Evaluation of Gut Associated Extracellular Enzyme-producing and Pathogen Inhibitory Microbial Community as Potential Probiotics in Nile Tilapia, *Oreochromis niloticus*

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**Abstract**

The present study aimed at evaluation of gut associated bacteria and yeasts in Nile tilapia (*Oreochromis niloticus*) as novel probiotics depending on extracellular digestive (amylase, protease and lipase) and degradation (cellulase, phytase, and xylanase) enzymes-producing ability, pathogen inhibition and bio-safety. The gastrointestinal (GI) tracts were taken out, separated into proximal and distal segments, homogenized, and enrichment culture was done on selective media plates for isolation of bacteria. Yeasts were isolated on yeast extract-peptone-dextrose media supplemented with antibiotics (150 mg L⁻¹). Both, bacteria and yeasts were detected in the GI tracts of Nile tilapia. Diverse enzyme-producing microbial populations were higher in the distal segment than the proximal segment. Ten out of 97 bacteria and 5 out of 32 yeast strains were primarily selected. The bacterium ONF1P and the yeast strain ONF7.1C were noticed as the efficient enzyme producing strains. Both the isolates were antagonistic against ≥2 tested fish pathogens. Both the strains were tolerant to diluted bile juice, capable to grow in fish mucus (intestinal) and compatible with previously isolated autochthonous fish gut bacteria. The isolates didn’t induce any pathological lesions or mortality in *O. niloticus* fingerlings. The strains ONF1P and ONF7.1C were identified as *Bacillus licheniformis* (KT362744) and *Pichia kudriavzevii* (KT582009), respectively, through 16S/18S rRNA gene fragment analyses. Extracellular enzyme-producing gut bacteria and yeast may restrain the growth of some fish pathogens and tolerate conditions within the GI tract. Further research should be directed to determine their in vivo effects on growth and disease resistance in *O. niloticus*.

**Keywords**

Nile tilapia; *Bacillus*; *Pichia*; Antagonism; Co-culture; Probiotics

**1 Background**

Tilapia, the second most common farmed fish in the world, is native to Africa and the Middle East (FAO, 2017). Ease of spawning (multiple spawner), efficient feed conversion, tolerance to adverse environmental conditions, resistance to disease, rapid growth rate and acceptability to the consumers make it a candidate species for culture (El-Saidy and Gaber, 2005). At present, many tropical and subtropical countries have focused on tilapia-culture to maximize aquaculture production (Lin et al., 2008). Tilapia has established a secure position in a number of water impoundments of India. At present, *O. niloticus* contributes more than 80% of tilapia aquaculture production globally and its performance in the ponds and reservoirs of India is much better than *O. mossambicus*. Production of tilapia is expanding in India following government approved responsible aquaculture. Exports of frozen tilapia augmented from 1133 tonnes in 2013 to 7738 tonnes in 2014 during the period of January-September (FAO, 2015). The rapid growth in the tilapia culture industry has led to the emergence of a number of pathogens e.g., *Aeromonas* spp., *Streptococcus* spp., *Flexibacter* spp. In consequence of high stocking density, intensive fish production often results in poor water quality explicating reduced growth and increased disease. Besides causing environmental hazards (Martinez, 2009), the excessive and inappropriate use of antibiotics has resulted in emergence of resistant strains that could be pathogenic in nature (Nomoto, 2005). Therefore, alternative ways of combating diseases need to be developed. Application of probiotics is suggested to be an environment-friendly method to improve nutrient utilization and combat pathogens in aquaculture (Vaseeharan and Ramasamy, 2003; Zheng et al., 2011; Sorrozo et al., 2012).
The specific micro-ecological system in the digestive tract of every species consists of diverse species of both, bacteria and yeasts. Amongst them, bacteria have been reported as the principal microbial colonizers within the GI tract of fish (MacDonald et al., 1986; Pond et al., 2006). Although, presence of yeasts have also been indicated to a lesser extent (Andlid et al., 1998; Gatesoupe, 2007; Mandal and Ghosh, 2013a; Das and Ghosh, 2014; Banerjee and Ghosh, 2014). In fact, it is a general trend in several of the earlier studies to consider fish gut microflora with special emphasis only on bacteria, characteristically the aerobic bacterial component, leaving out the eukaryotes (Austin, 2002). Previously, GI tract of the *Oreochromis* spp. have been evaluated in several studies in course of enumerating extracellular enzyme-producing (Sugita et al., 1997; Bairagi et al., 2002; Saha et al., 2006; Ray et al 2007; Sarkar and Ghosh, 2014; Sasmal and Ray, 2015), or pathogenic bacteria (Plumb, 1997; Son et al., 1997; Marathe et al., 2016). Enzymatic potential of gut bacteria isolated from various fish species have been displayed as the likely function that probiotics might accomplish. However, enzymatic properties together with anti-pathogenic potential in a backdrop of developing probiotics have been less studied for tilapia.

Consequently, the presently reported study intended to isolate and screen novel probiotics from Nile tilapia, *Oreochromis niloticus* with ability to produce diverse extracellular enzymes, inhibitory activity against potential fish pathogens, and bio-safety to the target fish.

2 Materials and Methods

2.1 Collection of fish and processing of gut sample

Specimens of tilapia, *Oreochromis niloticus* (Linnaeus) were procured from 3 composite culture ponds located at or around Burdwan (23°24’N, 87°86’E), West Bengal, India. Specimens were carried to the wet-laboratory in oxygen-packed bags. From each of the collection ponds, 3 specimens were collected (altogether 9 fish; average weight 100± 7.34 g) and kept in glass aquaria for 7 days for acclimatization. While in the culture pond, the fish were fed a mixture of fish meal, rice bran and mustard oil cake (crude protein: 30%) as supplementary feed along with natural feeding. The ranges of limnological parameters during the collection period were: temperature 27.1-28.3°C, pH 7.1-7.5 and dissolved oxygen 5.8-6.9 mg L⁻¹.

