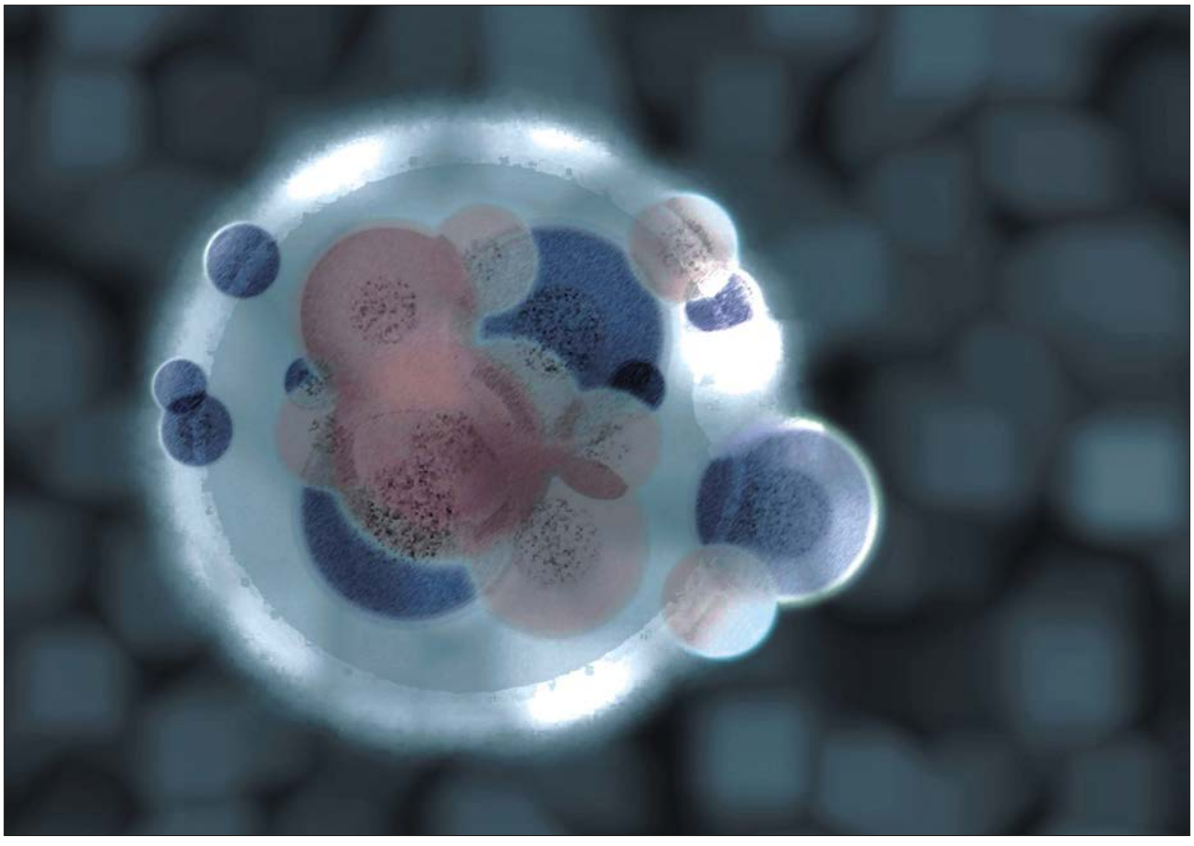

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 13 (8): 691-700, 2013
ISSN 1727-3048 / DOI: 10.3923/jbs.2013.691.700

Evaluation of Phytase-producing Ability by a Fish Gut Bacterium, *Bacillus subtilis* subsp. *subtilis*

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Abstract: Phytases produced by the symbiotic bacteria might increase the availability of phosphorus and other nutrients in farmed animals. Unlike terrestrial animals, phytase-producing fish gut bacteria are insufficiently studied. The present study was intended to evaluate phytase-producing gut bacteria in 6 freshwater carps, to identify the most promising one by 16SrDNA and to optimize phytase production by the strain. Homogenates of proximal (PI) and distal (DI) intestinal segments were spread onto Modified Phytase Screening Media (MPSM) plates to enumerate phytase-producing microbiota. Data were presented as Log Viable Counts g^{-1} intestine (LVC). Phytase producing microbiota was highest in the DI region of *Cirrhinus mrigala* (LVC = 3.85) followed by the DI region of *Labeo rohita* (LVC = 3.35). Out of 73 phytase-producing isolates, 14 primarily selected isolates detected through qualitative assay were further screened by quantitative assay using MPSM broth. The strain CM7 isolated from DI of *C. mrigala* showed highest phytase activity (2.31±0.01 U mL^{-1}) followed by the strain LR2 (2.21±0.01 U mL^{-1}) isolated from DI of *L. rohita*. Nucleotide homology analysis revealed that the isolate CM7 was similar to *Bacillus subtilis* subsp. *subtilis* (Accession no. JX292128). Highest phytase activity by the isolate in submerged culture was recorded as 2.61±0.04 U mL^{-1} under optimized conditions (pH 7, 35°C, 2.5% inoculum, 4% NaCl, 1% starch, 2% ammonium sulphate, 6 days incubation). Phytase-producing ability of the fish gut bacteria detected in the present study might expand the scope of their use to improve nutrient utilization in fish or other animals.

Key words: Phytase, fish, carps, gut bacteria, *Bacillus subtilis*

INTRODUCTION

Phytase (myoinositol hexakisphosphate phosphohydrolase, EC 3.1.3.8), an acid phosphorylase, is an important feed additive to increase the availability of phosphorus and other nutritionally important dietary components (e.g., minerals, trace elements, amino acids and energy) in monogastric animals by virtue of enzymatic hydrolysis of the phytic acid, the major Phosphorus (P) storage compound present in most plant feedstuffs (Maga, 1982; Reddy *et al*., 1989; Oatway *et al*., 2001). Owing to its chelation with various metals and proteins, phytic acid (myoinositol 1, 2, 3, 4, 5, 6-hexakis-dihydrogen phosphate) is considered as an antinutritional factor (Sabu *et al*., 2002). Incorporation of exogenous phytase in poultry (Lei and Stahl, 2000), pig (Han *et al*., 1997) and fish (Rodehutscord and Pffer, 1995; Li and Robinson,1997; Forster *et al*., 1999; Van Weerd *et al*., 1999; Baruah *et al*., 2007; Debnath *et al*., 2005a, b; Baruah *et al*., 2007; Sardar *et al*., 2007; Cao *et al*., 2008) diets has been reported to improve nutrient utilization and contribute significantly toward environmental protection by reducing phosphorus excretion.

