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

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INTRODUCTION

Chitin (C\textsubscript{6}H\textsubscript{12}O\textsubscript{12}N\textsubscript{3}) 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; Koushab 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, Diaptomus etc.) comprising natural food for the carp fry and fingerlings has been reported to range from 21 to 95 mg g\textsuperscript{-1} by dry weight (Bämmel, 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 (Ringo 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 is 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 autochthonous 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 microorganisms 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 (KF454035), respectively by 16S rRNA partial gene sequence analysis. Optimization of various fermentation parameters (e.g., temperature, pH, inoculum 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°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

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, Clonopharyngodon 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, inoculum 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°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.
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 anesthetized 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 (Ringo et al., 1995; Spanggaard et al., 2000; Ringo 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 Sellotremikof (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 20 min, 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, pH 7.0) and crude enzyme (0.5 mL) was incubated for 1 h at 40 °C following Vaghmare et al. (2010). The reducing sugars produced reacted with di-nitro saliclyc acid (DNSA) and expressed as N-acetyl-D-glucosamine standards to demonstrate the chitinase activity (Miller, 1959). Enzyme activity (U) was defined as the µg of N-acetyl-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´-AGAGTTTGATCCTTACGACT-3´) and 1492r (5´-GTTACCTTGTAGGCACCT-3´). The PCR reactions were performed using PCR mix containing 200 µM of deoxyribonucleotides (dNTPs), 0.2 µM of each primer, 2.5 mM MgCl2, 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: 5 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 5.1 Beta4 software following the Minimum Evolution Method.

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

<table>
<thead>
<tr>
<th>Fish Species</th>
<th>Food habits*</th>
<th>Average fish live weight (g)</th>
<th>Average fish length (cm)</th>
<th>Average Gut weight (g)</th>
<th>Gut length (L2 cm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catla, Catla catla</td>
<td>Planktophagous</td>
<td>370±10.97</td>
<td>29.4±2.34</td>
<td>12.18±0.39</td>
<td>224.5±7.76</td>
</tr>
<tr>
<td>Rohu, Labeo rohita</td>
<td>Omnivorous mostly plant matter</td>
<td>260±13.44</td>
<td>30.5±2.61</td>
<td>11.32±0.62</td>
<td>271.7±8.51</td>
</tr>
<tr>
<td>Mrigal, Cirrhitus mirigala</td>
<td>Detrivorous</td>
<td>330±12.33</td>
<td>30.7±2.70</td>
<td>8.29±0.57</td>
<td>431.3±10.27</td>
</tr>
<tr>
<td>Silver carp, Hypophthalmichthys molitrix</td>
<td>Planktophagous</td>
<td>440±14.42</td>
<td>26.6±3.84</td>
<td>8.38±0.68</td>
<td>218.3±8.68</td>
</tr>
<tr>
<td>Grass carp, Hypophthalmichthys molitrix</td>
<td>Detrivorous</td>
<td>450±10.88</td>
<td>28.9±2.21</td>
<td>16.7±0.55</td>
<td>63.2±8.39</td>
</tr>
<tr>
<td>Common carp, Cyprinus carpio</td>
<td>Detrivorous</td>
<td>375±13.44</td>
<td>27.4±2.37</td>
<td>7.81±0.58</td>
<td>47.3±9.81</td>
</tr>
</tbody>
</table>

Data are means ± S.D. of three determinations.