Prior to sacrifice, the fish specimens were starved (48 h) so as to clear their GI tracts. Tricaine methanesulfonate (MS-222) was applied as an anaesthetizing agent (0.03%); ventral surfaces of the fish were rubbed with 70% ethanol and dissected aseptically to take out the intestine (Ghosh et al., 2010). The GI tract was separated into proximal (PI) and distal (DI) segments and processed as described by Mandal and Ghosh (2013a) to isolate autochthonous gut-microorganisms. Gut segments from 3 specimens were pooled together to form one replicate (region-wise), thereby, there were three replicates in the study. Pooled samples were used to rule out conflicting conclusions owing to individual difference in gut microbiota (Ringo et al., 1995).

2.2 Isolation of microbial strains

For isolation of gut bacteria, homogenized samples of the gut regions were serially (1:10) diluted (Beveridge et al., 1991) up to 10⁻⁵ and each diluted sample (0.1 mL) was poured aseptically onto sterilized Soybean Casein Digest Agar (Tryptone Soya Agar, TSA; HiMedia, Mumbai, India) plates to determine the aerobic heterotrophic or facultative anaerobic culturable bacteria population.

Isolation of yeasts was done as described by Hirimuthugoda et al. (2007) and modified after Banerjee and Ghosh (2014). Briefly, homogenized samples of the two gut regions were serially diluted (1:10, w/v), homogenized samples (2 mL) were placed in liquid YPD (1% yeast extract, 2% peptone, 2% dextrose) culture medium (20 mL) supplemented with antibiotics (i.e., chloramphenicol, 150 mg L⁻¹; tetracycline, 150 mg L⁻¹). As chloramphenicol and tetracycline typically inhibit the growth of gram-positive and gram-negative bacteria, respectively, isolates grew on the YPD media were likely to be yeasts (Andlid et al., 1995). The broth culture medium was incubated for 5 days (pH 5-7, 30°C) and cells grown in YPD broth were transferred on YPD plates containing agar. Pure yeast colonies thus appeared were transferred to YPD slants for subsequent study.
For isolation and enumeration of different extracellular enzyme-producing bacteria (e.g., amylase, cellulase, lipase, phytase, protease and xylanase), the diluted gut homogenates were spread onto starch (ST), carboxymethylcellulose (CMC), tributyrin (TB), modified phytase screening (MPS) media, peptone-gelatin (PG) and xylan (XY) plates, respectively. ST, PG, TB and CMC media were prepared following (Bairagi et al., 2002), where as XY and MPS media were prepared following (Ninawe et al., 2006) and (Howson et al., 1983), respectively. After incubation (30°C, 48 h), appearance of bacterial colonies were counted following dilution plate count method and presented as log viable counts g⁻¹ GI tract (LVC) (Mandal and Ghosh, 2013a). Number of colonies reported in the present study was an average of three replicates. The prominent colonies were collected at random, streaked separately on respective media plates and repeatedly sub-cultured to get pure cultures. Pure cultures were preserved on slants in a refrigerator (4°C) for subsequent study.

2.3 Determination of yeast or bacterial strains
Colonies with different morphological appearances (e.g. configuration, colour, margin, opacity and surface) were selected on TSA plates and respective media plates for assessment of diverse extracellular enzyme-producing capacities (e.g. amylase, cellulase, lipase, phytase, protease and xylanase) and YPD plates were stained to determine the isolates as bacteria or yeast. Gram staining was applied to detect isolates as bacteria and yeast strains were determined by staining the isolates by Lacto phenol cotton blue.

2.4 Composition of the media used
Amylase media (g/L): Yeast extract 5; Peptone 5; NaCl 5; soluble starch 2; Agar 20; Cellulase media (g/L): Carboxymethylcellulose 5; Yeast extract 5; Peptone 5; NaCl 5; Agar 20; Lipase media (g/L): Tributyrin agar base 23.00 grams in 990 mL distilled water; Tributyrin 10 mL ; Modified phytase screening media (MPSM) (g/L): Glucose 10; (NH₄)₂SO₄ 1.0; Urea 10; Citric acid 3.0; Sodium citrate 2.0; MgSO₄·7H₂O 1.0; Sodium phytate 3.0; FeSO₄·7H₂O 0.01; Agar 20; Protease media (g/L): Peptone 5; Gelatin 4; Yeast extract 5; Agar 20; Xylanase media (g/L): Peptone 5; Yeast extract 2; MgSO₄·7H₂O 0.5; NaCl 0.5; CaCl₂ 0.15; Birch wood xylan 20; Agar 20.

2.5 Extracellular enzyme production: qualitative assay
Selective media plates were used to determine extracellular enzyme-producing capacities of the bacteria and yeast isolates. For yeasts, media plates were supplemented with the antibiotics as previously mentioned. Isolates were grown on ST and PG plates (30°C, 24 h) for detection of amylase- and protease-producing ability, respectively. Development of clear halo zone, while flooded with 1% Lugol’s iodine (ST plates) or 15% HgCl₂ solution (PG plates), indicated amylolytic and proteolytic activities, respectively (Jacob and Gerstein, 1960). Cellulolytic activity was determined on CMC plates flooded with Congo red dye prepared with 0.7% agarose, whereas, xylanolytic strains were detected on XY plates overlaid with 0.1% aqueous Congo red and repeated washing with 1M NaCl (Teather and Wood, 1982). Lipase producing strains showed halo surrounding their colony in 1% tributyrin plates (Sangiliyandi and Gunasekaran, 1996). Colonies grown on MPSM plates with clear zone of utilization indicated phytase activity of the isolates (Howson and Davis, 1983). There were 3 replicates for each experimental set. A brief description on determination of qualitative extracellular enzyme activities on the basis of measurement halo (diameter in mm) around the colony has been presented in Banerjee and Ghosh (2014). Scores were recorded as follows; 0, nil (no halo); 1, low (6-10 mm halo); 2, moderate (11-15 mm halo); 3, good (16-25 mm halo); 4, high (26-35 mm halo); 5, very high (≥35 mm halo).

