6,000 kg ha^{-1} $year^{-1}$ (T_3), cow dung at 10,000 kg ha^{-1} $year^{-1}$ (T_4), vermicompost at 10,000 kg ha^{-1} $year^{-1}$ (T_5) and vermicompost at 15,000 kg ha^{-1} $year^{-1}$ (T_6). In the beginning of the experiment, initial dose equal to 25% of the total amount of the manure type was applied and remaining amount was given in equal split doses at fortnightly intervals. Along with organic fertilizers, a supplementary diet was also added. The ingredients of supplementary diet, method of its preparation and provisioning have already been described by Godara *et al*. (2015a). The different ingredients and their relative proportions were: 35 parts fish meal (contributing 18% protein), 30 parts soybean (contributing 12.6% protein), 18 parts mustard oil cake (contributing 5.4% protein), 10 parts rice bran (contributing 1.3% protein), 6 parts wheat flour (contributing 0.6% protein) and 1 part salt+vitamins and mineral premix. Thus, the artificial diet contained about 38% (37.9%) protein.

Collection of bacterial material from the treated ponds: For bacteriological analysis, sediment samples were collected from the treated ponds after 30 days intervals in pre-sterilized glass bottles and processed within 6 h of collection. The suspension of sediment was prepared by mixing 1 mL of wet sediment in 99 mL of sterile distilled water. The analysis of bacterial samples was done in the laboratory.

Culture of collected bacteria: The water sediment samples taken from the ponds treated with different fertilizers were cultured in the Nutrient Agar (NA) medium by the conventional spread plate method (Fig. 1). For this, the sample was spread over the nutrient agar medium under aseptic conditions. The plates were incubated in B.O.D at 30±2°C for 18-24 h. Growth of bacteria on the nutrient agar plates was observed in all the treatments after 18-24 h. Pure colonies of bacteria were obtained by further sub-culturing the single colonies on nutrient agar plates. The samples were kept under laboratory conditions for isolation and identification of bacterial strains.

Identification of bacteria collected from the treated ponds: Isolated pure cultures were subjected to a number of primary and secondary biochemical tests (Krieg and Holt, 1984;

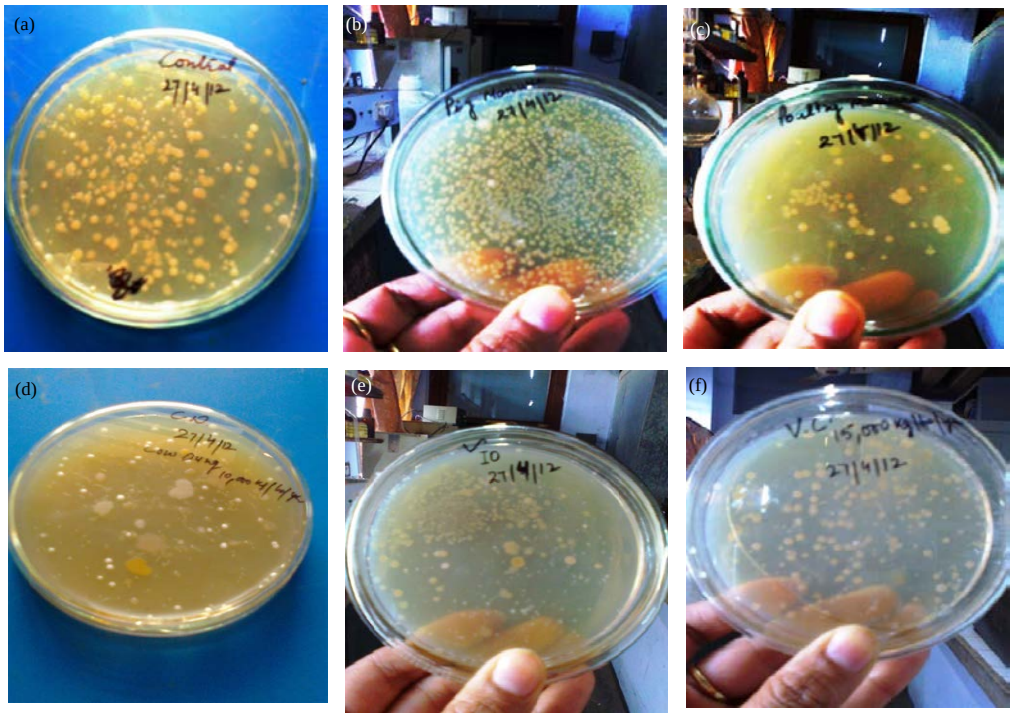

Fig. 1(a-f): Culture of bacteria raised from the sediments taken from the ponds treated with different fertilizers, (a) Control, (b) Pig manure, (c) Poultry manure, (d) Cow dung, (e) Vermicompost at 10,000 kg ha^{-1} $year^{-1}$ and (f) Vermicompost at 10,000 kg ha^{-1} $year^{-1}$

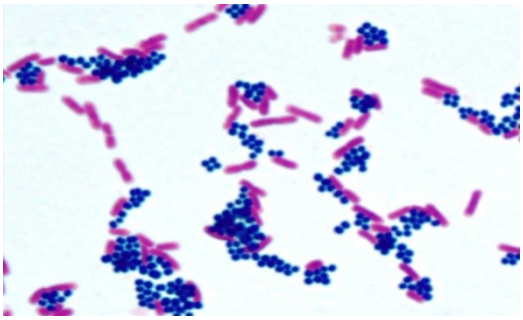

Fig. 2: Gram positive(blue), cocci shaped bacteria and gram negative (pink), rod shaped bacteria

Jakhar *et al*., 2010). For the primary classification and identification, bacterial shape and gram reaction were studied (Fig. 2 and 3 and Table 1-3). For the secondary and tentative identification several biochemical tests were performed (Fig. 3 and 4 and Table 1-3). On the basis of these tests, tentative identification of the bacteria was done with the help of a computer programme named PIBWin (Bryant, 2004). This programme enabled us to derive the ID scores of each bacterial isolate. These ID scores were then compared with the model scores and the bacterium present in the given isolate was tentatively identified (Table 1-3). The identification of each bacterial species