*adapted from Jangran, 1997
Optimization of enzyme production

Submerged fermentation was carried out by both of the strains, CMF2 and HMHI 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 KH₂PO₄; 0.3 g K₂HPO₄; 4 g NaCl; 0.5 g MgSO₄·7H₂O; 1 mg FeSO₄; 7H₂O; 0.1 mg ZnSO₄·7H₂O and 0.1 mg MnSO₄·7H₂O. The parameters studied were: incubation temperature (25°C – 50°C), initial pH of the media (5.5 - 9), inoculum volume (1% – 5%), surfactant (0.2%, v/v) (Tween 20, Tween 40, Tween 80, DMSO), colloidal chitin (0.5-3.0 g L⁻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. mirgala (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

<table>
<thead>
<tr>
<th>Fish Species</th>
<th>Log viable counts (g⁻¹ intestinal tissue)</th>
<th>Proximate intestine (PI)</th>
<th>Distal intestine (DI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fish species</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Labeo rohita</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Catla catla</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cirrhus mrigala</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypothalmichthys molitrix</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ctenopharyngodon idella</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Cyprinus carpio</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Log counts (g⁻¹ intestinal tissue)</td>
<td>Proximate intestine (PI)</td>
<td>Distal intestine (DI)</td>
</tr>
<tr>
<td></td>
<td>LRF1</td>
<td>5.84 ± 0.31</td>
<td>4.85 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>LRF6</td>
<td>5.25 ± 0.32</td>
<td>4.67 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>LRF2</td>
<td>4.85 ± 0.37</td>
<td>4.67 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>LRH1</td>
<td>4.37 ± 0.31</td>
<td>4.67 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>LRH8</td>
<td>4.11 ± 0.28</td>
<td>4.67 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>CCFl</td>
<td>7.83 ± 0.31</td>
<td>6.71 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>CCF7</td>
<td>8.32 ± 0.35</td>
<td>6.71 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>CCF1</td>
<td>7.18 ± 0.29</td>
<td>6.71 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>CCF2</td>
<td>7.18 ± 0.29</td>
<td>6.71 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>CCF4</td>
<td>6.88 ± 0.27</td>
<td>6.71 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>CMF2</td>
<td>10.82 ± 0.34</td>
<td>8.47 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>CMF3</td>
<td>8.01 ± 0.25</td>
<td>8.47 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>CMF4</td>
<td>7.77 ± 0.23</td>
<td>8.47 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>CMF5</td>
<td>9.58 ± 0.39</td>
<td>8.47 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>CMF6</td>
<td>7.84 ± 0.25</td>
<td>8.47 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>CMFH</td>
<td>9.13 ± 0.27</td>
<td>8.47 ± 0.37</td>
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<tr>
<td></td>
<td>CMFH</td>
<td>8.47 ± 0.37</td>
<td>8.47 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>CMHH</td>
<td>8.11 ± 0.28</td>
<td>8.47 ± 0.37</td>
</tr>
</tbody>
</table>

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 HMHI and CMF2 were Bacillus pumilus (GenBank Accession no. KF454036) and Bacillus flexus (GenBank Accession no. KF454035), respectively. The isolate HMHI 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).
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Figure 1 Dendrogram 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 EU364818.1 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 2c. 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 2d. 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 (%), (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 chitinolytic bacteria within the GI tracts of marine fish species were well documented (Roy 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 grey mullets (Hamid et al., 1979), while, chitinase producing *Aeromonas* and *Vibrio* were isolated from the GI tract of tilapia (Sakata and Koreeda, 1986). 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°C. Previous reports by Narayana et al. (2009) and
Sudhakar and Nagarajan (2011) also documented maximum chitinase production at 35°C by soil isolates Streptomyces sp. ANU6277 and Trichoderma harzianum, respectively. In another study, Bacillus laterosorus produced high chitinase activity at 35°C (Shannugaliah 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. laterosorus (Shannugaliah et al., 2008), Micrococcus sp. AG84 (Annamalai et al., 2010), Aeromonas sp. JK1 (Ahmadi et al., 2008) and B. pabuli (Frandsberg and Schmurer, 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 agastic 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 and limiting nutrient imbalance (Ramachandran et al., 2005; Roppesh et al., 2006). Surfaceactants 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 (Sasiırık 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 WYEY108 (Monreal and Reese, 1969), Acremonium oblativum (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 (Andronopoupolou 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 chitinase 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 (Ringuo 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 microbota 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 chit 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 chitin waste and also for production of different products of hydrolyzed chitin for various applications.

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