2.6 Extracellular enzyme production: quantitative assay
Based on the qualitative assay, 15 exo-enzyme producing isolates (5 yeast isolates and 10 bacterial isolates) were selected and further studied through quantitative assay. Exo-enzymes were produced in respective selective broth media following Banerjee and Ghosh (2014). Seed cultures were grown (30°C, 48 h) either in YPG broth (yeast) or in nutrient broth (bacteria), inoculated into the liquid production medium (25 mL) and the culture flasks were incubated in a rotary shaker at 150 rpm (30±1°C, 72 h). Subsequently, the contents were centrifuged (10,000 g, 10 min, 4°C) and the cell-free supernatant was used as the source of enzyme.
Quantitative determination of exo-enzymes production (amylase, cellulase, lipase and protease) were carried out after the standard methods portrayed by Bernfeld (1955), Denison and Koehn (1977), Bier (1955) and Walter (1984), respectively, and a detailed description for which has been presented by Bairagi et al. (2002). Quantitative assay of phytase and xylanase activities were measured following Yanke et al. (1999) and Bailey (1992), respectively. Protein content in the enzyme sample was measured after Lowry et al. (1951) and unit (U) activity has been presented.

2.7 Fish pathogens and culture maintenance
Six fish pathogenic strains Aeromonas hydrophila MTCC-1739 (AH), Aeromonas salmonicida MTCC-1945 (AS), Aeromonas sobria MTCC-3613 (AB), Bacillus mycoides MTCC-7538 (BM), Pseudomonas putida MTCC-1072 (PP) and Pseudomonas fluorescens MTCC-103 (PF) were obtained from the Microbial Type Culture Collection, Chandigarh, India. In addition, Aeromonas veronii (AV) and Pseudomonas sp. (P) were isolated from diseased fish. The fishes were suffering from pale gills, bloated appearance, skin ulcerations and hemorrhage. Experimental onset of disease by intraperitoneal injection to O. niloticus confirmed pathogenicity of the isolated strains. Pathogenic strains used in the study were maintained in the laboratory (4°C) on TSA (HiMedia, Mumbai, India) slants. Stock cultures were stored at -20°C in 0.9% NaCl with 20% glycerol in tryptone soya broth (TSB) to provide consistent inoculums during the study (Sugita et al., 1998).

2.8 Assay for pathogen inhibitory activity and Co-culture test
Inhibitory activity of the isolated strains against the said eight fish pathogens was primarily noticed through ‘cross-streaking’ (Madigan et al., 1997).

Co-cultured activity of selected bacterial and yeast isolates were tested with previously isolated ten autochthonous fish gut bacterial isolates, e.g. Bacillus subtilis (JX292128), Bacillus atrophaeus (HM246635), Bacillus pumilus (KF454036), Bacillus flexus (KF454035), Bacillus subtilis (HM352551), Bacillus methylitrophicus (KF559344), Bacillus subtilis subsp. Spizizenii (KF559346), Enterobacter hormaechei (KF559347), Bacillus amyloliquefaciens (KF623209) and Bacillus sonorensis (KF623291). At first, the isolates from O. niloticus were streaked and grown (30°C, 24 h) on nutrient agar plate. Afterward, autochthonous fish gut isolates (pure cultures) were streaked on the same plate at a 90° angle with the growth line of the strains from O. niloticus keeping a hairline gap (0.1 mm). Following incubation (30°C, 24 h), growth of microbiota was checked with previously streaked bacteria and disappearance of the gap indicated compatibility of the yeast and bacteria strains with the autochthonous fish gut bacteria.

2.9 Growth on fish mucus
Fish gut mucus was collected from live O. niloticus and thereafter processed following Dutta and Ghosh (2015). Growth on mucus was determined at 30°C by counting the number of bacterial cells with a Petroff-Hausser counting chamber at 24 h, 48 h and 72 h intervals. OD at 600 nm was taken after 24 h, 48 h and 72 h cultures. Sterilized uninoculated mucus was served as the control.

2.10 Bile tolerance
Bile tolerance of the selected gut isolates was evaluated through determination of minimum inhibitory concentration (MIC). Crude bile juice (pH 5.6) was collected from dissected gall bladder in aseptic condition, sterilized by passing through filter papers (0.8 μm and 0.22 μm pore) (HiMedia, Mumbai, India) and stored at -20°C until use. Cultures grown in TSB (30°C, 24 h) were centrifuged (10,000 g, 10 min, 4°C) and microbial suspensions were prepared in PBS. Sterile PBS (control) or sterile PBS supplemented with 5-100% (v/v) fish bile juice was inoculated (10⁷ CFU mL⁻¹) with the microbial suspension. Following incubation (1.5 h, 30°C), the microbial samples were serially diluted in sterile PBS and viable counts were determined on TSA media plates.