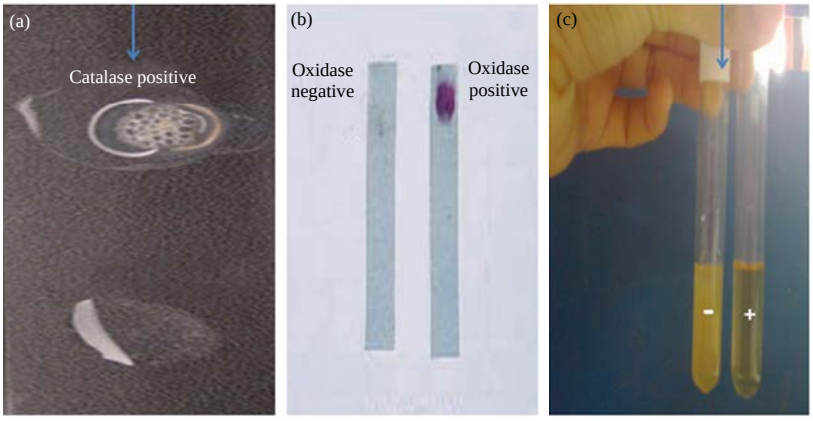

Fig. 3(a-c): (a) Catalase and (b) Oxidase producing bacteria gave positive reactions in these tests and (c) Growth in nutrient broth shows whether the bacterium is an aerobic, anaerobic or facultative

Table 1: Tentative identification of bacteria isolated from ponds treated with different manures*

Biochemical tests	Bacterial isolates 1-6	Bacterial isolates7-12	Bacterial isolates 13-18	Bacterial isolates 19-24
Gram reaction	-	-	-	-
Shape	Rods	Rods	Rods	Rods
Colour of colony	White	White	White	Cream
Aerobic	+	+	+	+
Anaerobic	-	+	-	-
Catalase	+	+	+	+
Oxidase	+	-	-	+
Glucose fermentation	+	+	+	-
Urease	-	-	-	-
Simmon citrate	-	-	+	+
Starch hydrolysis	-	-	-	-
Ehrlich indole	+	+	-	-
Nitrate-Nitrite	+	+	+	-
Adonitol	-	-	+	-
Xylose	-	+	+	+
Glucose acid	+	+	+	-
Sucrose	+	-	+	-
Sorbitol	-	+	+	-
Malonate	-	-	+	-
Arginine dihyrolase	+	-	-	+
Lactose	-	+	+	-
Maltose	+	+	+	-
Mannitol	+	+	+	+
Cellobiose	-	-	+	-
Glycerol	+	+	+	+
Inositol	-	-	+	+
Methyl red 37°C	+	+	-	-
Voges Proskauer 37°C	-	-	-	-
Growth at 37°C	+	+	+	+
Bacterial identified	*Aeromonas hydrophila*	*Escherichia coli*	*Enterobacter aerogenes*	*Pseudomonas fluorescens*
ID score	0.99219	0.99987	0.95521	0.99595
ID Model score	1.00000	1.00000	1.00000	1.00000

+: Showed growth, -: Showed no growth, *for the convenience, original numbers were re-designated

was then confirmed by growing the isolated bacterium on the specific medium (Fig. 5 and Table 4). Only those bacterial cultures were retained for further testing which have already been reported as heterotrophic pathogens.

Table 2: Tentative identification of bacteria isolated from ponds treated with different manures*

Biochemical tests	Bacterial isolates 25-30	Bacterial isolates 31-36	Bacterial isolates 37-42
Gram reaction	-	-	-
Shape	Rods	Rods	Rod
Colour of colony	Cream	Cream	Orange
Aerobic	+	+	-
Anaerobic	-	-	+
Catalase	+	+	+
Oxidase	-	+	-
Glucose fermentation	-	-	-
Urease	+	+	-
Simmon citrate	+	+	-
Starch hydrolysis	+	-	-
Enrlich indole	+	-	+
Nitrate-Nitrite	+	+	+
Adonitol	+	-	-
Xylose	+	+	-
Glucose acid	+	-	+
Sucrose	+	-	-
Sorbitol	+	-	+
Malonate	+	+	-
Arginine dihydrolase	-	+	-
Lactose	+	-	-
Maltose	+	+	-
Mannitol	+	+	+
Cellobiose	+	-	-
Glycerol	+	+	-
Inositol	+	-	-
Methyl red 37°C	+	-	+
Voges Proskauer 37°C	-	-	-
Growth at 37°C	+	+	+
Bacterial identification	*Klebsiella oxytoca*	*Pseudomonas aeruginosa*	*Shigella* spp.
ID score	0.96612	0.99753	0.99701
ID model score	1.00000	1.00000	1.00105

+: Showed growth, -: Showed no growth, *for the convenience, original numbers were re-designated

Testing of isolated bacteria for their pathogenicity: Whether an isolated bacterium type is a pathogen or not, was confirmed by the *in vitro* and *in vivo* pathogenicity tests.

***In vitro* pathogenicity test:** *In vitro* pathogenicity test of different bacteria was done by streaking pure culture of isolated bacteria on blood (of three species of carps) agar plates. The plates were incubated in B.O.D. at 30-20°C for 24 h and pathogenicity of bacteria was confirmed by determining α-β zones of haemolysis by growing bacteria on the plates (Ryan *et al*., 2004).

***In vivo* pathogenicity tests:** *In vivo* pathogenicity test was carried out following Keskin *et al*. (2004).

The healthy 8 months old carp fishes were brought to the laboratory and were acclimated at 25°C for one week in flat bottomed circular 30 L plastic tubs. The tubs were filled with de-chlorinated tap water which was daily removed and was also properly aerated. The fish were fed the above mentioned laboratory prepared diet. Only the healthy fish showing normal activities were selected for further experimentation. In this experiment, a dose of known viable count of an isolated and identified pathogenic bacterium taken from its pure culture in 200 μL of physiological buffer saline was administered interperitonially to the test fishes. There were four treatments viz. fish inoculated with viable count at (i) 1.00×10^8, (ii) 2.00×10^8, (iii) 3.00×10^8 and (iv) the control fish inoculated with only 200 μL of physiological buffer saline. These doses were selected keeping in view roughly the median number of bacteria present in the control waters. Appearance of morbidity

Table 3: Tentative identification of bacteria isolated from ponds treated with different manures*

