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EVALUATION OF CHITINOLYTIC GUT MICROBIOTA IN SOME CARPS AND OPTIMIZATION OF CULTURE CONDITIONS FOR CHITINASE PRODUCTION BY THE SELECTED BACTERIA

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ABSTRACT

Present study was aimed at isolation of autochthonous chitinase-producing bacteria from the gastrointestinal tracts of 3 Indian Major Carps (*Labeo rohita, Catla catla, Cirrhinus mrigala*) and 3 exotic carps (*Hypophthalmichthys molitrix, Ctenopharyngodon idella, Cyprinus carpio*). Altogether, 119 bacteria were isolated from both the proximal and distal intestine and screened for chitinolytic activity. On the basis of chitin hydrolysis zone, 63 isolates were primarily selected for chitinase production, from which 34 potent strains were further studied for quantitative enzyme assay. Amongst them, the strains HMH1 and CMF2 exhibited potent chitinolytic activity and were identified as *Bacillus pumilus* (KF454036) and *Bacillus flexus* (KF454035), respectively by 16S rRNA partial gene sequence analysis. Optimization of various fermentation parameters (e.g., temperature, pH, inoculums size, surfactant, colloidal chitin concentration, incubation time, carbon sources, organic and inorganic nitrogen sources) were carried out in chitinase production medium. Incubation for 72 h at 35^{0}C and initial pH 7.5 revealed optimum chitinase productions by *B. pumilus* HMH1 in the media supplemented with colloidal chitin 0.1% (w/v), maltose 2% (w/v), ammonium sulphate 1.0% (w/v) and Tween-80 0.2% (v/v). However, *B. flexus* CMF2 required 48 h incubation at 35°C and initial pH 8.0 with colloidal chitin 0.15% (w/v), sucrose 1% (w/v), yeast extract 2.0% (w/v) and Tween-20 0.2% (v/v) supplementation for optimum yield. The results indicate that there is ample scope for further research to appraise fish gut microorganisms for chitinase production or as probiotics to improve feed efficiency in fish.

Keywords: *Bacillus pumilus*, *Bacillus flexus*, chitinase, fish gut bacteria

INTRODUCTION

Chitin $(C_8H_{13}O_5N)_n$ has been estimated as the second most abundant biomass in the world after cellulose forming structural component of many fish food organisms, including fungi, crustaceans, coelenterates, protozoan and green algae **(Rinaudo, 2006; Khoushab and Yamabhai, 2010)**. Chitin has been reported to make up 3.6% (wet weight) of the stomach contents of juvenile black sea bream, *Acanthopagrus schlegeli* **(Om *et al.*, 2003)** that indicates feeding of chitin rich organisms in fish. The ability to degrade chitin is considered to involve principally the action of the enzyme chitinase (EC 3.2.11.14) that hydrolyzes insoluble chitin to its oligo and monomeric components. Chitinases are present in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants and animals playing important physiological and ecological roles **(Cody *et al.*, 1990)**. To the authors' knowledge, the first investigation on chitin degradation by bacteria was made by **Benecke (1905)**, who reported chitinolytic *Bacillus chitinovorus* from the polluted waters of Kiel harbour.

Freshwater carps cultured in India mostly feed on plankton, algae, aquatic organisms and detritus representing omnivorous feeding aptitude **(Jhingran, 1997)**. The chitin content of various copepods (e.g., *Cyclops*, *Diaptomous* etc.) comprising natural food for the carp fry and fingerlings has been reported to range from 21 to 95 mg g^{-1} by dry weight **(Båmstedt, 1986)**. Being rich in nutrients, the micro-environment of fish gut confers a favourable niche for the microorganisms **(Kar and Ghosh, 2008)**, and the gut microbiota in fish is closely related to the food that they use to consume **(Han *et al.*, 2010)**. These distinct microbial communities may contribute uniquely to the nutrient cycling in the system **(Ringø *et al.*, 2012)**. Therefore, feeding on chitin rich components might suggest likely occurrence of the chitinase-producing gut microorganisms in fish. However, in comparison to the comprehensive work conducted on different enzyme-producing gut microorganisms in fish, information on the chitinolytic gut microorganisms are scarce (**Ray *et al.*, 2012**). Studies have indicated that fish feeding on chitin rich diets have higher chitinase activity **(Danulat, 1986; Gutowska *et al.*, 2004)**. Apart from such sporadic information, likely occurrence of chitinolytic bacteria in fish gut and their significance in feed utilization of the host species are inconclusive and contradictory.

Previous studies conducted with Indian major carps indicated beneficial aspects of gut associated enzyme-producing microbiota in the host fish with regard to nutrition **(Ghosh *et al.*, 2002a; 2002b; Ray *et al.*, 2010)**. Application of autochthonous chitinase-producing gut bacteria as probiotics or supplementation of bacterial chitinase as feed additive might be assumed as a strategy for effective utilization of the chitin rich natural feedstuffs in fish. However, screening and characterization of chitinase-producing autochthonus fish gut microorganisms can be viewed as a prerequisite for their likely application in fish. Microbial production of chitinase has drawn global attention not only because of its extensive application, but also for the need of effective producer organisms. Therefore, the present study aimed at (1) isolation and enumeration of chitinase-producing gut microorganisms in 3 Indian Major Carps and 3 exotic carps, (2) identification of the most promising chitinase-producing micro-organisms by 16S rRNA partial gene sequence analysis, and finally (3) optimization of the various process parameters that influence chitinase production by the promising bacterial strains, *Bacillus pumilus* HMH1 (KF454036) and *Bacillus flexus* CMF2 (KF454035).