2.11 Bio-safety evaluation
Bio-safety evaluation of two selected isolates was carried out through in vivo studies conducted in 350 mL glass aquaria using 30 healthy O. niloticus fingerlings (Average body weight: 35±5.1 g). The fishes were acclimatized...
in the laboratory condition for 2 weeks and divided into three equal groups (two experimental, one control) with three replicates in each case. The candidate isolates were grown in TSB (30°C, 24 h), centrifuged (2800 g, 15 min, at 4°C) and cell pellets were suspended in sterile 0.9% saline. Each experimental fish received intra-peritoneal (IP) injection (1.0 mL) of microbial suspension (10^9 cells mL^-1). The fish in control group were injected with sterile 0.9% saline (Mesalhy et al., 2008). Fish were fed ad libitum with a diet containing approximately 35% crude protein having fish meal as the chief protein source. All groups were kept under observation for 21 days and health status was checked every day for development of any disease symptom.

2.12 Identification of isolates by 16S rRNA and 18S rRNA gene sequence analysis
The most promising two microbial isolates were identified through either 16S rRNA (bacteria) or 18S rRNA (yeast) partial gene sequence analyses after isolation and PCR amplification following the methods described in Dutta et al. (2015) and Gadanho et al. (2003), respectively, with minor modifications. The gene encoding 16S rRNA was amplified using 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') universal primers by polymerase chain reaction (PCR).

For 18S rRNA sequence analysis, an InstaGene Matrix (BIO-RAD) was used for extraction of genomic DNA samples. The ‘Internal Transcribed Spacer’ (ITS) region in the 18S rDNA fragment was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCTTCCGCTTATTGATATGC-30') as the forward and reverse primers. The amplification products were purified with a multiscreen filter plate (Millipore Corp, Bedford, MA) and sent to a commercial house for Sanger sequencing (ABI Prism 3730XL DNA analyzer; Applied Biosystems, Foster City, CA).

Sequenced data were edited (BioEdit Sequence Alignment Editor, Version 7.2.0), aligned and analyzed to find out the closest homolog using National Centre for Biotechnology Information (NCBI) GenBank database, and deposited to the NCBI GenBank. Phylogenetic trees were prepared using MEGA5.1 Beta 4 software following the Minimum Evolution Method.

2.13 Statistical analysis
Statistical analysis of the quantitative enzyme activity data was performed by the one-way analysis of variance (ANOVA), followed by Tukey’s test according to Zar (1999) using SPSS Version 10 software (Kinnear and Gray, 2000).

3 Results
Analysis of microbiological community in the GI tract revealed that considerable number of culturable aerobic or facultative anaerobic heterotrophic bacterial and yeast population were present on both PI and DI regions of *O. niloticus* (Table 1). Heterotrophic and diverse extracellular enzyme-producing microbial populations were found to be highest in the DI region than that of the PI region. While considering extracellular enzyme-producing bacteria, proteolytic population was dominated in the DI region (LVC = 5.36), which was followed by amylolytic bacteria (LVC = 4.87) and cellulolytic bacteria (LVC = 4.11). Phytate-degrading population was noticed to be the lowest (LVC = 3.25).

Altogether 97 bacteria were isolated, out of which 10 extracellular enzyme producing strains (4 from PI and 6 from DI) were primarily selected through qualitative enzyme assay. The intensity of transparent zone (halo) produced by the isolates are presented as score. Maximum and minimum scores of bacterial isolates were 27 and 17, respectively (Table 2). In case of yeast, out of the 32 yeast isolates, 5 extracellular enzyme-producing strains were selected primarily through qualitative enzyme assay, with maximum and minimum scores being 16 and 11, respectively which were further evaluated by quantitative enzyme assay to select the most promising isolates (Table 2). The results of quantitative estimation of extracellular amylase, protease, lipase, cellulase, phytase and xylanase production by the microbial strains are depicted in Table 3. The results revealed significant differences in the enzyme activities between different microbial isolates.
### Table 1 Log viable counts (LVC g⁻¹ GI tract) of autochthonous bacteria and yeasts isolated from the proximal intestine (PI) and distal intestine (DI) of tilapia (*Oreochromis niloticus*)

|                         | PI   | DI   |
|-------------------------|------|------|
| Total Heterotrophic Count in YPD plate for yeast | 3.85 | 4.17 |
| Total Heterotrophic Count for bacteria          | 5.83 | 6.18 |
| Amylolytic bacteria          | 4.24 | 4.87 |
| Proteolytic bacteria         | 4.81 | 5.36 |
| Lipolytic bacteria           | 3.02 | 3.75 |
| Cellulolytic bacteria        | 3.76 | 4.11 |
| Phytate degrading bacteria   | 2.94 | 3.25 |
| Xylanolytic bacteria         | 3.1  | 3.56 |

### Table 2 Qualitative extracellular enzyme activity of some microbial strains isolated from the GI tracts of tilapia (*Oreochromis niloticus*). Enzyme activities were presented as scores as described in the text

| Enzyme activity (U) | Strains | Proximal Intestine | Distal Intestine | Yeast strains | Proximal Intestine | Distal Intestine |
|---------------------|---------|--------------------|------------------|---------------|--------------------|------------------|
| Amylase¹             | ONF4L   | 3                  | 4                | ONH15.1B      | 3                  | 3                |
| Protease²            | ONF1X   | 5                  | 3                | ONF14.1C      | 2                  | 3                |
| Lipase³              | ONF1P   | 5                  | 5                | ONF15.1B      | 3                  | 3                |
| Cellulase⁴           | ONF1T   | 5                  | 3                | ONH8.1A       | 2                  | 3                |
| Phytase⁵             | ONH1Ph  | 3                  | 4                | ONF19.1C      | 2                  | 3                |
| Xylanase⁶            | ONH1A   | 2                  | 4                | ONF21.1B      | 3                  | 3                |

Note: With pure culture of isolates. Three replicates for each experimental set. ¹ On starch (SA) plate; ² On peptone-gelatin plate; ³ On tributyrin-agar (TA) plate; ⁴ On carboxy-methylcellulose (CMC) plate; ⁵ On sodium-phytate plate; ⁶ On xylan plate.