Microorganisms are the best sources for commercial production of phytases because of their easy cultivation and high yields of the enzyme (Li *et al*., 2008). Although, phytases have been detected in several species of bacteria, yeasts and fungi (Greiner and Konietzny, 2006; Li *et al*., 2008; Roy *et al*., 2009; Khan and Ghosh, 2012a), considerable research has been conducted on the soil fungus, *Aspergillus* which is predominantly being utilized for industrial production of phytase (Ullah, 1988a, b; Volfova *et al*., 1994). However, bacterial phytases might be alternative to the fungal enzymes due to some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency (Konietzny and Greiner, 2004). During the last decade there has been an improved understanding on the importance of commensal intestinal microbiota in fish (Ringø *et al*., 2010). The gut microbiota may be

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categorized as either autochthonous (adherent) or allochthonous (transient) depending upon its ability to adhere and colonize the mucus layer in the digestive tract (Ringø and Birkbeck, 1999; Ringø *et al*., 2003). Like ruminants, complementary enzymes produced by the symbiotic bacteria might help in digestion and assimilation of the plant feedstuffs in fish (Ray *et al*., 2012). Autochthonous phytase producing gut bacteria in fresh water fishes have been reported very recently (Roy *et al*., 2009; Khan *et al*., 2011; Khan and Ghosh, 2012a). To our knowledge, phytase-producing fish gut bacteria are still insufficiently studied for enzyme production (Khan and Ghosh, 2012b).

In view of effective utilization of the phytate rich plant feedstuffs as animal feed ingredients, supplementation of phytase from autochthonous microorganisms or use of phytase-producing gut bacteria as microbial feed additives might be more suitable than the added phytase from allochthonous microorganisms. Microbial enzymes have the advantage of large scale production by established fermentation techniques. To establish a successful fermentation process, it is necessary to make the environmental and nutritional conditions favorable for over production of the desired metabolite by the microorganism (Ray *et al*., 2007). Therefore, the present study aimed at (1) Re-validation of the occurrence of phytase-producing gut bacteria in some freshwater fishes, (2) Identification of the most promising phytase-producing bacteria by 16SrDNA partial sequence analysis and finally (3) Optimization of the various process parameters to influence phytase production by the promising bacterial strain, *Bacillus subtilis* subsp. *subtilis* (Gen bank Accession no: JX292128) .

MATERIALS AND METHODS

Fish species examined: Average length, weight and average weight of the gut of the 6 carp species (*Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*, *Hypophthalmichthys molitrix*, *Cyprinus carpio*, *Puntius japonicus*) examined in the present study are presented in Table 1. The specimens were sampled by gill net from 3 local culture ponds and transported to the laboratory at Golapbag, Burdwan, West Bengal, India within oxygen packed plastic bags.

Isolation of phytase-producing gut bacteria: To isolate phytase-producing gut bacteria, gut segments were processed following Khan and Ghosh (2012a). The GI tracts were divided into PI (proximal part of the intestine) and DI (distal part of the intestine), cut into pieces and flushed carefully three times with 0.9% sterile saline solution using an injection syringe in order to remove non-adherent (allochthonous) microbiota. The gut segments were homogenized with sterilized pre-chilled 0.9% NaCl solution (w/v, 1:10) as described elsewhere (Das and Tripathi, 1991). Pooled samples of 3 fish were used for each replicate to avoid erroneous conclusions due to individual variations in gut microbiota (Ringø *et al*., 2006; Khan and Ghosh, 2012a). Homogenate of the pooled intestinal segments of each of the 3 replicates for each fish species and each part of gut was used separately after appropriate serial (1:10) dilutions (Beveridge *et al*., 1991). Diluted samples (0.1 mL) were poured aseptically (each in triplicate) within a laminar airflow on sterilized tryptone soy agar [(TSA), Himedia, India] and incubated at 37°C for 48 h to determine culturable heterotrophic autochthonous bacteria. To determine phytase producing bacteria same inoculums (0.1 mL) were given on sterilized Modified Phytase Screening Media (MPSM) plates and incubated at 37°C for 72 h after (Howson and Davis, 1983) with some modifications. The composition of MPSM was (g L^{-1}): glucose, 10; $(NH_4)_2SO_4$, 1; urea, 10; citric acid, 3.0; sodium citrate, 2; $MgSO_4.7H_2O$, 1; sodium phytate, 3; $FeSO_4.7H_2O$, 0.01 and agar 20, pH 7. The Colony Forming Units (CFU) $unit^{-1}$ sample volume of gut homogenate were determined by multiplying the number of colonies formed on each plate by the reciprocal dilution. Data is presented as Log Viable Counts (LVC) g^{-1} intestine. Colonies appeared on MPSM plates were streaked separately to obtain pure cultures and kept in a refrigerator (4°C) until further study.

Screening of isolates by qualitative and quantitative assay for extra-cellular phytase production: Primarily the bacterial isolates were screened qualitatively for extracellular phytase production following Yanke *et al*. (1999). The bacterial colonies in the MPSM plates were partially washed and flooded with 2% (w/v) cobalt chloride solution. Subsequently, cobalt chloride solution

Table 1: Average live weight, fish length (standard length) and gut weight of the fishes examined

Fish species	Average fish live weight (g)	Average fish length (cm)	Average gut weight (g)
Rohu, *Labeo rohita*	95.00±2.030	18.21±3.150	3.19±0.070
Catla, *Catla catla*	89.67±3.630	21.93±2.710	3.08±0.150
Mrigal, *Cirrhinus mrigala*	115.00±4.230	20.34±3.550	5.29±0.580
Silver carp, *Hypophthalmichthys molitrix*	150.21±7.210	31.60±4.310	3.55±0.620
Common carp, *Cyprinus carpio*	155.35±6.140	23.30±2.070	5.64±0.050
Puntius japonicus	63.52±3.850	17.25±2.280	2.19±0.060

Each data represents Mean±SD of nine specimens

was removed and a solution containing equal volume of 6.25% (w/v) ammonium molybdate solution and 0.42% ammonium vanadate solution was added. The appearance of transparent zones around the bacterial colonies indicated utilization of phytate by the extracellular bacterial phytase.

Out of the 73 phytase-producing bacterial strains isolated from the fish species examined; 14 primarily selected isolates (20% of total isolates) were further evaluated for quantitative phytase assay. Quantitative phytase assay of the crude enzyme was done with MPSM broths after Yanke *et al*. (1999) using sodium phytate as the substrate. The colour developed due to phytase activity was determined with a spectrophotometer (Shimadzu UV1800) at 700 nm. One phytase Unit (U) was defined as the amount of enzyme per milliliter of supernatant that released 1 μg of inorganic phosphorus per minute. Soluble protein content of the crude enzyme was determined by Lowry *et al*. (1951) using bovine serum albumin as standard.