MATERIALS AND METHODS

Experimental fishes

Three species of Indian major carps (rohu, *Labeo rohita*; catla, *Catla catla*; mrigal, *Cirrhinus mrigala*) and three species of exotic carps (silver carp,

Hypophthalmichthys molitrix; common carp, *Cyprinus carpio*; grass carp, *Ctenopharyngodon idella*) were examined in this study. Three specimens of each species were collected from three composite carp culture ponds located surrounding Burdwan (23°14′N, 87°39′E), West Bengal, India. Altogether, nine specimens of each species were used in the present study. Their food habits, average weight and length, average weight of the gut and average length of gut are presented in Table 1. The collection ponds were free of sewage release or other anthropogenic activities. The specimens were sampled by a gill-net and transported to the laboratory at Golapbag, Burdwan inside oxygen-packed plastic bags. Ranges of the water quality parameters during the collection period were: dissolved oxygen 6.5–7.8 mg L^{-1}, temperature 26.2–27.8 °C, and pH 6.8–7.2.

Table 1 Food habits, average live weight, average fish length, average gut weight and gut length of the fishes examined

Fish Species	Food habits*	Average fish live weight (g)	Average fish length (cm)	Average Gut weight (g)	Gut length (L_G) (cm.)
Catla, *Catla catla*	Planktophagous	370±10.97	29.4±2.34	12.18±0.59	224.5±7.76
Rohu, *Labeo rohita*	Omnivorous, mostly plant matter	260±13.44	30.5±2.61	11.32±0.62	271.7±8.51
Mrigal, *Cirrhinus mrigala*	Detrivorous	330±12.33	30.7±2.70	8.29±0.57	431.3±10.27
Silver carp, *Hypophthalmichthys molitrix*	Planktophagous	440±14.42	26.6±3.84	8.38±0.68	218.3±8.68
Grass carp, *Ctenopharyngodon idella*	Herbivorous, mostly macrophytes	450±10.88	28.9±2.21	16.7±0.55	63.2±8.39
Common carp,*Cyprinus carpio*	Detrivorous	375±13.44	27.4±2.37	7.81±0.58	47.3±9.81

Data are means ± S.D. of three determinations.
*adapted from Jhingran, 1997

Processing of specimens

The fishes were kept separately in de-chlorinated tap water in 100L fibre-glass aquaria according to their source and species. The fish were starved for 48 h to clear their gastro-intestinal (GI) tracts before being dissected and to remove most of the allochthonous microbiota associated with digesta. The fish were anaesthetized by applying 0.03% of tricaine methanesulfonate (MS-222). The ventral surface of each fish was surface sterilized by scrubbing with 1% iodine solution **(Trust and Sparrow, 1974)**. The fish were dissected aseptically on ice tray and their GI tracts were removed. The GI tracts were divided into PI (proximal part of the intestine including intestinal bulb) and DI (distal part of the intestine) parts of the intestine, cut into pieces and opened by a longitudinal incision, transferred to sterile Petri-dishes and flushed carefully 3 times with 0.9% sterile saline solution using an injection syringe in order to remove non-adherent (allochthonous) microbiota **(Ghosh *et al.*, 2010; Khan and Ghosh, 2012)**. Gut segments from three specimens of a species collected from the same pond were pooled together region-wise for each replicate, and there were three replicates for each gut segment. Gut segments were homogenized with sterilized pre-chilled 0.9% sodium chloride solution (1:10; w/v) **(Beveridge *et al.*, 1991)**. Pooled samples of 3 fish were utilized for each replicate to avoid erroneous conclusions due to individual inconsistency in gut microorganisms, as described somewhere else **(Ringø *et al.*, 1995; Spanggaard *et al.*, 2000; Ringø *et al.*, 2006)**.

Microbial Culture

Homogenate of the pooled gut segments of each of the three replicates for each fish species and each region of gut was used separately after serial (1:10; up to 10^{-5}) dilutions **(Beveridge *et al.*, 1991)**. Diluted samples (100 µL) were spread aseptically within a laminar airflow on sterilized tryptone soy agar (TSA; HiMedia, India) plates and incubated at 30 °C for 48 h to determine culturable heterotrophic autochthonous microbiota. Chitinase producing microorganisms were isolated by spreading the diluted homogenate (100 µL) on sterilized colloidal chitin agar plates and incubated at 30 °C for 72 h. It was assumed that the microbiota, which had formed colonies on the selective colloidal chitin agar plates, had chitin degrading activity. Colony forming units (CFU) per unit sample volume of gut homogenate were determined by multiplying the number of colonies formed on each plate by the reciprocal dilution **(Rahmatullah and Beveridge, 1993)** and data were transformed as log viable counts (LVC). Colloidal chitin was prepared from the chitin flakes (Hi Media, India) following the modified method of **Roberts and Selitrennikoff (1988)**. Chitin flakes were ground to powder, 5g of powder was added slowly to 90 mL concentrated HCl and stirred vigorously for 2 h. Ice-cold 95% ethanol (500 mL) was added to it under vigorous stirring for 30min, kept overnight at 25 °C and stored at -20 °C until use. The precipitate was collected by centrifugation at 10,000 × *g* for 15 min and washed with 0.1 M sodium phosphate buffer (pH 7) until the colloidal chitin became neutral (pH 7.0) **(Ahmadi *et al.*, 2008)**. The well-separated colonies appeared on colloidal chitin agar plates were randomly picked and streaked separately on TSA plates to obtain pure cultures. Isolates were individually cultured on the colloidal chitin agar plates at 30 °C for 5 days and appearance of clear zone (due to chitin degradation) surrounding the colonies indicated positive result of chitinase production. Isolates (colony size: 14.5±4.5 mm) that produced a halo ≥25 mm (in excess of microbial colony) were selected for quantitative enzyme activity.