Quantitative assay of enzymes portrayed that both amylase and protease activities were the highest in ONF1P (191.46 ± 4.05U and 64.55 ± 2.39U respectively), isolated from the PI of Nile tilapia. Considerable lipase producing ability was exhibited by all of the 10 primarily selected bacterial isolates. Maximum lipase activity was recorded with the strain ONF4L isolated from the PI region (4.02 ± 0.14U), which was followed by ONF1P (3.99 ± 0.07U). The strain ONF1P also showed maximum cellulose, phytase and xylanase activities (53.33 ± 2.21U, 51.11 ± 1.63U and 8.69 ± 0.75U, respectively). In contrast, minimum xylanase activity was noticed with the strain ONH1Ph (2.88 ± 0.06U). Overall examination of the six different extracellular enzyme activities revealed that the strain ONF1P was the most efficient among the 10 primarily selected bacterial strains.

Among the yeasts, maximum activities for 5 out of the 6 tested exo-enzymes were recorded with the strain ONF7.1C isolated from the PI region of *Oreochromis niloticus*. The strain ONF7.1C revealed maximum amylase (135.13±3.26U), protease (33.42±1.87U), lipase (3.58±0.05U) and cellulase (39.87±2.14U) activities. ONF7.1C
was also recorded as the highest phytase-producing strain (4.71±0.06U) among yeast strains evaluated. Strain ONF8.1A produced highest xylanolytic activity (1.15±0.09U), which was followed by ONF7.1C (0.92±0.08U). Considering these facts, the isolates ONF7.1C was finally selected as the most efficient yeast strain.

Table 3 Enzyme activity (unit activity, U) by the primarily selected microbial strains isolated from the GI tracts of tilapia (Oreochromis niloticus). Data are Mean ± Standard error (n=3)

| Strains          | Amylase\(^{a}\) | Protease\(^{b}\) | Lipase\(^{c}\) | Cellulase\(^{d}\) | Phytase\(^{e}\) | Xylanase\(^{f}\) |
|------------------|-----------------|-----------------|---------------|-----------------|----------------|-----------------|
| **Bacterial strains** |                 |                 |               |                 |                |                 |
| Proximal Intestine |                 |                 |               |                 |                |                 |
| ONF4L            | 142.28±3.25\(^{g}\) | 52.69±2.23\(^{g}\) | 4.02±0.14\(^{h}\) | 36.51±1.88\(^{g}\) | 42.58±1.57\(^{i}\) | 3.52±0.08\(^{c}\) |
| ONF1X            | 177.62±4.36\(^{h}\) | 51.08±2.48\(^{g}\) | 3.95±0.09\(^{h}\) | 39.43±1.59\(^{g}\) | 37.50±1.41\(^{h}\) | 4.29±0.18\(^{f}\) |
| ONF1P            | 191.46±0.05\(^{i}\) | 64.55±2.39\(^{b}\) | 3.99±0.07\(^{i}\) | 53.33±2.21\(^{i}\) | 51.11±1.63\(^{b}\) | 8.69±0.75\(^{b}\) |
| ONF1T            | 170.48±4.77\(^{h}\) | 47.33±2.86\(^{i}\) | 3.78±0.05\(^{b}\) | 32.55±1.59\(^{g}\) | 46.35±1.54\(^{c}\) | -               |
| **Distal Intestine** |                 |                 |               |                 |                |                 |
| ONH1Ph           | 141.05±3.56\(^{f}\) | 38.49±1.84\(^{d}\) | 3.84±0.06\(^{b}\) | 20.06±1.12\(^{g}\) | 13.67±0.94\(^{d}\) | 2.88±0.06\(^{d}\) |
| ONH1A            | 101.44±2.55\(^{g}\) | 53.69±2.35\(^{g}\) | 3.44±0.02\(^{a}\) | 35.28±1.61\(^{d}\) | 20.16±1.14\(^{i}\) | -               |
| ONH2A            | 143.56±3.39\(^{g}\) | 29.61±1.46\(^{g}\) | 3.64±0.04\(^{a}\) | 33.62±1.68\(^{d}\) | 21.47±1.10\(^{g}\) | 5.06±0.69\(^{f}\) |
| ONH2Ph           | 149.63±3.50\(^{g}\) | 49.90±2.98\(^{g}\) | 3.55±0.03\(^{f}\) | 36.92±1.58\(^{g}\) | 17.77±1.08\(^{g}\) | -               |
| ONH1C            | 188.40±4.05\(^{i}\) | 37.45±1.41\(^{c}\) | 3.89±0.06\(^{a}\) | 48.73±1.79\(^{c}\) | 23.58±1.19\(^{g}\) | -               |
| ONH2C            | 139.73±3.01\(^{f}\) | 55.88±2.36\(^{a}\) | 3.41±0.02\(^{a}\) | 45.66±1.80\(^{c}\) | 6.93±0.74\(^{c}\) | -               |
| **Yeast strains** |                 |                 |               |                 |                |                 |
| Proximal Intestine |                 |                 |               |                 |                |                 |
| ONF7.1C          | 135.13±3.26\(^{c}\) | 33.42±1.87\(^{c}\) | 3.58±0.05\(^{b}\) | 39.87±2.14\(^{d}\) | 4.71±0.06\(^{c}\) | 0.92±0.09\(^{b}\) |
| ONF8.1A          | 90.28±1.98\(^{b}\) | 28.15±1.21\(^{c}\) | 3.12±0.08\(^{g}\) | 36.42±2.74\(^{g}\) | -               | 1.15±0.09\(^{b}\) |
| ONF14.1C         | 84.21±1.65\(^{a}\) | 23.71±1.12\(^{b}\) | 1.96±0.07\(^{g}\) | 34.64±2.61\(^{g}\) | 3.97±0.08\(^{b}\) | 0.73±0.07\(^{a}\) |
| ONF21.1B         | 119.61±2.89\(^{d}\) | 18.38±1.16\(^{e}\) | 2.84±0.06\(^{a}\) | -               | 3.78±0.07\(^{a}\) | 0.86±0.08\(^{ab}\) |
| **Distal Intestine** |                 |                 |               |                 |                |                 |
| ONH15.1B         | 103.58±2.51\(^{c}\) | 24.23±1.09\(^{h}\) | 2.35±0.02\(^{c}\) | 26.53±1.23\(^{a}\) | -               | -               |

Note: Values with the same superscripts in the same vertical column are not significantly different (P<0.05).