Identification of the most promising isolate by 16SrDNA sequence analysis: One isolate with high enzyme-producing ability was further characterized by the analysis of 16SrDNA partial sequence as described by Roy *et al*. (2009). Sequenced data were aligned and analyzed for finding the closest homolog of the isolate using a combination of NCBI GenBank and RDP database. Phylogenetic tree was constructed in MEGA 4.1 software using the neighbour joining method with bootstrap analysis to obtain information on their molecular phylogeny.

Optimization of fermentation parameters for phytase production: The most promising isolate CM7 (*Bacillus subtilis* subsp. *subtilis*) was used for optimization of the process parameters for phytase production under submerged fermentation. Inoculums were prepared from a freshly raised 5-d-old slant culture in MPSM broth grown at 37°C for 48 h. The inoculants thus obtained contained 4.8×10^7 cells mL^{-1}. The protocol adopted for optimization of various process parameters was to evaluate the effect of an individual parameter and to incorporate it at the optimized level in the experiment before optimizing the next parameter. The parameters studied were: incubation temperature (25- 50°C), initial pH of the media (3-9), incubation period (2-10 d), inoculum volume (1-5 mL) and NaCl (1%-5%). In addition, the medium was supplemented with different carbon sources (1% w/v) (glucose, sucrose, lactose, maltose, starch) and nitrogen sources (1% w/v), both organic and inorganic (Ammonium nitrate, tyrosine, ammonium sulfate, tryptophan, peptone, yeast extract). Further, the selected carbon and nitrogen sources (starch and ammonium sulphate, respectively) were varied within a narrow range (1-5%) to optimize phytase production. The production flasks were incubated at 37°C for 72 h unless otherwise mentioned. All the experiments were carried out in triplicate. Phytase assay of the crude enzyme was done with culture broths after Yanke *et al*. (1999) as described in the previous section.

Statistical analysis: Statistical analysis of the data pertaining quantitative phytase assay of the primarily selected strains were performed according to Zar (1999) using SPSS Ver10 (Kinnear and Gray, 2000) software. All the data are given as Means±SE, n = 3.

RESULTS

Isolation and identification of the most promising phytase-producing bacteria: Enumeration of microbial community in the GI tracts of the 6 fish species studied revealed that autochthonous culturable heterotrophic and phytase-producing microbiota were present in both PI and DI regions in all the fish species studied (Table 2). Population levels of culturable autochthonous heterotrophic aerobic/facultative anaerobic bacteria were highest in the DI region of all the fish species studied. Highest number of culturable heterotrophic microbiota

Table 2: Log values of culturable autochthonous aerobic heterotrophic (grown on TSA plates) and phytase-producing (grown on MPSM plates) bacteria isolated from the GI tracts of 6 different fish species

	Log viable counts (g^{-1} intestinal tissue)			
	Proximal intestine		Distal intestine	
Fish species	TSA	MPSM	TSA	MPSM
Rohu, *Labeo rohita*	5.76	2.93	6.81	3.35
Catla, *Catla catla*	5.14	2.19	6.11	2.31
Mrigal, *Cirrhinus mrigala*	6.78	3.11	8.35	3.85
Silver carp, *Hypophthalmichthys molitrix*	4.57	2.10	6.46	2.48
Common carp, *Cyprinus carpio*	4.87	2.15	5.75	2.21
Puntius japonicus	5.37	2.29	6.25	2.95

TSA: Tryptone soy agar, MPSM: Modified phytase screening medium

Table 3: Primarily selected bacterial isolates from fish gut with their quantitative extra-cellular phytase activity

	Proximal intestine		Distal intestine	
Fish species	Bacterial isolates	Phytase activity (U)•	Bacterial isolates	Phytase activity (U)•
Labeo rohita	LR1	2.07±0.03^{c}	LR2	2.21±0.01^{a}
			LR3	1.05±0.02^{e}
Catla catla	CC1	1.02±0.02^{e}	CC3	1.22±0.01^{d}
Cirrhinus mrigala	CM4	2.12±0.01^{b}	CM7	2.31±0.01^{a}
	CM5	1.19±0.02^{d}		
Hypophthalmichthys molitrix	HM1	1.14±0.02^{d}	HM4	1.06±0.01^{e}
Cyprinus carpio	CP3	1.11±0.01^{d}	CP5	0.95±0.02^{e}
Puntius japonicus	PJF3	1.91±0.03^{c}	PJH1	2.15±0.02^{b}

Data are Means±SE of three determinations. Means with same superscript does not vary significantly ($p<0.05$), * Micrograms of inorganic phosphorus liberated mL^{-1} of enzyme extract min^{-1}

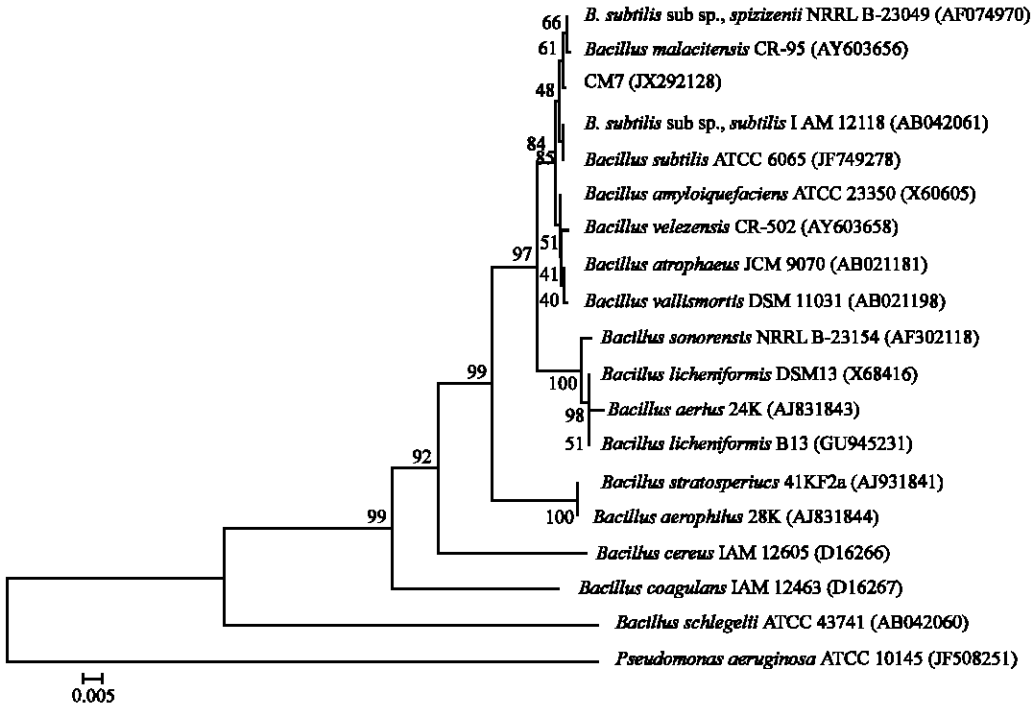

Fig. 1: Dendogram showing phylogenetic relations of the bacterial strain *Bacillus subtilis* subsp. *subtilis* CM7 with other closely related strains referenced to accession numbers