Evaluation of quantitative chitinase activity

Quantitative chitinase assay was carried out using colloidal chitin as substrate. Growth in colloidal chitin broth was centrifuged at 5,000 × *g* for 5 min at 4 °C and the cell-free supernatant was used as the crude enzyme. The assay mixture containing 0.5% colloidal chitin (1 mL), 25 mM sodium phosphate buffer (0.5 mL, pH7.0) and crude enzyme (0.5 mL) was incubated for 1 h at 40 °C following **Waghmare *et al.* (2010)**. The reducing sugars produced reacted with di-nitro salicylic acid (DNSA) and expressed as N-acetyl-β-D-glucosamine standards to demonstrate the chitinase activity **(Miller, 1959)**. Enzyme activity (U) was defined as the µg of N-acetyl-β-D-glucosamine liberated mg^{-1} protein min^{-1}. Protein content of the enzyme extract was measured using bovine serum albumin as standard **(Lowry *et al.*, 1951)**.

Identification of Isolates by 16S rRNA gene Sequence Analysis

The most promising two chitinase producing strains were identified through 16S rRNA partial gene sequence analysis after isolation and PCR amplification following the methods described in **Das *et al.* (2014)**. The gene encoding 16S rRNA was amplified from the isolates by polymerase chain reaction (PCR) using universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR reactions were performed using PCR mix containing 200 µM of deoxynucleotides (dNTPs), 0.2 µM of each primer, 2.5 mM $MgCl_2$, 1 × PCR buffer and 0.2 U of Taq DNA polymerase (Invitrogen). To extract genomic DNA for obtaining template DNA from it, colonies were suspended in sterilized saline, centrifuged and the pellet suspended in InstaGene Matrix (Bio-Rad, USA). The cycle used for PCR reaction was: 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 1 min, extension at 72 °C for 1 min **(Lane, 1991)**. PCR products were purified by using Montage PCR Clean up kit (Millipore, USA). Sequencing of the purified PCR products were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an automated DNA sequencing system (Applied BioSystems 3730XL, USA). Sequenced data were edited using BioEdit Sequence Alignment Editor (Version 7.2.0), aligned and analyzed for finding the closest homolog using National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP) databases. Sequences were deposited to the NCBI GenBank and accession numbers were obtained. Phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of the closest type strains using MEGA 5.1Beta4 software following the Minimum Evolution Method.

Optimization of enzyme production

Submerged fermentation was carried out by both of the strains, CMF2 and HMH1 to optimize various process parameters influencing chitinase production. Optimization of various process parameters were carried out in chitinase production medium containing inorganic salts (g/L): 0.7 g KH_2PO_4; 0.3 g K_2HPO_4; 4 g NaCl; 0.5 g $MgSO_4$, $7H_2O$; 1 mg $FeSO_4$, $7H_2O$; 0.1 mg $ZnSO_4$, $7H_2O$ and 0.1 mg $MnSO_4$, $7H_2O$. The parameters studied were: incubation temperature (25^0C - 50^0C), initial pH of the media (5 - 9), inoculum volume (1% – 5%), surfactant (0.2%, v/v) (Tween 20, Tween 40, Tween 80, DMSO), colloidal chitin (0.5-3.0 gL^{-1}) as substrate and incubation period (24 h – 120 h). Further, the medium was supplemented with different carbon sources (1%, w/v) (glucose, sucrose, lactose, maltose and starch) and organic/inorganic nitrogen sources (1%, w/v) (ammonium sulfate, ammonium nitrate, peptone, yeast extract, ammonium chloride and tyrosine). The selected carbon and nitrogen sources were varied within a narrow range (1%-5%) to optimize chitinase production.

Statistical Analysis

Statistical analysis of the quantitative enzyme activity data was performed by the analysis of variance (ANOVA) followed by Tukey's test according to **Zar (1999)** using SPSS Ver10 **(Kinnear and Gray, 2000)**.

RESULTS

Enumeration of gut microbial community in the 6 fish species studied revealed that autochthonous culturable heterotrophic and chitinase producing microorganisms 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 and chitinase- producing bacteria were highest in the DI regions of all the fish species studied. Maximum counts of chitinase-producing bacteria were noticed in the DI region of silver carp, *H. molitrix* (LVC=2.35 g^{-1} intestinal tissue) followed by the DI region of mrigal, *C. mrigala* (LVC=2.27 g^{-1} intestinal tissue).

Table 2 Log values of culturable autochthonous aerobic / facultative anaerobic heterotrophic (grown on TSA plates) and chitinase-producing (grown on colloidal chitin agar plates) bacteria isolated from the GI tracts of 3 Indian Major Carps and 3 exotic carps

Fish Species	Log viable counts (g^{-1} intestinal tissue)			
	Proximate intestine(PI)		Distal intestine(DI)	
	TSA	CCA	TSA	CCA
C. catla	5.84	1.46	6.87	1.95
L. rohita	5.25	1.92	6.33	2.05
C. mrigala	5.33	2.14	6.17	2.27
H. molitrix	4.85	1.96	6.44	2.35
C. idella	5.11	1.25	6.39	1.39
C. carpio	4.76	1.65	5.94	2.23

TSA-Tryptone soy agar; CCA- Colloidal Chitin Agar

Out of the 119 randomly selected isolates, 63 chitinase-producing bacteria were primarily selected by qualitative enzyme assay, which were further evaluated by quantitative enzyme assay. Determination of chitinase activity led to select 34 strains (21 from PI and 13 from DI), results of which are depicted in Table 3. The maximum chitinolytic activity was noticed with the strain HMH1 (11.95 ± 0.34 U) isolated from DI of *H. molitrix*, followed by the strain CMF2 (10.82 ± 0.31 U) isolated from the PI of *C. mrigala.* Therefore, considering the results of chitinase activity, the isolates HMH1 and CMF2 were finally selected for identification and studied for chitinase production under submerged fermentation for likely use in future.