Biochemical tests	Bacterial isolates 43-48	Bacterial isolates 49-54	Bacterial isolate 55-60
Gram reaction	+	+	+
Shape	Cocci in clusters	Cocci in clusters	Cocci in chains
Colour of colony	Yellow-orange	White-cream or Yellow-orange	White - cream
Aerobic	+	+	-
Anaerobic	-	+	-
Catalase	+	+	-
Oxidase	+	-	-
Glucose Fermentation	-	+	+
Urease	+	+	-
Simmon citrate	+	-	-
Starch hydrolysis	-	-	+
Ehrlich indole	-	-	-
Nitrate-Nitrite	-	+	-
Adonitol	-	-	+
Xylose	-	-	+
Sucrose	-	+	-
Sorbitol	-	+	+
Arginine dihyrolase	-	+	-
Lactose	-	+	+
Maltose	-	+	+
Mannitol	-	+	+
Cellobiose	-	-	+
Glycerol	-	+	+
Inositol	-	-	-
Tryptophan	-	+	-
Glucose	+	+	+
Fructose	+	+	+
Insulin	-	-	-
Voges Proskauer 37ºC	+	+	+
Galactose	-	+	+
Bacterial identified	*Micrococcus luteus*	*Staphylococcus aureus*	*Streptococcus* spp.
ID score	0.99620	0.90954	0.99964

+: Showed growth, -: Showed no growth, *for the convenience, original numbers were re-designated

in a fish was taken for its being a diseased individual. For each treatment, 30 fish were taken and each treatment had four replications. Observations were recorded daily on the number of fish surviving and hence the longevity of each species was recorded under the given treatment.

Determination of Colony Forming Unit (CFU) of isolated bacteria and estimation of bacterial populations in the treated fish ponds: Colony Forming Unit (CFU mL^{-1}) is used to identify the number of viable microorganisms in a fixed amount of liquid. The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony. Thus, the number of colonies should give the number of bacteria that can grow under the employed incubation conditions. The bacterial sample was diluted by factors of 10 and plated on agar medium. After incubation, the sample showing number of colonies on a dilution plate between 30-300 was determined. A plate having 30-300 colonies was chosen because this range is considered statistically significant. The cfu of a bacterium was determined by the following serial dilution steps.

Initially, 18-24 h old bacterial culture was taken. Then, 1 mL of well mixed culture (broth) was added to 9 mL of sterilized Phosphate Buffer Saline (PBS) contained in a test tube. The contents were mixed thoroughly by micropipette. Then, 1 mL of bacterial culture in PBS obtained from the first step was transferred to the second test tube containing 9 mL PBS. Following the similar method, 1 mL of culture obtained from the second tube was transferred to the third tube containing

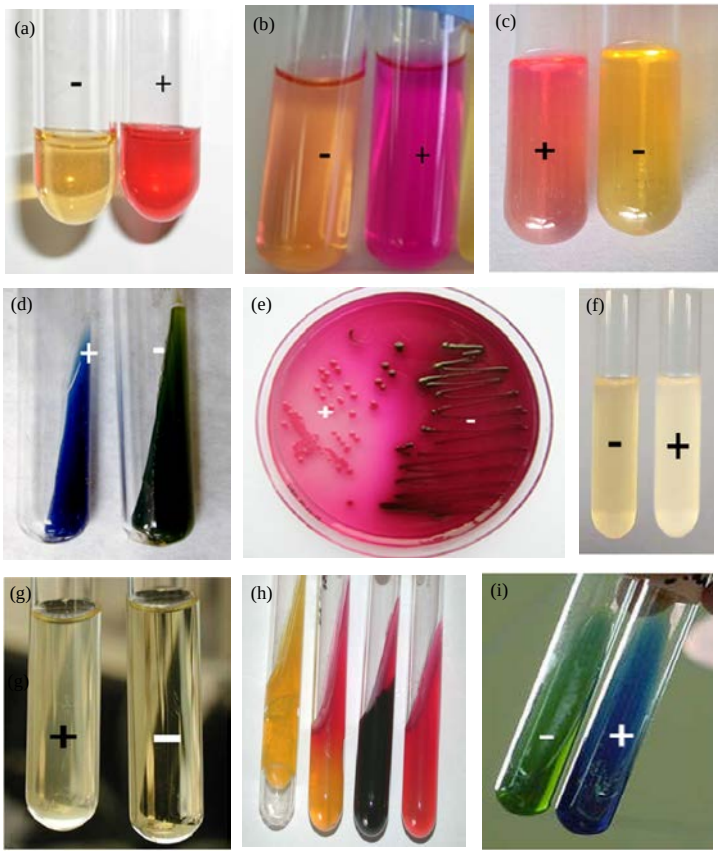

Fig. 4(a-i): (a) Methyl red test, (b) Vogues Proskauer test, (c) Indole/urese test, (d) Citrate test, (e) Endo Agar test for the selective isolation, cultivation and differentiation of coliform and other enteric bacteria, (f) Growth in Glucose broth, (g) Growth in sucrose broth, (h) TSI test; lactose fermenters resulted in yellow colour whereas non-lactose fermenters resulted in reddish pink colour; blackening of the butt was due to H_2S production and (i) Malonate utilization test showing green colour at neutral pH in an uninoculated medium that turned blue when pH was raised high by the presence of bacterium showing a positive reaction

Table 4: Confirmatory identification of bacteria isolated from ponds treated with different manures

Selective media	Bacterial isolate No.	-ve/+ve	Confirmation of bacterium
Rimler-Shott medium base	1-6	+ve	*Aeromonas hydrophila*
Luria agar base	7-12	+ve	*Escherichia coli*
EMB medium	13-18	+ve	*Enterobacter aerogens*
Pseudomonas agar F Base	19-24	+ve	*Pseudomonas fluorescens*
Blood agar	25-30	+ve	*Klebsiella oxytoca*
Antibiotic Assay Medium No. 38	31-36	+ve	*Pseudomonas aeruginosa*
S.S. Agar absent	37-42	+ve, colony orange, no pink colony	*Shigella* spp. present, *Salmonella* absent
Hugh Leifson glucose medium	43-48	+ve	*Micrococcus luteus*
Antibiotic Assay Medium. C	49-54	+ve	*Staphylococcus aureus*
Blood agar	55-60	+ve	*Streptococcus* sp.