was noticed in the DI region of *C. mrigala* followed by DI region of *L. rohita* (LVC = 8.35 and 6.81 g^{-1} intestinal tissue, respectively). However, phytase producing microbiota detected on MPSM plate was highest in the DI region of *C. mrigala* followed by the DI region of *L. rohita* (LVC = 3.85 and 3.35 g^{-1} intestinal tissue, respectively).

Out of the 73 phytase-producing isolates (32 isolates from the PI region and 41 isolates from the DI region), 14 isolates were primarily selected through qualitative phytase assay and were further evaluated by quantitative phytase assay to pick the most promising isolate. Primarily selected isolates with their sources and phytase activity were given in Table 3. The strain CM7 isolated from *C. mrigala* showed highest phytase activity (2.31±0.01 U mL^{-1}) followed by the strain LR2 (2.21±0.01 U mL^{-1}) isolated from *Labeo rohita* (Table 3). Therefore, the isolate CM7 was finally selected for identification and studied for phytase production under submerged fermentation.

Nucleotide homology and phylogenetic analysis of the 16SrDNA partial sequence by nucleotide blast in the National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP) revealed that the strain CM7 was similar to *Bacillus subtilis* subsp. *subtilis*. The isolate CM7 showed 99% similarity with *B. subtilis* subsp. *subtilis* IAM 12118 (Accn. No. AB042061) The phylogenetic relations of the isolate (CM7) with other closely related bacteria are presented in the dendogram (Fig. 1). Partial sequence of the 16SrDNA from the selected isolate (CM7) was

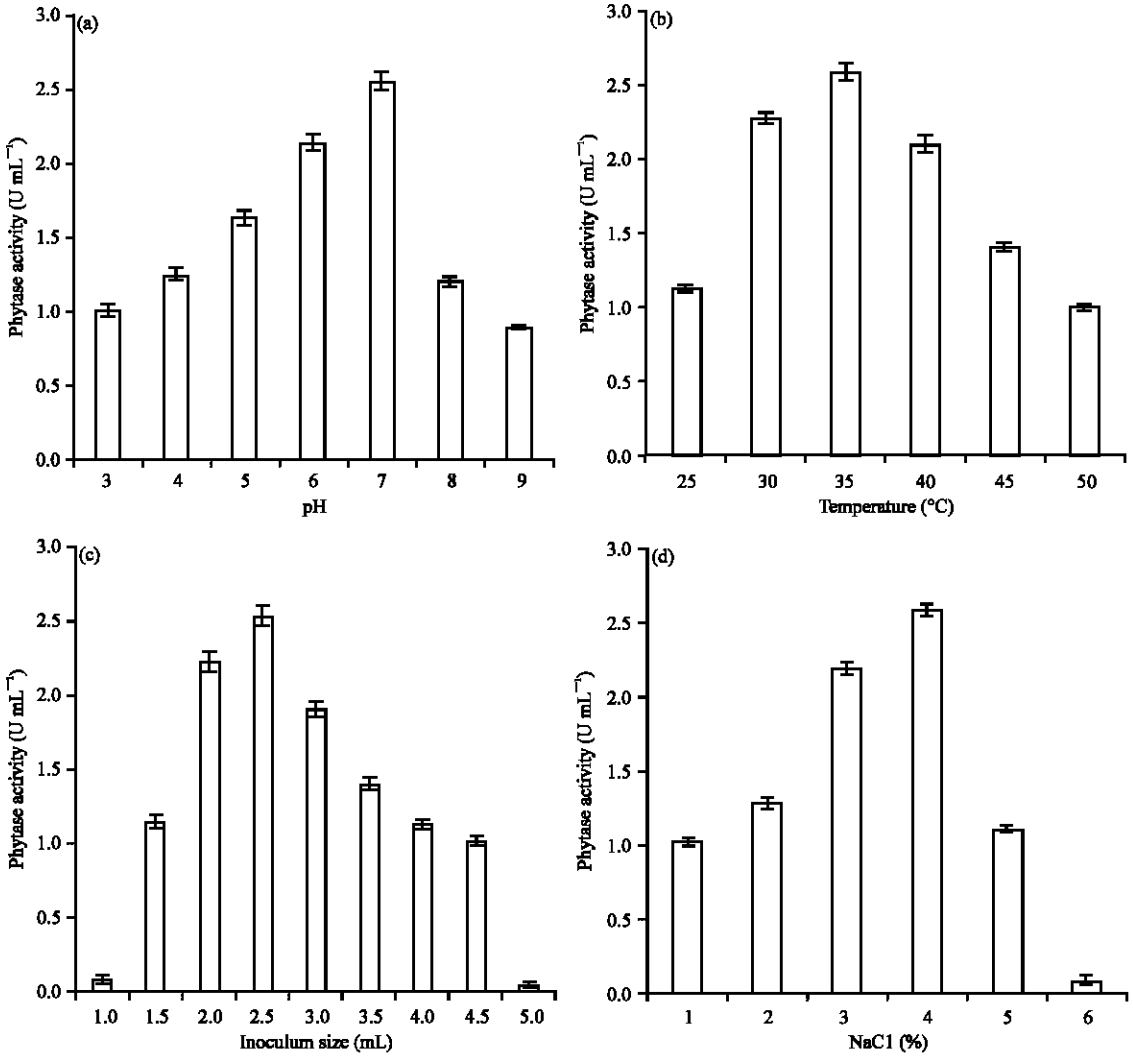

Fig. 2(a-d): (a) Effect of pH, (b) Temperature, (c) Inoculum size and (d) NaCl (%) on phytase production in submerged fermentation by *Bacillus subtilis* subsp. *subtilis* CM7

deposited in the NCBI GenBank database to obtain accession number (Genbank Accession No.: JX292128).