\(^{a}\)μg of maltose liberated mg\(^{-1}\) protein min\(^{-1}\); \(^{b}\)μg of tyrosine liberated mg\(^{-1}\) protein min\(^{-1}\); \(^{c}\)μg of free fatty acid liberated mg\(^{-1}\) protein min\(^{-1}\); \(^{d}\)μg of glucose liberated mg\(^{-1}\) protein min\(^{-1}\); \(^{e}\)μg of inorganic phosphate liberated mg\(^{-1}\) protein min\(^{-1}\); \(^{f}\)μg of D-xylene liberated mg\(^{-1}\) protein min\(^{-1}\)

The primarily selected 15 extracellular enzyme-producing isolates (10 bacteria and 5 yeasts) were further screened for antagonism against said eight fish pathogens through ‘cross-streaking’ method and the result has been shown in Table 4. Out of the 15 isolates, 6 strains (3 bacteria and 3 yeasts) were noticed to inhibit at least one of the tested fish pathogens. Bacillus mycoides can be inhibited by most of the isolates (4 bacteria and 3 yeasts), the highest inhibition zone being 8 mm by the strain ONF1P. Pseudomonas sp. and Pseudomonas fluorescense can be inhibited by 6 bacterial strains, however, no yeast strain was able to inhibit this pathogen. Aeromonas veronii was inhibited by only one bacterial isolate, ONH2A and none of the yeast strains could antagonize this pathogen. Aeromonas hydrophilia was inhibited by another bacterial isolate, ONF4L. It appeared that the strain ONF1P could inhibit 5 fish pathogens producing distinct inhibition zones, and the yeast strain ONF7.1C could inhibit two fish pathogens including Aeromonas hydrophilia. The strains ONF1P and ONF7.1C were able to produce considerable amount of exo-enzymes and were also noticed to be antagonistic against 7 tested fish pathogens. Therefore, these promising strains (ONF1P and ONF7.1C) were finally selected for identification in view of possible future use.

Co-culture test (in vitro) against ten autochthonous fish gut bacteria revealed that the selected yeast strain (ONF7.1C) could not affect the growth of the autochthonous gut bacteria, however, selected bacterial strain (ONF1P) couldn’t grow with Enterobacter hormaecheii (KF559347). Therefore, the selected extracellular enzyme-producing yeast strain was mostly compatible with the commonly occurring autochthonous fish gut bacteria. Both ONF1P and ONF7.1C showed tolerance against diluted bile juice. The selected bacterial strains
survived after 24 h exposure to different concentrations of bile juice. With gradual decrease in the concentration of bile, an increase in the number of viable colonies was observed. Table 5 depicts the result of bile tolerance.

Table 4 Inhibition area (mm) produced by the selected gut bacteria in cross streaking method against the tested fish pathogens

| Strains | AH | AS | AB | AV | P | BM | PP | PF |
|---------|----|----|----|----|---|----|----|----|
| **Bacterial strains** | | | | | | | | |
| Proximal Intestine | | | | | | | | |
| ONF4L | 6 | - | 5 | - | - | - | - | - |
| ONF1X | - | - | - | - | 3 | - | - | 6 |
| ONF1P | - | 5 | - | - | 7 | 8 | 5 | 7 |
| ONF1T | - | 2 | - | - | 4 | 4 | 4 | 5 |
| Distal Intestine | | | | | | | | |
| ONH1Ph | - | 2 | - | - | - | - | 4 | - |
| ONH1A | - | - | - | - | 4 | - | - | - |
| ONH2A | - | - | 4 | 3 | 2 | 3 | - | - |
| ONH2Ph | - | - | - | - | - | - | 2 | - |
| ONH1C | - | - | 3 | - | 3 | 1 | - | 4 |
| ONH2C | - | - | - | - | - | - | 3 | - |
| **Yeast strains** | | | | | | | | |
| Proximal Intestine | | | | | | | | |
| ONF7.1C | 2 | - | - | - | - | 3 | - | - |
| ONF8.1A | - | - | - | - | - | - | 3 | - |
| ONF14.1C | - | 2 | - | - | - | - | 2 | - |
| ONF21.1B | - | - | - | - | - | 2 | - | - |
| Distal Intestine | | | | | | | | |
| ONH15.1B | - | - | - | - | - | 2 | - | - |

Note: AH=Aeromonas hydrophila; AS=Aeromonas salmonicida; AB=Aeromonas sobria; AV=Aeromonas veronii; P=Pseudomonas sp.; BM=Bacillus mycoides; PP=Pseudomonas putida; PF=Pseudomonas fluorescens