9 mL PBS. By using this procedure, dilutions were made up to 9-10 times. One milliliter of the bacterial solution was taken from 7th, 8th, 9th and 10th dilutions and spread on freshly prepared

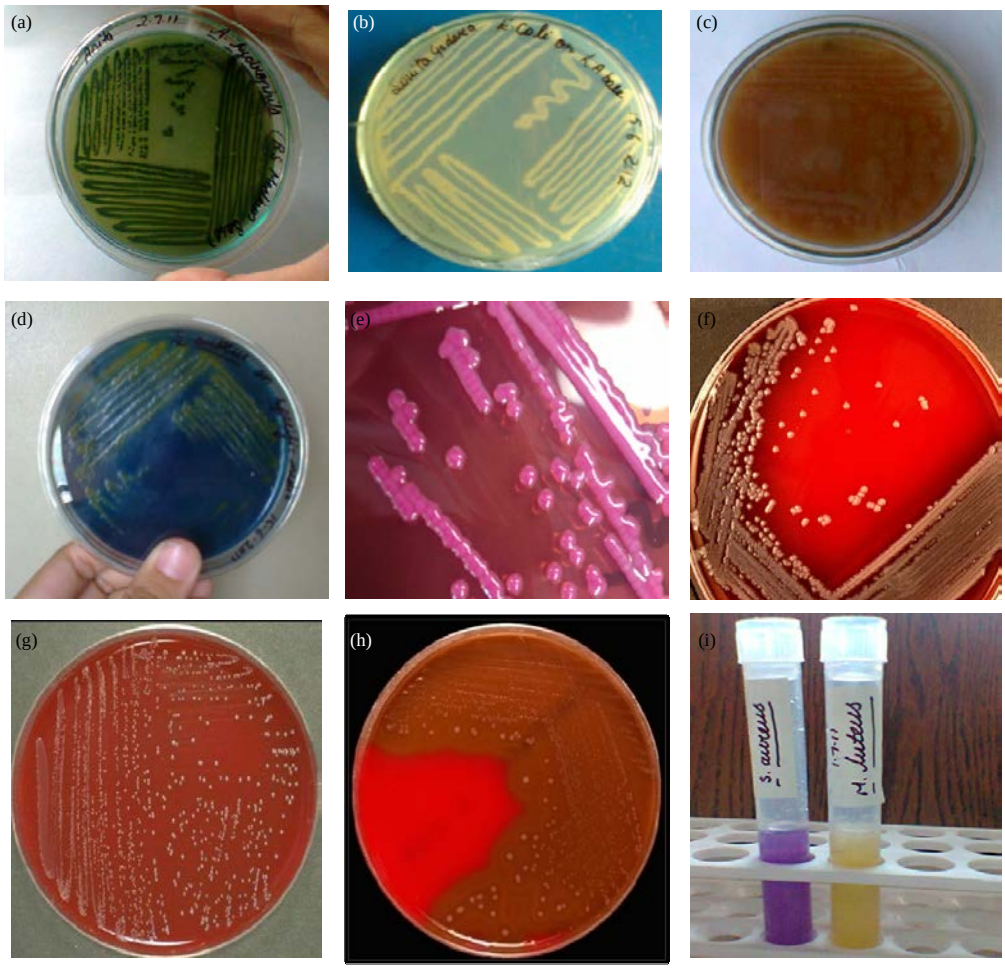

Fig. 5(a-i): (a) *Aeromonas hydrophila* on Rimler-Shott medium, (b) *Escherichia coli* on Luria agar base, (c) *Enterobacter aerogens* on EMB agar, (d) *Pseudomonas fluorescence* on *Pseudomonas* agar base, (e) *Klebsiella oxytoca* on blood agar, (f) *Pseudomonas aeruginosa* on blood agar, (g) *Staphylococcus aureus* on antibiotic assay medium, (h) *Streptococcus* spp. on blood agar and (i) *Staphylococcus aureus* on Antibiotic Assay Medium. C and *Micrococcus luteus* on Hugh Leifson glucose medium

Nutrient Agar (NA) plates. Plates were made in triplicates and incubated for 24 h at 30±2°C. On the next day, number of colonies on each NA plate was counted and Colony Forming Unit (CFU) was recorded by the following formula:

$$\text{CFU mL}^{-1} = \text{No. of colonies dilution factor}$$

Statistical analysis: The experiments were laid down in Completely Randomized Design for Analysis of Variance (ANOVA). The values of Least Significant Differences (LSD) were derived and the treatment means were compared at 5 per cent level of significance (Snedecor and Cochran, 1989).

RESULTS

Identification of isolated bacterial strains collected from ponds treated with different manures: On the basis of a number of physical and biochemical tests performed on the isolated pure cultures of bacteria, ten bacterial isolates were identified as separate colonies from fish ponds treated with different organic manures from August, 2011 to August, 2012. These included seven gram negative and three gram positive bacterial strains (Fig. 2 and 3 and Table 1-3). The PIB Win programme based on biochemical tests (Fig. 3 and 4) tentatively identified these seven gram negative isolates as *Aeromonas hydrophila*, *Escherichia coli*, *Enterobacter aerogens*, *Pseudomonas fluorescens*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Shigella* sp., whereas, three gram positive as *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus* sp. (Table 1-3). The specific media test confirmed the identification of these bacteria ((Fig. 5 and Table 4), which was identical to that tentatively done by the PIB Win Programme (Fig. 5 and Table 1-3).

Pathogenicity of isolated bacteria: The results of *in vitro* test revealed that all the 10 bacteria isolated from the aquatic medium successfully grew on the blood of three species of Indian major carps and showed α-β zone of hemolysis. Therefore, all the ten bacteria seemed to be pathogenic in nature. This contention was fully confirmed by the *in vivo* pathogenicity tests (Table 5).