Table 3 Chitinase activity of the gut bacterial strains selected through quantitative enzyme assay

Fish species	Strains	Chitinase (U)	Fish species	Strains	Chitinase (U)
Labeo rohita	LRF1	3.55 ± 0.14^{cd}	*Hypophthalmichthys molitrix*	HMF1	10.66 ± 0.34^{hi}
	LRF6	7.08 ± 0.21^{e}		HMF5	10.23 ± 0.39^{h}
	LRF2	7.11 ± 0.29^{e}		HMF7	9.71 ± 0.23^{g}
	LRH4	4.23 ± 0.16^{d}		HMF2	9.46 ± 0.28^{g}
	LRH8	6.96 ± 0.28^{e}		HMH6	10.77 ± 0.33^{hi}
Catla catla	CCF6	9.78 ± 0.41^{h}		HMH1	11.95 ± 0.34^{j}
	CCF7	7.83 ± 0.33^{f}	*Ctenopharyngodon idella*	CtIF6	3.03 ± 0.13^{bc}
	CCF1	8.32 ± 0.35^{fg}		CtIF5	2.84 ± 0.11^{b}
	CCF2	7.18 ± 0.29^{e}		CtIH1	3.11 ± 0.14^{c}
	CCF4	6.88 ± 0.27^{e}		CtIH2	3.19 ± 0.19^{c}
Cirrhinus mrigala	CMF2	10.82 ± 0.31^{i}	*Cyprinus carpio*	CyCF2	2.32 ± 0.11^{a}
	CMF3	8.01 ± 0.25^{f}		CyCF3	3.35 ± 0.18^{c}
	CMF4	7.77 ± 0.23^{f}		CyCH6	4.17 ± 0.18^{d}
	CMF5	9.58 ± 0.39^{g}		CyCH4	3.76 ± 0.11^{d}
	CMF6	7.84 ± 0.25^{f}		CyCH1	4.42 ± 0.17^{d}
	CMH8	9.13 ± 0.27^{g}		CyCH9	2.83 ± 0.06^{b}
	CMH9	8.47 ± 0.37^{fg}			
	CMH11	8.11 ± 0.28^{f}			

Data are means ± S.E. of three determinations. Means with same superscript in the same column do not vary significantly ($P<0.05$).
U= µg N-acetyl-β-D-glucosamine liberated mg^{-1} protein min^{-1}

Nucleotide homology and phylogenetic analysis of the 16S rRNA partial gene sequences by nucleotide blast in the National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP) databases revealed that the strains HMH1 and CMF2 were *Bacillus pumilus* (GenBank Accession no. KF454036) and *Bacillus flexus* (GenBank Accession no. KF454035), respectively. The isolate HMH1 showed 98% similarity with *Bacillus pumilus* (GenBank Accession no. NR112637), while the isolate CMF2 showed 99% similarity with *Bacillus flexus* (GenBank Accession no. NR024691). Phylogenetic relation of the two identified chitinolytic bacteria with other closely related type strains retrieved from the RDP database are presented in the dendogram (Figure 1).