Optimization of fermentation conditions: Submerged fermentation was carried out to optimize the important physical, chemical and nutritional parameters that influence phytase production. Optimum initial pH of the medium required for maximum phytase production by the strain *Bacillus subtilis* subsp. *subtilis* CM7 was evaluated at various pH levels (3-9) (Fig. 2a). Phytase production was highest at pH 7.0 (2.56±0.06 U mL^{-1}). Result on the effect of temperature (25-50°C) on phytase production by *Bacillus subtilis* subsp. *subtilis* indicated that the optimum temperature for phytase activity was 35°C (2.59±0.05 U mL^{-1}). Phytase production decreased beyond this temperature (Fig. 2b).

Effect of inoculum percentage on phytase production has been depicted in Fig. 2c. Phytase activity was gradually increased with inoculum concentration leading to maximum enzyme activity at 2.5 mL (2.54±0.07 U mL^{-1}) and then declined for further increase in the concentration. When different concentrations of NaCl were used for enzyme assay it was found that phytase production was highest (2.58 ±0.04 U mL^{-1}) in 4% of NaCl (Fig. 2d) concentration. Beyond this concentration phytase activity was decreased. In the present study, different carbon sources were supplemented in the production medium (Fig. 3a). Among all the carbon sources tested, starch supplementation (1%) increased (Fig. 3b) phytase production (2.53±0.06 U mL^{-1}) and glucose was the second better carbon source

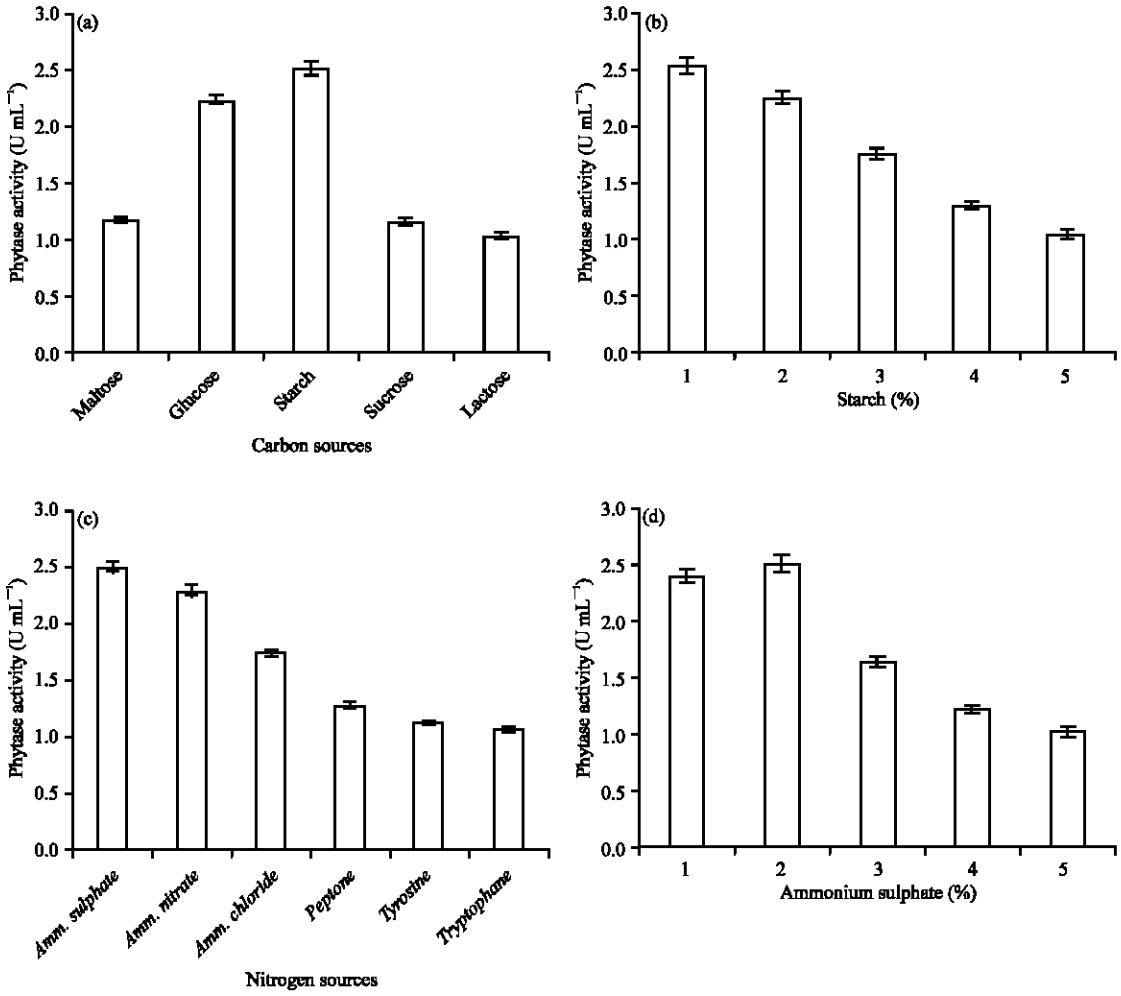

Fig. 3(a-d): (a) Effect of carbon sources, (b) Varying levels of the selected carbon source, (c) Nitrogen sources and (d) Varying levels of the selected nitrogen source on phytase production in submerged fermentation by *Bacillus subtilis* subsp. *subtilis* CM7

for phytase production. When different nitrogen sources were used for enzyme assay it was found that ammonium sulfate was the potent nitrogen source for phytase production (2.57±0.06 U mL^{-1}) followed by ammonium nitrate as shown in (Fig. 3c). Further study revealed 2% ammonium sulfate as an optimum concentration for phytase production (Fig. 3d).

Phytase production at different time intervals with optimized parameters was shown in Fig. 4. Phytase production increased gradually with the incubation period and maximum production was obtained after 6 days (2.61±0.04 U mL^{-1}). However, enzyme yield declined during further incubation.