Table 5: Longevity of Indian major carps inoculated with various doses of bacteria isolated from the pond waters treated with different manures during nine week experiment

Bacterium specie	Control	1.00×10^8	2.00×10^8	3.00×10^8
Catla (Catla catla)				
Aeromonas hydrophila	63±0	14.3±0.8	9.6±0.4	3.3±0.1
Escherichia coli	63±0	17.3±0.9	12.3±0.5	5.0±0.2
Enterobacter aerogens	63±0	17.3±0.9	12.6±0.7	4.6±0.1
Pseudomonas fluorescens	63±0	29.6±1.6	16.6±0.8	8.6±0.4
Klebsiella oxytoca	63±0	22.6±1.2	12.6±0.7	5.3±0.2
Pseudomonas aeruginosa	63±0	22.3±1.8	14.6±0.8	7.3±0.6
Shigella sp.	63±0	16.6±0.6	12.0±0.7	5.6±0.2
Micrococcus luteus	63±0	18.6±0.7	14.3±0.7	8.3±0.3
Staphylococcus aureus	63±0	18.6±0.5	13.3±0.8	5.6±0.3
Streptococcus sp.	63±0	19.3±1.9	12.6±0.7	6.3±0.2
Rohu (Labeo rohita)				
Aeromonas hydrophila	63±0	15.6±12	11.0±0.8	4.3±0.3
Escherichia coli	63±0	18.0±1.4	14.3±1.1	6.3±0.2
Enterobacter aerogens	63±0	19.6±.1.3	13.3±1.1	6.0±0.8
Pseudomonas fluorescens	63±0	31.3±1.8	18.0±1.4	10.0±0.7
Klebsiella oxytoca	63±0	24.0±1.6	14.0±1.2	6.3±0.3
Pseudomonas aeruginosa	63±0	26.6±2.4	16.3±1.4	8.6±0.9
Shigella sp.	63±0	18.0±1.4	13.3±1.0	7.0±0.3
Micrococcus luteus	63±0	22.3±1.6	16.0±1.2	9.6±0.3
Staphylococcus aureus	63±0	20.0±1.5	14.6±1.1	7.0±0.4
Streptococcus sp.	63±0	20.6±1.8	14.0±1.1	7.3±0.3
Mrigal (Cirrhinus mrigala)				
Aeromonas hydrophila	63±0	17.3±1.2	12.6±0.6	5.6±0.2
Escherichia coli	63±0	19.3±1.1	15.6±0.8	7.0±0.3
Enterobacter aerogens	63±0	20.6±1.2	15.3±0.9	7.6±0.3
Pseudomonas fluorescens	63±0	33.3±2.3	19.3±1.1	11.3±0.5
Klebsiella oxytoca	63±0	25.3±1.2	15.3±0.9	8.6±0.5
Pseudomonas aeruginosa	63±0	24.0±1.8	14.6±1.2	7.3±0.6
Shigella sp.	63±0	19.3±1.0	14.0±1.1	6.3±0.3
Micrococcus luteus	63±0	23.6±1.1	17.3±1.2	11.0±0.5
Staphylococcus aureus	63±0	21.3±1.1	15.6±0.8	8.3±0.4
Streptococcus sp.	63±0	21.0±1.2	15.3±0.7	8.6±0.4

*Mean s.d. of 120 observations (30 fishes×4 replications), LSD dose ($p<0.05$) = 3.8, LSD bacteria ($p<0.05$) = 1.2, LSD fish ($p<0.05$) = 0.2

Aeromonas hydrophila, seemed to be the most deadly pathogen of Indian major carps followed by *Enterobacter aerogens, Escherichia coli*, *Klebsiella oxytoca*, *Staphylococcus aureus*, *Shigella* sp., *Streptococcus* sp., *Pseudomonas aeruginosa, Micrococcus luteus* and *Pseudomonas fluorescens*. All these bacteria seemed to be the serious pathogens of all the three fish species under all the three inoculation doses. However, significant differences existed in their pathogenic efficacy as is evident from the longevity of the Indian major carps ($p<0.05$, ANOVA, Table 5). The differences between the doses were significant and the order of pathogenic efficiency was: $3.00\times10^8>2.00\times10^8>1.00\times10^8$ ($p<0.05$, ANOVA, Table 5), the higher dose showed the lowest longevity and no mortality, however, was observed in the fishes kept under the control treatment (Table 5). Likewise, there were significant differences in the susceptibility of three fish species as reflected by their longevity under different bacterial inoculations; *Catla catla* was the most susceptible followed by *Labeo rohita* and *Cirrhinus mrigala* ((LSD, $p<0.05$, ANOVA, Table 5). Interestingly, all the fishes under the control treatment remained alive during the entire period of 63 days of this experiment.

Viable counts of isolated heterotrophic pathogenic bacterial strains and their abundances in the pond waters treated with different manures: All the ten pathogenic heterotrophic bacteria were present in waters treated with these manures (Table 6). However, minimal number was observed in the pond waters treated with (T_5) vermicompost at 10,000 kg ha^{-1} year^{-1}, followed, in increasing order, by (T_6) vermicompost at15,000 kg ha^{-1} year^{-1} and under the control treatment for all the bacterial isolates but variable for other remaining treatments (Table 7). For example, in waters treated with cow dung manure, *Aeromonas hydrophila* was most abundant followed by *Pseudomonas fluorescens*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Streptococcus* sp., *Enterobacter aerogens*, *Shigella* sp., *Staphylococcus aureus*, *Micrococcus luteus* and *Escherichia coli.* In waters treated with poultry manure, *Pseudomonas fluorescens* was most abundant followed by *Pseudomonas aeruginosa, Aeromonas hydrophila*, *Streptococcus* sp., *Klebsiella oxytoca*, *Staphylococcus aureus*, *Micrococcus luteus*, *Enterobacter aerogens* and *Shigella* sp. Likewise, in waters treated with piggery manure *Pseudomonas aeruginosa* was most abundant followed by *Pseudomonas fluorescens, Streptococcus* sp., *Aeromonas hydrophila*, *Klebsiella oxytoca, Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, *Enterobacter aerogens* and *Shigella* sp. The differences between the bacterial abundances were extremely wide/significant (Table 7). On the basis of pathogenic microbial abundances, the treatment (T_5) vermicompost at 10,000 kg ha^{-1} year^{-1} seemed to be the safest among the five organic fertilizers.