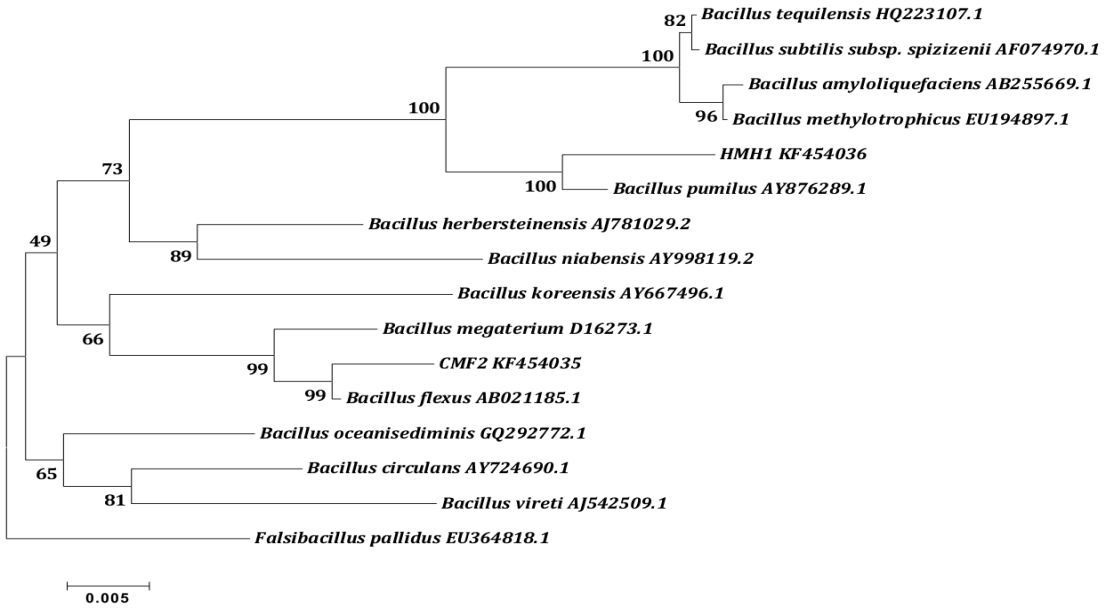

Figure 1 Dendogram showing phylogenetic relations of the two most promising chitinase producing bacterial strains, *Bacillus pumilus* HMH1 (KF454036) and *Bacillus flexus* CMF2 (KF454035) with other closely related type strains retrieved from NCBI GenBank. GenBank accession numbers of the reference strains are shown in parentheses. Horizontal bars in the dendogram represent branch length. Similarity and homology of the neighbouring sequences are shown by the bootstrap values. Distance matrix calculated by Tamura 3-parameter following Minimum Evolution Method. Scale bar=0.005 substitutions per nucleotide position. *Falsibacillus pallidus* EU36818.1 served as an out-group.

Optimum temperature for chitinolytic activity by the both strains, *B. pumilus* HMH1 and *B. flexus* CMF2 were noticed to be 35°C (13.65 ± 0.31 and 10.21 ± 0.18 U, respectively) (Figure 2a). Further increase in temperature resulted in decrease in the enzyme yield. Initial pH of the medium required for chitinase production by the strains was evaluated at various pH levels (5.0-9.0). Within the tested pH range, pH 7.5 was optimum for chitinase production by *B. pumilus* HMH1 (14.08 ± 0.25 U), whereas, chitinase yield was the maximum by *B. flexus* CMF2 (11.48 ± 0.21 U) at pH 8 (Figure 2b).

Effect of percentage of inoculum on chitinase production has been depicted in Figure 2.c. Chitinase production gradually increased with increase of inoculum percentage leading to maximum enzyme yield at 3.0% for *B. pumilus* HMH1 (14.23 ± 0.26 U) and 2.5% for *B. flexus* CMF2 (11.87 ± 0.19 U), thereafter declined with further increase in the concentration. Influence of various surfactants on chitinase production was determined by adding different surfactants viz. Tween 20, Tween 40, Tween 80, DMSO in the production medium at fixed volume (0.2%, v/v) and presented in Figure 2.d. The results evidenced maximum chitinase production by *B. pumilus* HMH1 with Tween 80 supplementation (14.55 ± 0.21 U), although, Tween 20 was the best for *B. flexus* CMF2 (11.75 ± 0.21 U).

Colloidal chitin was used in the production media as the substrate, as well as the carbon source. Among the tested levels, 0.1% and 0.15% of colloidal chitin supported maximum chitinase production by *B. pumilus* HMH1 (14.64 ± 0.23 U) and *B. flexus* CMF2 (11.96 ± 0.21 U), respectively (Figure 2e). Chitinase productions at different time intervals are presented in Figure 2f. Enzyme production increased gradually with incubation time, and maximum production was obtained after 72 h (15.12 ± 0.26 U) and 48 h (12.25 ± 0.21 U) in *B. pumilus* HMH1 and *B. flexus* CMF2, respectively.