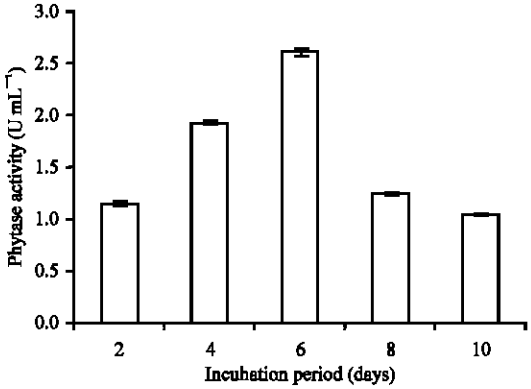

Fig. 4: Effect of incubation period on phytase production in Submerged Fermentation by *Bacillus subtilis* subsp. *subtilis* CM7

DISCUSSION

Phytases have a wide distribution in plants, microorganisms and in some animal tissues (Kim *et al*., 1999; Vohra and Satyanarayana, 2003). Reports on phytase activity in fish (LaVorgna, 1998; Ellestad *et al*., 2003) were contradictory and confusing. Presence of endogenous phytase activity in hybrids of tilapia *O. niloticus*×*O. aureus* (LaVorgna, 1998) and striped bass *Morone chrysops*×*M. sexatilis* (Ellestad *et al*., 2003) have been reported. Although, the apparent presence of intestinal phytase activity in fish might be capable of digesting only a tiny portion of the phytate-phosphorus present in the diet. Therefore, phytases produced by the symbiotic microorganisms might augment the phytase activity within the GI tract as evidenced in ruminants (Lan *et al.,* 2011). In the present study, autochthonous bacterial symbionts were isolated from the PI and DI of six species of freshwater carps and some of the strains appeared as efficient phytase producers. Heterotrophic and phytase producing populations were recorded highest in the DI regions of all the fish species studied which was in general complied with the previous reports (Ray *et al*., 2010; Mondal *et al*., 2008 ; Ghosh *et al*., 2010). Previously, (Li *et al*., 2008) documented phytase-producing marine yeast strains from the gut of sea cucumber (*Holothuria scabra*) and marine fish (*Hexagrammos otakii* and *Synecogobius hasts*). However, only a few reports have endorsed phytase activity to autochthonous gut bacteria from freshwater fishes (Roy *et al*., 2009; Khan *et al*., 2011; Khan and Ghosh, 2012a). Roy *et al*. (2009) isolated two strains of *B. licheniformis* from the PI and DI regions of an Indian Major Carp (IMC), *Labeo rohita* while (Khan *et al*., 2011) isolated a phytase-producing *Rhodococcus* sp., from the gut of another IMC, *Catla catla*. Further, Khan and Ghosh (2012a), investigated the presence of phytase producing autochthonous bacteria in the GI tracts of 14 freshwater teleost fishes. Two efficient phytase producing strains isolated from *L. bata* and *Gudusia chapra* were identified as *B. subtilis* and *B. atropheus*, respectively. In the present study, phytase-producing strains were evaluated through quantitative phytase assay and the most promising strain (CM7) was identified as *B. subtilis* subsp. *subtilis* based on 16SrDNA sequence analysis as suggested elsewhere (Roy *et al*., 2009; Ghosh *et al*., 2010; Mondal *et al*., 2010; Ray *et al*., 2010). To the authors' knowledge, different strains of exo-enzyme producing *Bacillus* spp., have been identified from the GI tract of freshwater teleosts previously (for review see Ray *et al*., 2012). *Bacillus* spp., might hold added interest in nutrition and probiotics studies as they can be kept in the spore form and therefore stored for a long lime (Hong *et al*., 2005).

An effort was made in the present study to optimize the important physical, chemical and nutritional parameters that influence phytase production by the most promising phytase-producing fish gut bacterium, *Bacillus subtilis* subsp. *subtilis* (Genbank Accession No.: JX292128). Previous reports indicated maximum phytase production by most of the microorganisms in acidic pH, although, *Bacillus* spp., were reported to have optimum activity at neutral/alkaline pH (Greiner and Konietzny, 2006). The optimal cultivation condition for phytase production (2.61±0.04 U mL^{-1}) by the bacteria was pH 7 which was consistent with (Khan and Ghosh, 2012b). Optimum phytase production at pH 7 in the present study might be due to the fact that the bacterial symbiont used in the SSF was isolated from the gut of an agastric fish (*Cirrhinus mrigala*) and the bacterium was adapted to the neutral/alkaline pH therein (Khan and Ghosh, 2012b). The study registered sharp decline in enzyme production at a pH which was higher or lower than pH 7.0 (Fig. 2a). A pH beyond the optimum level may interfere with the amino acid composition of the enzyme and thereby decreases the enzyme activity (Esakkiraj *et al*., 2009).

Optimum temperature for phytase production was 35°C which was favourable temperature for fresh water as well as fish gut system. Khan and Ghosh (2012b) also reported 35°C as the optimum temperature for phytase production under solid state fermentation by *B. subtilis* isolated from a minor carp, *Labeo bata*. As enzyme is a secondary metabolite produced during exponential growth phase, incubation at higher temperature (>35°C) could lead to poor growth and low enzyme yield (Sabu *et al*., 2002). In the present study, phytase activity was gradually increased with inoculum concentration up to 2.5 mL, but then declined for further concentration. Higher concentrations of inoculum were inhibitory for phytase production giving minimum enzyme yield (Sabu *et al*., 2002) at highest inoculum size (5 mL). Reduced enzyme production at higher concentrations might be due to increased competition for nutrient uptake and exhaustion of nutrients creating nutrient imbalance (Ramachandran *et al*., 2005; Roopesh *et al*., 2006). On the contrary, lower concentrations may not be sufficient to effect maximal enzyme production (Sabu *et al*., 2002). It is very important to examine the effects of different concentrations of NaCl in the production medium. Li *et al*. (2008) mentioned that added NaCl was the most suitable for phytase production by the marine yeast and also indicate that the marine yeast strain could produce a high

level of phytase in the production medium prepared with seawater. In the present study added NaCl also suitable for phytase production, although the bacterial strain was isolated from a fish taken from freshwater habitat. The strain CM7 produced large amount of phytase under 4% NaCl concentration which indicates that it has high salt tolerance ability and it is suitable for industrial application.

Type and nature of carbon and nitrogen sources are among the most important factors for any fermentation process (Pandey *et al*., 2001). Carbon source represents the energy source that will be available for the growth of the microorganism (Roopesh *et al*., 2006). Here, 1% starch exhibits maximum enzyme activity (Fig. 3b). Starch has been reported to enhance phytase production by *Aspergillus niger* (Vats and Banerjee, 2002) and *Mucor racemosus* (Roopesh *et al*., 2006). Li *et al*. (2008) reported glucose was the best carbon source in submerged culture with the marine yeast strain *Kodamea ahmeri* BG3 isolated from the gut of a marine fish, *Hexagrammes otakii*. Among the nitrogen sources supplemented ammonium sulphate showed best phytase production (Fig. 3c). Li *et al*. (2008) also documented ammonium sulfate to increase phytase production by the marine yeast strain *Kodamea ohmeri* BG3. In the present study, maximum phytase production was yielded after 6 days incubation (Fig. 4). However, enzyme yield declined during further incubation probably due to reduced nutrient level in the medium. Otherwise, it could also be the result of poisoning and denaturation of the enzyme by interaction with other components in the medium (Ramesh and Lonsane, 1987).