Table 6: Presence/absence of bacteria isolated from ponds treated with different manures

	Treatments					
Bacterium and Bacterial isolate	T_1	T_2	T_3	T_4	T_5	T_6
Gram negative						
Aeromonas hydrophila (Isolate 1-6)	+	+	+	+	+	+
Escherichia coli (Isolate 7-12)	+	+	+	+	+	+
Enterobacter aerogens (Isolate 13-18)	+	+	+	+	+	+
Pseudomonas fluorescens (Isolate 19-24)	+	+	+	+	+	+
Klebsiella oxytoca (Isolate 25-30)	+	+	+	+	+	+
Pseudomonas aeruginosa (Isolate 31-36)	+	+	+	+	+	+
Shigella sp. (Isolate 37-42)	+	+	+	+	+	+
Gram positive						
Micrococcus luteus (Isolate 43-48)	+	+	+	+	+	+
Staphylococcus aureus (Isolate 49-54)	+	+	+	+	+	+
Streptococcus sp. (Isolate 55-60)	+	+	+	+	+	+

T_1: Control, T_2: Pig manure at 4,000 kg ha^{-1} year^{-1}, T_3:Poultry manure at 6,000 kg ha^{-1} year^{-1}, T_4: Cow dung at 10,000 kg ha^{-1} year^{-1}, T_5: Vermicompost at 10,000 kg ha^{-1} year^{-1}and T_6: Vermicompost at 15, 000 kg ha^{-1} year^{-1}, +: Bacterium present, -: Bacterium absent

Table 7: Viable counts of isolated heterotrophic pathogenic bacterial strains from ponds treated with different manures

	Viable counts treatments (CFU mL^{-1})					
Inoculated bacteria	T_1	T_2	T_3	T_4	T_5	T_6
Gram negative						
Aeromonas hydrophila	1.01×10^{6}	2.30×10^{8}	2.08×10^{9}	1.80×10^{10}	0.45×10^{6}	0.50×10^{6}
Escherichia coli	1.30×10^{7}	1.60×10^{8}	2.01×10^{8}	1.50×10^{7}	0.31×10^{7}	0.44×10^{7}
Enterobacter aerogens	1.80×10^{7}	2.33×10^{7}	2.75×10^{7}	2.73×10^{7}	0.83×10^{7}	0.90×10^{7}
Shigella sp.	1.20×10^{7}	1.90×10^{7}	1.85×10^{7}	2.21×10^{7}	0.30×10^{7}	0.60×10^{7}
Klebsiella oxytoca	1.09×10^{8}	1.66×10^{8}	2.10×10^{8}	1.87×10^{8}	0.18×10^{8}	0.45×10^{8}
Pseudomonas fluorescens	2.07×10^{7}	2.10×10^{9}	2.70×10^{10}	1.90×10^{8}	1.47×10^{7}	1.70×10^{7}
Pseudomonas aeruginosa	2.37×10^{7}	2.25×10^{9}	2.80×10^{9}	1.71×10^{8}	1.55×10^{7}-	1.87×10^{7}-
Gram positive						
Micrococcus luteus	3.51×10^{7}	2.70×10^{7}	2.80×10^{7}	1.75×10^{7}	0.51×10^{7}	0.90×10^{7}
Staphylococcus aureus	3.89×10^{7}	2.90×10^{7}	3.60×10^{7}	2.00×10^{7}	0.89×10^{7}	0.94×10^{7}
Streptococcus sp.	4.10×10^{6}	3.60×10^{8}	4.00×10^{8}	3.20×10^{7}	0.53×10^{6}	0.67×10^{6}

*Mean of 4 replications, T_1: Control, T_2: Pig manure at 4,000 kg ha^{-1} $year^{-1}$, T_3:Poultry manure at 6,000 kg ha^{-1} $year^{-1}$, T_4: Cow dung at 10,000 kg ha^{-1} $year^{-1}$, T_5: Vermicompost at 10,000 kg ha^{-1} $year^{-1}$and T_6: Vermicompost at 15, 000 kg ha^{-1} $year^{-1}$

DISCUSSION

Both marine and freshwater fishes have specific indigenous microflora (Sakata, 1990; Ringo and Gatesoupe, 1998; Sihag and Sharma, 2012). Earlier reports reveal that the aquatic animals carried microflora consisting mainly of gram-negative aerobic, obligate anaerobic and facultative anaerobic bacteria, the composition of which may change with environmental stresses like diet (Ringo and Strom, 1994) and fish age (Bergh *et al*., 1994). The isolated bacterial strains belonged to *Aeromonas* spp., *Pseudomonas* spp., *Shigella* spp. and members of the family Enterobacteriaceae. Jakhar *et al*. (2013) isolated seven species of bacteria from the tissues of diseased prawn (*Macrobrachium rosenbergii*). These included *Aeromonas hydrophila, Pseudomonas fluorescence, Mirococcus luteus*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Enterobacter cloacae* and *Klebsiella pneumoniae*; first two were pathogenic and remaining five were non-pathogenic. Ravikumar *et al*. (2010) isolated 10 bacterial types from diseased ornamental fish, 5 g negative bacteria viz. *Escherichia coli, Klebsiella pneumoniae*, *Shigella flexneri*, *Proteus myxofaciens* and *Vibrio* sp. and five gram positive bacterial types viz. *Streptococcus pyogens*., *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus cereus* and *Clostridium* sp. In the present study, 10 pathogenic bacterial types were isolated from the waters treated with different manures. These included, seven gram negative viz. *Aeromonas hydrophila, Escherichia coli*, *Enterobacter aerogens*, *Pseudomonas fluorescens*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Shigella* sp. and three gram positive viz. *Micrococcus luteus*, *Staphylococcus aureus* and *Streptococcus* sp.

Interspecies competition for the resources and space is very common in the ecosystems; pond ecosystem should be no exception. Bacteria do compete for the nutrients as well as space. This fact has given the birth of science of probiotics (Sihag and Sharma, 2012). The beneficial bacteria in the pond ecosystem must be playing a role in controlling the abundances of the pathogenic bacteria. Such beneficial bacteria must be in larger numbers in the pond waters treated with vermicompost than those treated with other fertilizers. This hypothesis opens many new areas of investigation for the aquatic microbiologists. What are the bacterial profiles of different kinds of fertilizers? Does vermicompost vis-à-vis other fertilizers carry beneficial bacterial types in larger numbers and with higher diversity or harmful bacterial types in smaller numbers and with lower diversity? How do these bacteria compete for the resources and space in the pond ecosystem? These are some of the subjects for future studies and investigations that may lead to understanding of better management of pond waters for higher aquaculture production.