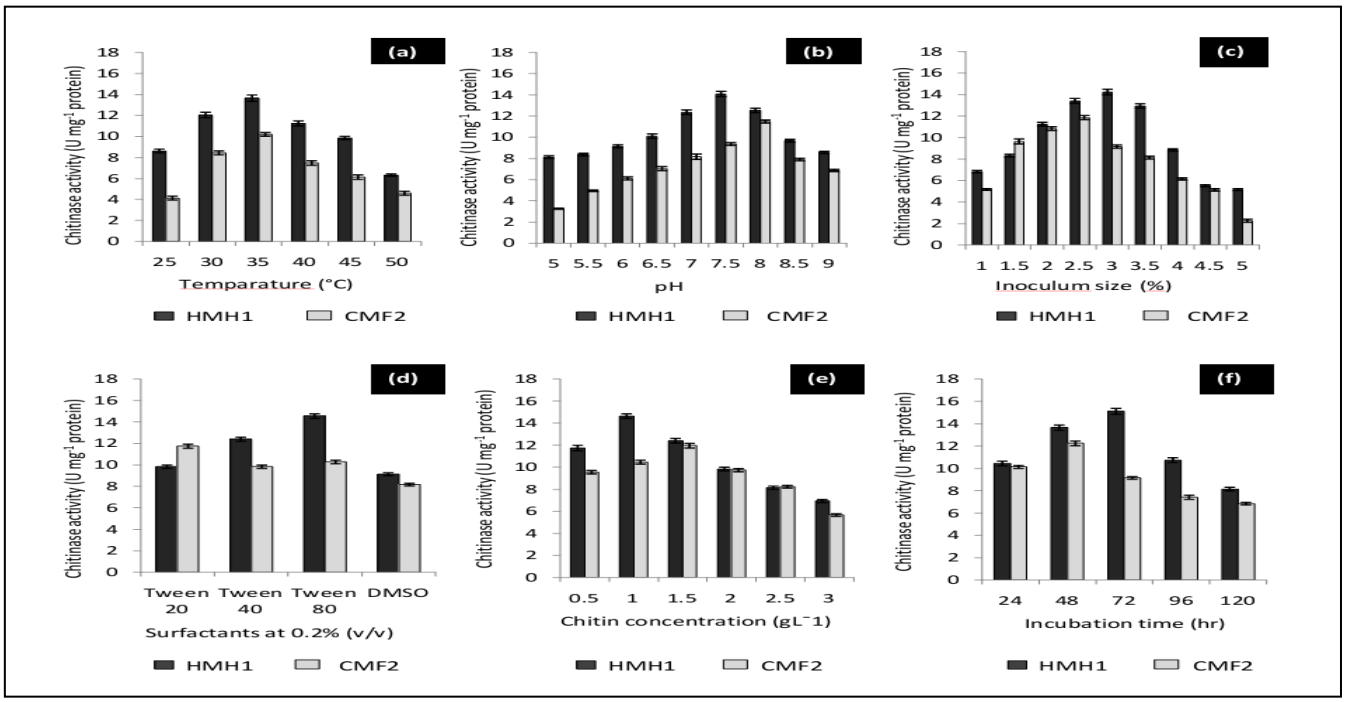

Figure 2 Effect of (a) temperature, (b) pH, (c) inoculum size (%, v/v), (d) surfactants (0.2%, v/v) (e) colloidal chitin (substrate) and (f) incubation period on chitinase production by *Bacillus pumilus* HMH1 and *Bacillus flexus* CMF2.

Optimization of various supplemented carbon sources (1%, w/v) revealed that maltose was the most effective carbon source for chitinase production by *B. pumilus* HMH1 (16.87 ± 0.06 U), while sucrose produced the best result for *B. flexus* CMF2 (14.53 ± 0.06 U) (Figure 3a). Although, further increase in the sucrose level diminished chitinase production by *B. flexus* CMF2, supplementation of 2% maltose was noticed as optimum for *B. pumilus* HMH1 (16.97 ± 0.06 U) (Figure 3b,c).

Amongst the diverse organic and inorganic nitrogen sources (1%, w/v) evaluated, ammonium sulfate and yeast extract sustained maximum chitinase production by the strains, *B. pumilus* HMH1 (16.91 ± 0.06 U) and *B. flexus* CMF2 (14.42 ± 0.06 U), respectively (Figure 3d). Moreover, supplementation of additional ammonium sulfate (>1%) reduced chitinase production by *B. pumilus* HMH1. However, supplementation of yeast extract at 2% could maximize chitinase production by *B. flexus* CMF2 (14.59 ± 0.11 U) (Figure 3e, f). Finally, optimization of the fermentation parameters with *B. pumilus* HMH1 resulted in 41.5% increase in chitinase production over the initial value, whereas, chitinase production was increased by 42.9% in *B. flexus* CMF2.

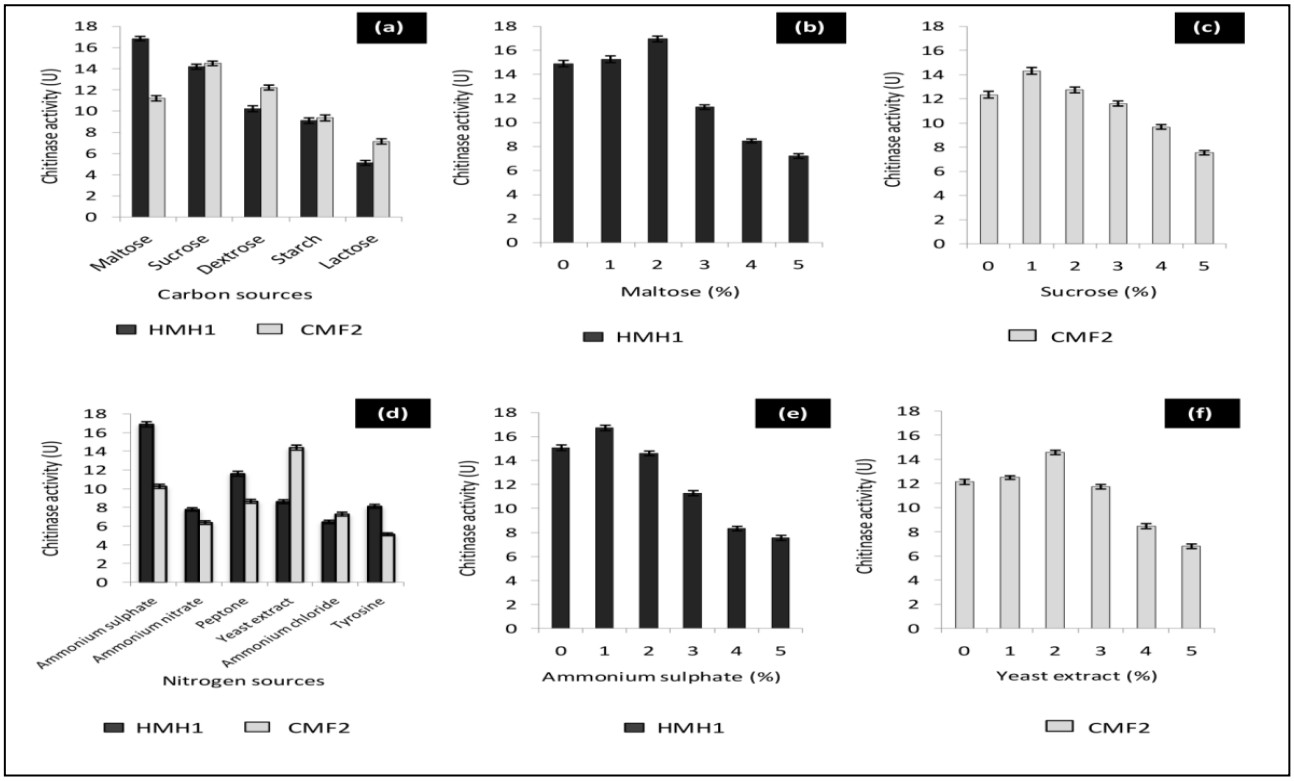

Figure 3 Effect of carbon sources (a) levels of the selected carbon sources (b, c) nitrogen sources (d) and levels of the selected nitrogen sources (e, f) on chitinase production by *Bacillus pumilus* HMH1 and *Bacillus flexus* CMF2.