CONCLUSION

This study led to conclude that pH, temperature, carbon and nitrogen sources play a most vital role in phytase production by fish gut bacteria, *Bacillus subtilis* subsp. *subtilis*. Therefore, submerged fermentation was conducted to optimize the medium for highest phytase production by this bacterial strain. Microorganisms are the best sources for commercial phytase production because of their easy cultivation and high yield (Li *et al*., 2008). Phytases have a wide range of applications in animal and human nutrition as it can reduce phosphorus excretion of monogastric animals by replacing inorganic phosphates in the animal diet, contribute significantly toward environmental protection and lead to improved availability of minerals, trace elements, amino acids and energy (Vats and Banerjee, 2002). Incorporation of phytase into commercial fish diets has also been reported to achieve above mentioned presumed benefits (Sardar *et al*., 2007; Cao *et al*., 2008). However, still fish gut bacteria is an untouched bioresource for enzyme production. This present study reports production of phytase in submerged fermentation by the selected strain of *Bacillus subtilis* subsp. *subtilis* CM7 isolated from the gut of a freshwater major carp, *Cirrhinus mrigala*. Our study revealed that fish gut bacteria can produce a large amount of extracellular phytase which was in agreement with the previous reports (Roy *et al*., 2009). Such phytase producing microorganisms might aid in degradation of phytate within the guts of the host animal (Li *et al*., 2008). Therefore, apart from the likely application of exogenous phytase, possibilities for direct inclusion of such organisms as probiotics should be emphasized in future studies to improve availability of essential minerals and nutrients from the plant ingredient incorporated diets.

ACKNOWLEDGMENTS

The authors are grateful to the Head, Department of Zoology, The University of Burdwan, West Bengal, India; The Department of Science and Technology (FIST Programme), New Delhi, India and The University Grants Commission [UGC-SAP programme and Major Research Project F. No. 37-383/2009(SR)], New Delhi, India for providing research facilities and financial support. The first author is grateful to the UGC for awarding the Project Fellowship.

REFERENCES

Baruah, K., N.P. Sahu, A.K. Pal, K.K. Jain, D. Debnath and S.C. Mukherjee, 2007. Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. Aquacult. Res., 38: 109-120.

Beveridge, M.C.M., P.K. Sikdar, G.N. Frerichs and S. Millar, 1991. The ingestion of bacteria in suspension by the common carp *Cyprinus carpio* L. J. Fish Biol., 39: 825-831.

Cao, L., Y. Yang, W.M. Wang, A. Yakupitiyage, D.R. Yuan and J.S. Diana, 2008. Effects of pretreatment with microbial phytase on phosphorous utilization and growth performance of Nile tilapia (*Oreochromis niloticus*). Aquacult. Nutr., 14: 99-109.

Das, K.M. and S.D. Tripathi, 1991. Studies on the digestive enzymes of grass carp, *Ctenopharyngodon idella* (Val.). Aquaculture, 92: 21-32.

Debnath, D., A.K. Pal, N.P. Sahu, K.K. Jain, S. Yengkokpam and S.C. Mukherjee, 2005a. Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. Aquacult. Res., 36: 180-187.

Debnath, D., N.P. Sahu, A.K. Pal, K.K. Jain, S. Yengkokpam and S.C. Mukherjee, 2005b. Mineral status of *Pangasius pangasius* (Hamilton) fingerlings in relation to supplemental phytase: Absorption, whole body and bone mineral content. Aquacult. Res., 36: 326-335.

Ellestad, L.E., G. Dahl, R. Angel and H.J. Soares, 2003. The effect of exogenously administered recombinant bovine somatotropin on intestinal phytase activity and *in vivo* phytate hydrolysis in hybrid striped bass *Morone chrysops* x *M. sexatilis*. Aquacult. Nutr., 9: 327-336.

Esakkiraj, P., G. Immanuel, S.M. Sowmya, P. Iyapparaj and A. Palavesam, 2009. Evaluation of protease producing ability of fish gut isolate *Bacillus cereus* for aqua Feed. J. Food Bioprocess Technol., 2: 383-390.

Forster, I., D.A. Higgs, B.S. Dosanjh, M. Rowshandeli and J. Parr, 1999. Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (*Oncorhynchus mykiss*) held in 11°C fresh water. Aquaculture, 179: 109-125.

Ghosh, K., M. Roy, N. Kar and E. Ringø, 2010. Gastrointestinal bacteria in rohu, *Labeo rohita* (Actinopterygii: Cypriniformes: Cyprinidae): Scanning electron microscopy and bacteriological study. Acta Ichthyol. Piscatoria, 40: 129-135.

Greiner, R. and U. Konietzny, 2006. Phytase for food application. Food Technol. Biotechnol., 44: 125-140.

Han, Y.M., F. Yang, A.G. Zhou, E.R. Miller, P.K. Ku, M.G. Hogberg and X.G. Lei, 1997. Supplemental phytases of microbial and cereal sources improve dietary phytate phosphorus utilization by pigs from weaning through finishing. J. Anim. Sci., 75: 1017-1025.

Hong, H.A., L.H. Duc and S.M. Cutting, 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev., 29: 813-835.

Howson, S.J. and R.J. Davis, 1983. Production of phytate-hydrolysing enzyme by fungi. J. Enzyme Microbiol. Technol., 5: 377-382.

Khan, A., S. Mandal, D. Samanta, S. Chatterjee and K. Ghosh, 2011. Phytase-producing *Rhodococcus* sp. (MTCC 9508) from fish gut: A preliminary study. Proc. Zool. Soc., 64: 29-34.

Khan, A. and K. Ghosh, 2012a. Characterization and identification of gut-associated phytase-producing bacteria in some fresh water fish cultured in ponds. Acta Ichthyol. Pisc., 42: 37-45.

Khan, A. and K. Ghosh, 2012b. Evaluation of phytase production by fish gut bacterium, *Bacillus subtilis* for processing of *Ipomea aquatica* leaves as probable aquafeed ingredient. J. Aquat. Food Prod. Technol. (In press).

Kim, D.S., J.S. Godber and H.R. Kim, 1999. Culture conditions for a new phytaseproducing fungus. Biotechnol. Lett., 21: 1077-1081.

Kinnear P.R. and C.D. Gray, 2000. SPSS for Windows Made Simple. Release 10. Psychology Press, Sussex, UK.

Konietzny, U. and R. Greiner, 2004. Bacterial phytase: Potentioal application, *in vivo* function and regulation of its synthesis. Brazilian J. Microbiol., 35: 11-18.

LaVorgna, M., 1998. Utilization of phytate phosphorus by tilapia. Ph.D. Thesis, University of Maryland, Eastern Shore, Princess Anne, MD., USA.