In this study, among the manure treated waters, vermicompost at 10,000 kg ha^{-1} $year^{-1}$ had the minimal number of pathogenic bacteria followed invariably by those treated with vermicompost at 15,000 kg ha^{-1} $year^{-1}$ and the control. All other treated waters had variable number of bacteria. The possible reason for this situation may be the prevailing set of hydro-biological conditions and bacterial profiles in such waters. Our earlier studies categorically revealed significant differences in the quality parameters and fish growth in waters treated with different manures (Godara *et al*., 2015a, b). In those studies, vermicompost at 10,000 kg ha^{-1} $^{-}year^{1}$ gave the best ranges of hydro-biological parameters and maximal fish growth (Godara *et al*., 2015a, b). Other earlier studies also revealed that after manure application, significant differences existed in NH_4-N (Blackburn and Henriksen, 1983; Jana and Barat, 1983; Mei *et al*., 1995; Yao and Zhaoyang, 1997); depletion of dissolved oxygen (Boyd, 1990) and changes from alkaline to acidic pH (Nath *et al*., 1994) in the waters of a majority of the treatments. This could be the possible reasons of higher abundances of the heterotrophic pathogenic bacteria in some waters and in lower numbers in the others (Jha *et al*., 2004). In this study, among the pond waters treated with five organic fertilizers, those treated with vermicompost at 10,000 kg ha^{-1} $year^{-1}$ gave minimal counts of pathogenic bacteria followed, in ascending order, by vermicompost at 15,000 kg ha^{-1} $year^{-1}$ and the control, the waters treated with other fertilizers had higher number of heterotrophic pathogenic bacteria. Under the former treatment, the hydro-biological parameters were in the favourable ranges (Godara *et al*., 2015b) and the fish growth too was maximal (Godara *et al*., 2015a). Therefore, on the basis of these results, vermicompost at 10,000 kg ha^{-1} $year^{-1}$ seemed to be the best among the five organic fertilizer treatments in controlling the abundance of pathogenic bacteria in the treated pond waters.

CONCLUSION

Fish plays a vital role in supplementing the nutritional component in the human diet. Therefore, fish should become a part of the common man's diet. For that matter, fish production should be made cheap and cost effective. This could be done by the utilization of cheap and easily available resources and in puts like organic manures, as pond fertilizers. Livestock wastes and farm yard manures are such fertilizers. These wastes make the raw material for the production of vermicompost. Despite the known usefulness of the latter in agriculture as organic manure for higher crop yield, the information on the use of vermicompost as pond fertilizer in aquaculture is rather scanty. The present study reveals that among the five manures used, vermicompost at 10,000 kg ha^{-1} $year^{-1}$ had maximal check on the pathogenic bacteria. This contention is supported by our earlier study where fish growth was maximal and water quality parameters were the best. Hence, vermicompost with the latter dose is recommended as an organic fertilizer for the fertilization of fish ponds. Vermicompost at 10,000 kg ha^{-1} $year^{-1}$ can be used more effectively for manuring semi intensive carp culture ponds for keeping the hydro-biological parameters in favourable ranges and maximal fish growth.

ACKNOWLEDGMENTS

We are thankful to the Head, Department of Zoology, for providing necessary facilities. During the course of this study, Sunita Godara received financial assistance from Haryana Agricultural University in the form of a Merit stipend and DST/ INSPIRE scholarship as JRF.

REFERENCES

Abbas, S., I. Ahmed, M. Salim and Khalil-Ur-Rehman, 2010. Comparative effects of fertilization and supplementary feed on growth performance of three fish species. Int. J. Agric. Biol., 12: 276-280.

Bergh, O., K.E. Naas and T. Harboe, 1994. Shift in the intestinal microflora of Atlantic halibut (*Hippoglossus hippoglossus*) larvae during first feeding. Can. J. Fish Aquat. Sci., 51: 1899-1903.

Blackburn, T.H. and K. Henriksen, 1983. Nitrogen cycling in different types of sediments from Danish waters. Limnol. Oceanogr., 28: 477-493.

Boyd, C.E., 1990. Water Quality in Ponds for Aquaculture. 1st Edn., Auburn University Agricultural Experimentation Station, Auburn, AL., Pages: 482.

Bryant, T.N., 2004. PIBWin-software for probabilistic identification. J. Applied Microbiol., 97: 1326-1327.

Choo, P.S. and M.J. Williams, 2003. Fisheries production in Asia: Its role in food security and nutrition. NAGA: WorldFish Center Q., 26: 11-16.

Das, B.K., 2004. Bacterial Diseases of Fish and their Control. In: Disease Diagnosis and Health Management of Freshwater Fish and Shellfish, Swain, P., P.K. Sahoo and B.K. Das (Eds.). Central Institute of Freshwater Aquaculture, Bhubaneswar, pp: 45-52.

Dhawan, A. and S. Kaur, 2002a. Effect of pig dung on water quality and polyculture of carp species during winter and summer. Aquacult. Int., 10: 297-307.

Dhawan, A. and S. Kaur, 2002b. Pig dung as pond manure: Effect on water quality, pond productivity and growth of carps in polyculture system. NAGA: ICLARM Q., 25: 11-14.

Godara, S., R.C. Sihag and R.K. Gupta, 2015a. Effect of pond fertilization with vermicompost and some other manures on the growth performance of Indian major carps. J. Fish. Aqua. Sci., 10: 199-211.

Godara, S., R.C. Sihag and R.K. Gupta, 2015b. Effect of pond fertilization with vermicompost and some other manures on the hydrobiological parameters of treated pond waters. J. Fish. Aqua. Sci., 10: 212-231.

Hojovec, J., 1977. Health Effects from Waste Utilization. In: Animal Wastes, Taiganides, E.P. (Ed.). Applied Science Publishers Ltd., London, pp: 105-109.

Holt, J.G., 1984. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore, MD, USA., Pages: 355.

Hossain, M.A., D.C. Little and R.C. Bhujel, 2003. Nursing duration and pond fertilization level affect polycultures of Indian major carp (rohu *Labeo rohita* and mrigal *Cirrhina mrigala*) with monosex Nile tilapia *Oreochromis niloticus*. Aquacult. Res., 34: 765-775.

Hussain, S.A., 1986. Fish as a vital source of animal protein. Prog. Farming, 6: 13-13.