DISCUSSION

Chitinolytic enzymes are present in a wide range of organisms such as bacteria, fungi, yeasts, plants, actinomycetes, arthropods, and also in vertebrates **(Hamid *et al.*, 2013)**. There is a growing interest on chitin hydrolysis in aquaculture as fish consume green algae, crustaceans, zooplanktons, etc. as their food source that contain considerable amount of chitin. Chitinases in the GI tract of fishes may come from the fish itself, its prey and/or the enteric bacteria. To the authors' knowledge, chitinolytic bacteria in the intestine of fish were recorded for the first time in a marine teleost, *Lateolabrax* **(Okutani, 1966)**. Since then, occurrences of chtinolytic bacteria within the GI tracts of marine fish species were well documented (**Ray *et al.*, 2012**) in comparison to their freshwater counter parts. In the present investigation, microbial symbionts were isolated from the GI tracts of 6 freshwater carp species and some of the isolates exhibited exogenous chitinase activity. It may be mentioned that the fish species examined were starved for 48 h and their GI tracts were thoroughly washed with sterile chilled 0.9% saline prior to isolation of microorganisms. Therefore, it is assumed that the microorganisms isolated in the present study belong to the autochthonous microbiota as suggested elsewhere **(Ray *et al.*, 2010; Ghosh *et al.*, 2010)**. Appreciable quantity of chitinase-producing microflora detected in the PI and DI segments of the GI tracts in the fish species studied may signify their probable role in degradation of ingested chitin through the food. Previously, the fish gut isolates have been demonstrated to break down chitin *in vivo* to aid in the digestion process **(Goodrich and Morita, 1977; Danulat and Kausch, 1984; MacDonald *et al.*, 1986; Kono *et al.*, 1987)**. Further, it may be mentioned that microbial population was found highest in DI regions of all the fish species studied when compared to the PI regions, which is in conformity with the earlier reports **(Mondal *et al.*, 2008; Ray *et al.*, 2010; Ghosh *et al.*, 2010)**. Although, endogenous chitinases and chitinase genes have been detected in teleosts **(Kurokawa *et al.*, 2004)**, this may not rule out the presence of extracellular bacterial chitinases representing symbiotic relationships **(Gutowska *et al.*, 2004)**.

In the present study, chitinase-producing strains were noticed through quantitative chitinase assay and the two most promising strains (HMH1 and CMF2) were identified as *B. pumilus* (GenBank Accession no. KF454036) and *B. flexus* (GenBank Accession no. KF454035), respectively, based on the 16S rRNA partial gene sequence analysis as suggested elsewhere **(Roy *et al.*, 2009; Ghosh *et al.*, 2010; Mondal *et al.*, 2010; Ray *et al.*, 2010)**. Previous reports have also shown that *Bacillus* spp. can produce chitinolytic enzymes **(Wen *et al.*, 2002; Chen *et al.*, 2004; Driss *et al.*, 2005; Waldeck *et al.*, 2006; Chang *et al.*, 2007)**, however, present study is the first one reporting chitinolytic bacilli from fish gut. Moreover, diverse strains of extracellular enzyme producing *Bacillus* spp. have been identified from the GI tract of freshwater teleosts (for review see **Ray *et al.*, 2012**), which are in accordance with the present report. Amongst the teleosts, previously, chitinolytic *Enterobacter*, *Vibrio* and *Pseudomonas* were reported from gray mullets **(Hamid *et al.*, 1979)**, while, chitinase producing *Aeromonas* and *Vibrio* were isolated from the GI tract of tilapia **(Sakata *et al.*, 1980)**. In another study, **Sakata and Koreeda (1986)** reported chitin degrading gut bacteria isolated from intestinal contents of tilapia (*Sarotherodon niloticus*) belonging to *Plesiomonas shigelloides* and *Aeromonas hydrophila*. Therefore, available literatures suggest that chitinolytic bacteria in the Indian Major Carps (IMC) or other carp species were not detected/evaluated so far, except in the common carp, *Cyprinus carpio* **(Sugita *et al.*, 1999)**.

Optimization of the important physical, chemical and nutritional parameters were carried out under submerged fermentation to evaluate chitinase production potential of the two most promising chitinase-producing bacteria detected in the present study. Temperature affects a variety of bioprocesses, therefore, the growth of microorganisms and enzyme production are also affected with alteration in incubation temperature. The highest chitinase activity by both the strains was recorded at 35^{0}C. Previous reports by **Narayana *et al.* (2009)** and

Sudhakar and Nagarajan (2011) also documented maximum chitinase production at 35^{0}C by soil isolates *Streptomyces* sp. ANU6277 and *Trichoderma harzianum*, respectively. In another study, *Bacillus laterosporus* produced high chitinase activity at 35 °C **(Shanmugaiah *et al*., 2008)**. Further, considerable levels of chitinase production at 30 °C might indicate adaptability of both the strains at the tropical water condition.