Lan, G.Q., N. Abdullah, S. Jalaludin and W.Y. Ho, 2011. Purification and characterization of a phytase from *Mitsuokella jalaludinii*, a bovine rumen bacterium. Afr. J. Biotechnol., 10: 12766-12776.

Lei, X.G. and C.H. Stahl, 2000. Nutritional benefits of phytase and dietary deteminants of its efficacy. J. Applied Anim. Res., 17: 97-112.

Li, M.H. and E.H. Robinson, 1997. Microbial phytase can replace inorganic phosphorus supplements in channel catfish *Ictalurus punctatus* diets. J. World Aquacult. Soc., 28: 402-406.

Li, X., Z. Chi, Z. Liu, K. Yan and H. Li, 2008. Phytase production by a marine yeast *Kodamea ohmeri* BG3. Applied Biochem. Biotechnol., 149: 183-193.

Lowry, O.H., N.J. Rosendrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with folin-phenol reagent. J. Biol. Chem., 193: 265-275.

Maga, J.A., 1982. Phytate: Its chemistry, occurrence, food interactions, nutritional significance and methods of analysis. J. Agric. Food Chem., 30: 1-9.

Mondal, S., T. Roy, S.K. Sen and A.K. Ray, 2008. Distribution of enzyme-producing bacteria in the digestive tracts of some freshwater fish. Acta Ichthyol. Et Piscatorial, 38: 1-8.

Mondal, S., T. Roy and A.K. Ray, 2010. Characterization and identification of enzyme-producing bacteria isolated from the digestive tract of bata, *Labeo bata*. J. World Aquacult. Soc., 41: 369-377.

Oatway, L., T. Vasanthan and J.H. Helm, 2001. Phytic acid. Food Rev. Int., 17: 419-431.

Pandey, A., C.R. Soccol, J.A. Rodriguez-Leon and P. Nigam, 2001. Solid-State Fermentation in Biotechnology: Fundamentals and Applications. 1st Edn., Asiatech Publishers Inc., New Delhi, ISBN: 81-87680-06-7, pp: 221.

Ramachandran S., A. Bairagi and A.K. Ray, 2005. Improvement of nutritive value of grass pea (*Lathyrus sativus*) seed meal in the formulated diets for Rohu, *Labeo rohita* (Hamilton) fingerlings after fermentation with a fish gut bacterium. Bioresour. Technol., 96: 1465-1472.

Ramesh, M.V. and B.K. Lonsane, 1987. Solid state fermentation for production of amylase by *Bacillus megaterium* 16M. Biotechnol. Lett., 9: 323-328.

Ray, A.K., A. Bairagi, K.S. Ghosh and S.K. Sen, 2007. Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acta Ichthyologica et Piscatoria, 37: 47-53.

Ray, A.K., T. Roy, S. Mondal and E. Ringø, 2010. Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. Aquacult. Res., 41: 1462-1469.

Ray A.K., K. Ghosh and E. Ringø, 2012. Enzyme-producing bacteria isolated from fish gut: A review. Aquacult. Nutr., 18: 465-492.

Reddy, N.R., M.D. Pierson, S.K. Sathe and D.K. Salunkhe, 1989. Phytates in Cereals and Legumes. CCRC Press, BocaRaton, FL.

Ringø, E. and T.H. Birkbeck, 1999. Intestinal microflora of fish larvae and fry. Aquacult. Res., 30: 73-93.

Ringø, E., R.E. Olsen, T.M. Mayhew and R. Myklebust, 2003. Electron microscopy of the intestinal microflora of fish. Aquaculture, 227: 395-415.

Ringø, E., S. Sperstad, R. Myklebust, T.M. Mayhew and R.E. Olsen, 2006. The effect of dietary inulin on bacteria associated with hindgut of Arctic charr (*Salvelinus alpines* L.). Aquacult. Res., 37: 891-897.

Ringø, E., R.E. Olsen, T.O. Gifstad, R.A. Dalmo, H. Amlund, G.I. Hemre and A.M. Bakke, 2010. Prebiotics in aquaculture: A review. Aquacult. Nutr., 16: 117-136.

Rodehutscord, M. and E. Pffer, 1995. Effect of supplemental microbial phytase on phosphorus digestibilityand utilization in rainbow trout (*Oncorhynchus mykiss*). Water Sci. Technol., 31: 143-147.

Roopesh, K., S. Ramachandran, K.M. Nampoothiri, G. Szakacs and A. Pandey, 2006. Comparison of phytase production on wheat bran and oil cakes in solid-state fermentation by *Mucor racemosus*. Bioresour. Technol., 97: 506-511.

Roy, T., S. Mondal and A.K. Ray, 2009. Phytase producing bacteria in the digestive tracts of some freshwater fish. Aquacult. Res., 40: 344-353.

Sabu, A., S. Sarita, A. Pandey, B. Bogar, G. Szakacs and C.R. Soccol, 2002. Solid-state fermentation for production of phytase by *Rhizopus oligosporus*. Applied Biochem. Biotechnol., 102-103: 251-260.

Sardar, P., H.S. Randhawa, M. Abid and Prabhakar, 2007. Effect of dietary microbial phytase supplementation on growth performance, nutrient utilization, body compositions and haemato-biochemical profiles of *Cyprinus carpio* (L.) fingerlings fed soyprotein-based diet. Aquacult. Nutr., 13: 444-456.

Ullah, A., 1988a. Nonparametric estimation and hypothesis testing in econometric models. Empirical Econ., 13: 223-249.

Ullah, A., 1988b. Nonparametric estimation of econometric functionals. Can. J. Econ., 21: 625-658.

Van Weerd, J.H., Khalaf and T. Aartsen, 1999. Balance trials with African catfish *Clarias gariepinus* fed phytase-treated soybean meal based diets. Aquacult. Nutr., 5: 135-142.

Vats, P. and U.C. Banerjee, 2002. Studies on the production of phytase by a newly isolated strain of *Aspergillus niger* van Tieghem obtained from rotten wood-logs. Proc. Biochem., 38: 211-217.

Vohra, A. and T. Satyanarayana, 2003. Phytases: Microbial sources, production, purification and potential biotechnological applications. Crit. Rev. Biotechnol., 23: 29-60.

Volfova, O., J. Dvorakova, A. Hanzlkova and A. Jandera, 1994. Phytase from *Aspergillus niger*. Folia Microbiol., 39: 481-484.

Yanke, L.J., L.B.Selinger and K.J. Cheng, 1999. Phytase activity of *Selenomonas ruminantium*: A preliminary characterization. Lett. Applied Microbiol., 29: 20-25.

Zar, J.H., 1999. Biostatistical Analysis. 4th Edn., Pearson Education, Singapore, New Delhi, India.