Jakhar, V., S.K. Gahlawat and R.C. Sihag, 2010. Methodology for the isolation and identification of bacterial pathogens. Ecosphere, 1: 57-69.

Jakhar, V., R.C. Sihag and S.K. Gahlawat, 2013. Survey and characterization of diseases in giant freshwater prawn (*Macrobrachium rosenbergii* de Man) in Haryana. Indian J. Anim. Res., 47: 479-485.

Jana, B.B. and S. Barat, 1983. Development of heterotrophic and ammonifying bacterial populations as affected by the fish *Clarias batrachus* (Linn.) under different experimental conditions. Acta Hydrochimica Hydrobiologica, 11: 569-576.

Jha, P., K. Sarkar and S. Barat, 2004. Effect of different application rates of cowdung and poultry excreta on water quality and growth of ornamental carp, *Cyprinus carpio* vr. *koi,* in concrete tanks. Turk. J. Fish. Aquat. Sci., 4: 17-22.

Jinyi, D., G. Xianzhen, F. Xiuzhen and L. Meizhen, 1987. Preliminary studies on the effects of animal manure on bacterial disease of fish. Network of Aquaculture Center in Asia-Pacific, Bangkok, pp: 49.

Keskin, O., C.J. Tsai, H. Wolfson and R. Nussinov, 2004. A new, structurally nonredundant, diverse data set of protein-protein interfaces and its implications. Protein Sci., 13: 1043-1055.

Krieg, N.R. and J.G. Holt, 1984. Bergy's Manual of Systematic Bacteriology. Vol. 1, Williams and Wilkins Co., Baltimore.

Mei, Z., Z. Shi and X. Zhu, 1995. The Studies on the Nutritional Content and the Release of the Fish Pond Mud. In: The Studies of the Ecosystem in the Chinese Fish Ponds, Zhu, X. and Z. Shi (Eds.). Sanghai Science and Technology Press, Shanghai, pp: 29-36.

Muendo, P.N., A. Milstein, A.A. van Dam, E.N. Gamal, J.J. Stoorvogel and M.C.J. Verdegem, 2006. Exploring the trophic structure in organically fertilized and feed-driven tilapia culture environments using multivariate analyses. Aquacult. Res., 37: 151-163.

Nath, D., L.N. Mandal, S.D. Tripathi and H.C. Karmakar, 1994. Correlation between fish production and physico-chemical parameters of water and soil in semi-intensive carp culture ponds of West Bengal, India. J. Inland Fish. Soc. India, 26: 106-115.

Nath, S.S. and J.E. Lannan, 1992. Dry matter-nutrient relationships in manures and factors affecting nutrient availability from poultry manures. Tenth Annual Admin Report, 1991-1992, Office of International Research and Diversity, Corvallis, Oregon, pp: 110-119.

Quines, O.D., 1988. Microorganisms: Indicators of pollution in integrated livestock-fish farming systems. Environ. Int., 14: 531-534.

Ravikumar, S., G.P. Selvan and N.A.A. Gracelin, 2010. Antimicrobial activity of medicinal plants along Kanyakumari coast, Tamil Nadu, India. Afr. J. Basic Applied Sci., 2: 153-157.

Ringo, E. and E. Strom, 1994. Microflora of Arctic charr, *Salvelinus alpinus* (L.): Gastrointestinal microflora of free-living fish and effect of diet and salinity on intestinal microflora. Aquacult. Res., 25: 623-629.

Ringo, E. and F.J. Gatesoupe, 1998. Lactic acid bacteria in fish: A review. Aquaculture, 160: 177-203.

Ryan, K.J., C.G. Tay and J.C. Sherris, 2004. Sherris Medical Microbiology: An Introduction to Infectious Diseases. 4th Edn., McGraw Hill, New York, USA., ISBN-13: 9780838585290, Pages: 979.

Saini, V.K., R.C. Sihag, R.C. Sharma, S.K. Gahlawat and R.K. Gupta, 2008a. Biodegradation of water hyacinth, sugarcane bagasse and rice husk through vermicomposting. Int. J. Environ. Waste Manage., 2: 601-609.

Saini, V.K., R.C. Sihag, R.C. Sharma, S.K. Gahlawat and R.K. Gupta, 2008b. Relative efficacy of two methods of vermicomposting for bio-degradation of organic wastes. Int. J. Environ. Waste Manage., 2: 102-110.

Saini, V.K., R.C. Sihag, R.C. Sharma and S.K. Gahlawat, 2010. Relative efficacy of two species of earthworms in biodegradation of organic wastes under semi-arid subtropical conditions of North-West India. World Rev. Sci. Technol. Sustainable Dev., 7: 259-270.

Sakata, T., 1990. Microflora in the Digestive Tract of Fish and Shellfish. In: Microbiology of Poecilotherms, Lesel, R. (Ed.). Elsevier, Amsterdam, pp: 171-176.

Sandhu, G.S., 2005. A Textbook of Fish and Fisheries. Dominant Publishers and Distributors, New Delhi, India, ISBN-13: 9788178882741, pp: 39-40.

Schroeder, G.L., 1987. Carbon and nitrogen budgets in manured fish ponds on Israel's coastal plain. Aquaculture, 62: 259-279.

Sheikh, B.A. and S.A. Sheikh, 2004. Aquaculture and integrated farming system. Pak. J. Agric. Agric. Eng. Vet. Sci., 20: 52-58.

Sihag, R.C. and P. Sharma, 2012. Probiotics: The new ecofriendly alternative measures of disease control for sustainable aquaculture. J. Fish. Aquat. Sci., 7: 72-103.

Snedecor, G.W. and W.C. Cochran, 1989. Statistical Method. 8th Edn., Iowa State University Press, Ames, IA., USA., Pages: 502.

Sugita, H., T. Fushino, K. Oshima and Y. Deguchi, 1985. Microflora in the water and sediment of freshwater culture ponds. Bull. Jpn. Soc. Sci. Fish., 51: 91-97.

Yao, S. and H. Zhaoyang, 1997. Shrimp pond settlement-the quantity of nutrients dispersion and seasonal changes between water and mud contacting surfaces. Mar. Fish. Res., 18: 60-66.

Yildirim, O., 2008. Aquafeed industry in Turkey: Its aquafeed projections towards the year 2015. Turk. J. Fish. Aquatic Sci., 8: 93-98.