Initial pH of the production media not only helps in the chitinase production, but also plays an important role in cell growth **(Saima *et al.* 2013)**. The results revealed that pH 7.5 and 8 were optimum for chitinase production by *B. pumilus* HMH1 and *B. flexus* CMF2, respectively. Previous reports also suggested that *B. laterosporous* **(Shanmugaiah *et al*., 2008)**, *Micrococcus* sp. AG84 **(Annamalai *et al*., 2010)**, *Aeromonas* sp. JK1 **(Ahmadi *et al*., 2008)** and *B. pabuli* **(Frandberg and Schnurer, 1994)** were capable of producing a high amount of chitinase at alkaline condition. Optimum chitinase production at alkaline pH noticed in the present study might be due to the fact that the bacterial symbiont were isolated from the gut of agastric carps and the bacterium was adapted to the alkaline pH therein as evidenced for phytase-producing gut bacteria in some carp species **(Khan and Ghosh, 2013)**. A pH beyond the optimum level may alter the amino acid composition of the enzyme and thereby diminishes the enzyme activity **(Esakkiraj *et al*., 2009)**. Chitinase activity gradually increased with increase in inoculum concentration up to 2.5-3.0, and thereafter declined in further concentrations. Reduced enzyme production at higher concentrations of inoculum might be due to increased competition for nutrient uptake and exhaustion of nutrients creating nutrient imbalance **(Ramachandran *et al*., 2005; Roopesh *et al*., 2006)**. Surfactants might influence the growth and extracellular enzyme production of the microorganisms. In the present study, supplementation (0.2%, v/v) of Tween 80 and Tween 20 resulted in optimum chitinase production by *B. pumilus* and *B. flexus*, respectively. Several researchers have shown that incorporation of surfactants could induce the formation of smaller pellets leading to increase in the extracellular enzyme synthesis **(Sasirekha *et al.*, 2012)**, or increase the cell wall/cell membrane permeability leading to the concurrent increase in the secretion of biomolecules **(Das *et al.*, 2013)**.

Several studies have reported colloidal chitin (CC) as the best substrate for chitinase production by *Streptomyces viridificans* **(Gunaratna and Balasubramanian, 1994)**, *Streptomyces lydicus* WYEC108 **(Monreal and Reese, 1969)**, *Acremonium obclavatum* **(Gunaratna and Balasubramanian, 1994)** and *Aeromonas* spp. **(Saima *et al.*, 2013)**. The present study also noticed 0.1% and 0.15% of colloidal chitin to support optimum chitinase production by *B. pumilus* HMH1 and *B. flexus* CMF2, respectively, which were much lower than the observations made by **Souza *et al.* (2005), Karunya *et al.* (2011)** and **Saima *et al.*, (2013)**, who reported the maximum chitinase production at 0.3% colloidal chitin. Colloidal chitin has been reported to act as a sole carbon and nitrogen sources for chitinase production **(Faramarzi *et al.*, 2009)**. Conversely, presence of CC along with other carbon sources (e.g., sucrose) augmented chitinase production several folds by *Bacillus subtilis* **(Karunya *et al.*, 2011)** and *Thermococcus chitonophagus* **(Andronopoulou and Vorgias, 2004)**. Our study also revealed that 1% sucrose and 2% maltose supplementation improved chitinase production by *B. flexus* CMF2 and *B. pumilus* HMH1, respectively. Ammonium sulfate and yeast extract resulted in the maximum chitinase production by the strains, *B. pumilus* HMH1 and *B. flexus* CMF2, respectively. Previously, urea was found to be the suitable nitrogen source for chitinase production by *Paenibacillus* sp. D1 **(Singh *et al*., 2009)**. Whereas, **Saima *et al.* (2013)** reported that malt extract and yeast extract were the most favorable nitrogen source in *A. hydrophila* HS4 and *A. punctata* HS6, respectively. Optimization of the incubation period was done to see the cumulative effect of various process parameters. Our study revealed that 48-72 h of incubation supported the highest chitinasae production by *B. flexus* CMF2 and *B. pumilus* HMH1, respectively. Similar observations were also reported by **Wang and Hwang (2001)** that *B. cereus*, *B. alvei* and *B. sphaericus* produced highest chitinase after 48 h of incubation. Incubation for longer duration might cause decline in enzyme yield due to reduced nutrient level in the medium or 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

Diverse fish species appear to utilize chitin at different levels. Chitin is well utilized by many marine fish that may be linked to their natural diet as many fish species, such as Atlantic cod, eat chitin-rich prey like crabs **(Ringø *et al.*, 2012)**. In general, cyprinids (e.g., carps) utilize chitin relatively effectively, and in some cases, increased growth has been reported due to chitin supplementation **(Gopalakannan and Arul, 2006)**. Whether this is an evolutionary adaptation to the natural diets to regulate endogenous chitinase production, or symbiotic relation with the chitinolytic microorganisms that would benefit the host fish remains to be investigated. The present study is the first one reporting chitinase-producing microbiota in the GI tracts of the Indian major carps and exotic carps. Microorganisms were isolated in the present study by culture dependant methods, further study involving the PCR amplification technique for the *chiA* gene might be useful in detecting chitinolytic bacteria associated with fish GI tract as suggested by **Sugita and Ito (2006)**. Whether the gut microbiota isolated in the present study can contribute to the host's nutrition has not been dealt with and an assessment of their role should be given high precedence in future studies. Further, the efficient chitinase-producers detected in the present investigation may be useful for treatment of chitinous waste and also for production of different products of hydrolyzed chitin for various applications.

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