Table 5 Tolerance of the selected isolates at different concentrations of fish bile juice for 1.5 h at 30°C, viable count was determined on TSA plates inoculated with bile exposed bacterial suspension. Data are mean ± standard error (n = 3)

| Bile (%) | ONF1P | ONF7.1C |
|----------|-------|---------|
| 0        | 5.72±0.006 | 4.63±0.007 |
| 1        | 5.54±0.006 | 4.27±0.009 |
| 2        | 5.50±0.004 | 4.16±0.007 |
| 3        | 5.48±0.005 | 4.02±0.009 |
| 4        | 5.37±0.004 | 3.90±0.007 |
| 5        | 5.26±0.006 | 3.81±0.005 |
| 6        | 5.26±0.004 | 3.48±0.006 |
| 7        | 5.20±0.005 | 3.18±0.003 |
| 8        | 5.14±0.005 | 2.67±0.006 |
| 9        | 5.14±0.008 | 2.46±0.003 |
| 10       | 5.03±0.001 | 2.06±0.004 |
| 11       | 5.01±0.002 | - |
| 12       | 4.80±0.003 | - |
| 13       | 4.45±0.004 | - |

Study of growth in mucus revealed that strains ONF1P and ONF7.1C grew well in mucus of O. niloticus (Figure 1). As evident from the optical density, maximum growth was observed after 48h of incubation. Growth eventually started to decline after 72 h of incubation.
After 21 days of small-scale in vivo experiment, it was noticed that along with the control set intra-peritoneal injection of the isolates (ONF1P and ONF7.1C) did not induce any pathological signs/disease symptoms or mortalities in both treatment groups.

Based on the 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 RDP databases, strain ONF1P was identified as *Bacillus sonorensis* (Accession No. KT362744). On the other hand, based on the nucleotide homology and phylogenetic analysis of the 18S rRNA partial gene sequence by nucleotide blast in the NCBI GenBank, strain ONF7.1C was identified as *Pichia kudriavzevii* (Accession No. KT582009). Phylogenetic relationships of the identified bacteria (Figure 2) and yeast (Figure 3) isolates with other closely-related strains retrieved from NCBI GenBank are presented in the dendogram.

![Dendrogram showing phylogenetic relations of the bacterial strain, Bacillus sonorensis ONF1P, with other closely related strains retrieved from NCBI GenBank and RDP](image)
The GenBank accession numbers of the reference strains are shown besides the names. Horizontal bars in the dendrogram represent the branch length. Similarity and homology of the neighbouring sequences have been shown by bootstrap values. Distance matrix was calculated by Tamura-Nei model. The scale bar indicates 0.005 substitutions per nucleotide position. *Bacillus smithii* AB271749.1 served as an out group.

![Dendrogram](image)

**Figure 3** Dendrogram showing phylogenetic relations of the yeast, *Pichia kudriavzevii* ONF7.1C, with other closely related strains retrieved from NCBI GenBank

The GenBank accession numbers of the reference strains are shown besides the names. Horizontal bars in the dendrogram represent the branch length. Similarity and homology of the neighbouring sequences have been shown by bootstrap values. Distance matrix was calculated by Tamura-2 model. The scale bar indicates 0.005 substitutions per nucleotide position. *Candida mesorugosa* FJ768909.1 served as an out group.

### 4 Discussion

Numerous reports have been published during the last three decades describing bacteria (Ringo et al., 2003; 2007) and yeasts (Dash and Ghosh, 2013; Banerjee and Ghosh, 2014) attached to mucosa and microvilli of the intestine. Yeasts are ubiquitous microorganisms that grow in various environments where organic substrates are available (Gatesoupe, 2007). Bacteria are abundant in the environment in which fish live and it is ingested by the fish along with their diet. Later, they may adapt themselves to the environment of the GI tract and form a symbiotic association (Saha et al., 2006). Attention has been paid to identify autochthonous fish gut microbiota in order to gain information on their activities (Cail, 1990). It could be mentioned that the fish species examined in the presently reported study 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 may be advocated that the microorganisms isolated in the present study belong to the autochthonous microbiota as suggested elsewhere (Ray et al., 2010; Ghosh et al., 2010; Mukherjee et al., 2017). Several investigators have reported the presence of enzyme producing bacteria in the digestive tract of *Oreochromis* spp. (Sugita et al., 1997; Bairagi et al., 2002; Saha et al., 2006; Ray et al., 2007; Mondal et al., 2008; Sarkar and Ghosh, 2014; Sasmal and Ray, 2015). The present study is the first one demonstrating adherent bacteria and yeasts on the gut surfaces of *Oreochromis niloticus* along with antagonistic properties against the potential fish pathogens. Selection of strong extracellular enzyme producers was considered as the primary aim of this study. Microbial isolates detected in the present study represented their ability for production of both, extracellular digestive (amylase, protease, lipase) and degradation enzymes (cellulase, xylanase, phytase). The observations of Saha and Ray (1998), Ghosh et al. (2002) and Bairagi et al. (2002) were in agreement with the proposition that fish harbour cellulolytic as well as amylolytic and proteolytic bacteria in their intestinal tract. Previous study of Saha et al. (2006) revealed that *Bacillus circulans*, isolated from *Oreochromis mossambica*, was able to produce large amount of amylase, protease and cellulase. Two most
promising exo-enzyme producing strains ONF1P and ONF7.1C were capable of producing all of the six studied enzymes in considerable amount, which was determined by quantitative assay. ONF1P was identified as *B. sonorensis* (Accession No. KT362744) based on the 16S rRNA partial gene sequence analysis. Previous report by Mary et al. (1975) recorded that *Bacillus* and *Corynebacterium* were principal groups of microorganisms present in tilapia to tolerate the adverse effects of digestive enzymes. Moreover, previous studies have recorded *B. sonorensis* from the gut of Indian major carps, *Cirrhinus mrigala* (Dutta and Ghosh, 2015) and *Catla catla* (Dutta et al., 2015), although, occurrence of *B. sonorensis* depicted in the present study has not been reported previously. In the present investigation, considerable populations of amylolytic and proteolytic bacteria were detected, which was in agreement with Mondal et al. (2008) who reported amylolytic and proteolytic bacteria in the gastrointestinal tract of Nile tilapia correlating to its omnivorous feeding aptitude. Bairagi et al. (2002) isolated an efficient protease-producing strain, TP3A from the gut of *Oreochromis mossambica*. However, none of them made an attempt to identify the efficient enzyme-producing strains. Large number of cellulolytic bacteria was detected in this study. This result might indicate that cellulolytic bacteria exist in the GI tract of Nile tilapia and support the hypothesis that bacteria contribute to the exogenous production of cellulase in fish. Endogenous phytase activity has been reported in hybrid tilapia (*O. niloticus × O. aureus*) (LaVorgna, 1998). However, to the authors’ knowledge, occurrence of phytase and xylanase-producing microorganisms in Nile tilapia has not been reported previously.

Tilapia have a high level of immunity compared to other fresh water fishes that guard against the infection (Thillaimaharani et al., 2012). *In vivo* studies involving scanning electron microscopy indicated that yeast adhere firmly to the gut mucosal epithelia, which may resolve the colonization capacity of yeasts within the fish GI tract (Ofek and Beachey, 1980; Traore et al., 1994). In the present study, one promising extracellular enzyme-producing autochthonous yeast (ONF7.1C) was identified as *Pichia kudriavzevi* (Accession No. KT582009) based on 18S rRNA partial gene sequence analysis. *Pichia* was described as good exo-enzyme producer in *Cirrhinus mrigala* (Banerjee and Ghosh, 2014). Tannase producing *P. kudriavzevi* in fish gut has also been documented (Mandal and Ghosh, 2013a, b). Probiotic yeasts with phytase activity have been demonstrated in the GI tract of sea cucumbers (Hirimuthugoda et al., 2007). Present study also support considerable amount of phytase production by isolated yeast strains from *Oreochromis niloticus*. The presence of cellulase and xylanase producing yeasts in the GI tracts of this fish species might indicate their probable role in degradation of dietary non starch polysaccharides.

Demonstration of antagonistic properties against some fish pathogens was considered as the second criteria for the selection of candidate probiotics. Apart from nutritional benefits, the enzyme-producing gut bacteria in fish have been assumed to compete continuously with pathogens through the competitive exclusion or the production of antimicrobial compounds (Ray et al., 2012). Although several strains of Bacilli were demonstrated as probiotics for fish, antagonism of pathogens by the Bacilli has been seldom indicated (Laloo et al., 2010; Geraylou et al., 2014; Mukherjee and Ghosh, 2016). In the present study, most of the promising exo-enzyme producing bacteria and yeast strains displayed antagonism against at least two of the tested fish pathogens. To the best of the authors’ knowledge, exoenzyme-producing ability along with pathogen inhibitory potential of gut microflora from Nile tilapia depicted in the present study has not been reported previously.

It is essential for every probiotic microorganism to be capable of withstanding fish GI conditions. The gut microorganisms should be able to adhere to mucus of the GI tract and capable of tolerating bile juice in order to demonstrate its beneficial attributes. Both the selected microorganisms, ONF1P and ONF7.1C, grew well in fish mucus, although, minor differences were noted in growth rate of bacteria and yeast, which might be due to difference in specific nutritional requirements of prokaryotes and eukaryotes (Geraylou et al., 2014). In addition, as evident from the log viable count of bacteria and yeast inoculums in fish bile juice *B. sonorensis* ONF1P and *P. kudriavzevi* ONF7.1C were found to tolerate 12.5% and 7.5% of the diluted bile juice, respectively. In this context, it can be stated that the physiological concentration of bile in the fish GI tract was estimated to be approximately 0.4-1.3% (Balcázar et al., 2008). Therefore, it can be concluded that both the microorganisms were
capable of tolerating fish bile juice to a much higher concentration than that they normally encounter in the GI micro-environment. Thus, the study might suggest that extracellular enzyme-producing gut bacteria and yeast may restrain the growth of some fish pathogens and tolerate conditions within the GI tract. The present observation was in agreement to the previous reports establishing probiotic attributes of fish gut bacteria (Geraylou et al., 2014; Dutta and Ghosh, 2015; Dutta et al., 2015; Mukherjee and Ghosh, 2016). Although to the author’s knowledge, such attempt has never been made to characterize yeasts as likely probiotics.

Safety of the host is an important criterion for any probiotic organism as recommended elsewhere (Verschuere et al., 2000). In this study, the selected isolates were evaluated for safety measurement through small scale in vivo study and experimental results revealed that the isolates did not induce any pathological signs or mortalities in *O. niloticus*. In addition, co-culture of the selected strains with previously isolated autochthonous fish gut bacteria did not affect growth of the later. Thus, in accordance to some of the preceding reports (Banerjee and Ghosh, 2014; Banerjee et al., 2016), present study might corroborate the likely co-existence of a diverse autochthonous gut microflora with the putative probiotic strains selected in the present study.

In this study, we isolated gut microorganisms and tested different probiotic characteristics of them through in vitro methods. However, assumptions based on in vitro experiments might not conform exactly to in vivo conditions. Although, the autochthonous organisms isolated from the host fish might be better suited to survive and colonize within the GI tract of the same fish. Nevertheless, extensive in vivo studies are warranted with these autochthonous organisms to determine their effects on overall growth and disease resistance in *O. niloticus*. Therefore, an evaluation of their role in vivo should be given high precedence in future studies.

**Authors’ contributions**

KG: designed and supervised the study, contributed in the MS, carried out sequence analyses; SB: carried out pathogen inhibition, co-culture, gut stability and bio-safety studies, prepared the MS; UMM: isolated and maintained bacteria samples, carried out 16S sequencing works; HAK: isolated and maintained yeast samples, carried out 18S sequencing works; DD: carried out enzymological works, contributed in sequence analyses.

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