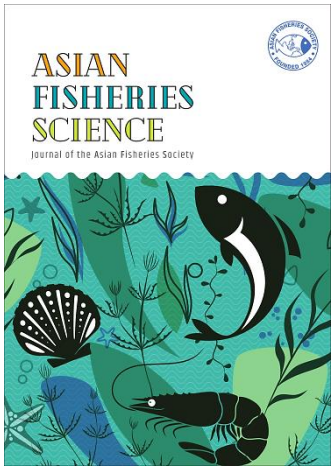

Characterisation of Extracellular Enzyme-Producing Microorganisms From the Gut of Nile Tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

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Abstract

The current priority in the aquaculture industry is to replace fish meal with cheaper but efficient protein sources like plant-based feedstuffs to support global expansion and sustainability. Hence, research has focused on finding novel ways to increase the digestibility of plant-based diets. Exogenous enzymes have emerged as an excellent alternative to improve nutrient digestibility of the diet in animals, including fish. The present study aims to explore exogenous enzyme-producing bacteria in the gastrointestinal (GI) tract of Nile tilapia (*Oreochromis niloticus* (Linnaeus, 1758)). Two Gram-negative enzyme-producing (amylase, xylanase, and lipase) bacterial strains viz. *Aeromonas veronii* ONKP1 (GenBank accession no. MN602971) and *Stenotrophomonas maltophilia* ONKP2 (GenBank accession no. MN602972) were identified by biochemical tests and 16s RNA analysis. They are capable of utilising citrate, maltose, glucose, and gelatine. Besides, *A. veronii* ONKP1 can utilise mannitol, sucrose, and lactose, whereas *S. maltophilia* ONKP2 is catalase and urease positive. *Aeromonas veronii* ONKP1 was superior in terms of enzyme production to *S. maltophilia* ONKP2. *Stenotrophomonas maltophilia* ONKP2 is a rarely reported strain, specifically from healthy fish. Amylase, xylanase, and lipase could be used as feed additives for fermenting plant products and producing prebiotics like xylooligosaccharides. Further, this study might help understand the role of gut-associated bacteria viz. *A. veronii* and *S. maltophilia*, in fish nutrition.

Keywords: *Aeromonas veronii*, *Stenotrophomonas maltophilia*, gastrointestinal (GI) bacteria, fish nutrition

Introduction

The aquaculture industry is facing challenges due to high feed costs and rapid expansion on a global scale. Major protein sources, like fish meal and fish oil, are very costly and limited in availability. Therefore, for sustainable aquaculture, environment-friendly and economically viable substitutes and growth promoters have become very important. Fishmeal can be partially or wholly replaced by cheaper and readily available plant protein sources (Gatlin III et al., 2007; Daniel, 2018; Jannathulla et al., 2019; Abbasi et al., 2020; Xie et al., 2021). But the presence of antinutritional factors like protease inhibitors, phytate, tannin, lignin, oligosaccharides, and non-starch polysaccharides in plant feedstuffs (Francis et al., 2001; Hajra et al., 2013; Prabu et al., 2017; Kokou and Fountoulaki, 2018) and the lack of enzymes for digesting most plant carbohydrates like cellulose and xylan in fish limit its use in aquafeed (Kumar and Chakravarty, 2018). In this regard, the application of exogenous enzymes like carbohydrases and phytases may be beneficial to increase feed utilisation and digestibility (Castillo and Gatlin III, 2015; Zheng et al., 2020). Moreover, feed enzymes have made a promising impact on the growth and nutrient utilisation of both non-ruminants (poultry and swine) (Kiarie et al., 2013) and ruminants (cow and cattle) (Sujani and Seresinhe, 2015) worldwide. In recent years, aquaculture researchers also started to elucidate the effects of supplementation of dietary enzymes viz. protease, carbohydrases (amylase, xylanases, and cellulase) and lipase as growth promoters and immunomodulators in aquatic species (Zheng et al., 2020). For isolation of enzymes, the indigenous gut

microbiota of fish has been extensively studied in the past few years (Ray et al., 2012; Egerton et al., 2018) due to their higher catalytic efficiencies, unique features, great diversity, variety and tolerance to extreme processing conditions, e.g., temperature, salts and pH (Singh et al., 2016). Besides, microorganisms possess bulk production capacity and the convenience of genetic manipulation (Liu et al., 2013).

Nile tilapia (*Oreochromis niloticus* (Linnaeus, 1758)) is a major commercial freshwater fish species in the world. It is a member of the Cichlidae family and contributing to world aquaculture since ancient Egyptian days. Tilapias are, in general, very tough, fast-growing, omnivorous and can tolerate a wide range of environmental conditions, which make their farming easy with low technology system. Due to their superior cultural adaptability, they are now being called "aquatic chicken" (Amal and Zamri-Saad, 2011). In many tropical and subtropical countries, including India, tilapia culture has become a focus of aquaculture to maximise production (Ghosh et al., 2017). Hence, knowledge about the microbial community found in the gut of tilapia is of great importance. Although the gut microbiota of many marines (Egerton et al., 2018) and freshwater fish (Ray et al., 2012) has been investigated during the last decades, few studies have been done on gut microbiota of *Oreochromis* spp. (Ghosh et al., 2017). This study evaluated the gut microbiota of marketed fresh Nile tilapia, *O. niloticus*, to understand their role in fish health and nutrition. This might help in the development of feed for aquatic species of commercial importance.

Materials and Methods

Isolation of bacteria

Ten live, apparently healthy Nile tilapia, *O. niloticus* (average weight 250 ± 9.50 g; length 12 ± 2.34 cm) were procured from fish markets of Kalyani, located in Nadia district in West Bengal, India and transported to the laboratory in oxygen-packed bags. The source of the fish was from nearby aquaculture ponds in Kalyani. The fish were acclimatised in glass aquaria for 7 days to eliminate the transient or allochthonous microbiota, which accumulated through the environment. Allochthonous microbiota is an incidental visitor and, thereby, rejected after some time without colonising in the gastrointestinal (GI) tract (Ray et al., 2012). During acclimatisation, the fish were fed the same feed formulation (a mixture of fish meal, rice bran and mustard oil cake, crude protein: 30 %) as provided by the farmers, corresponding to 5 % of their body weight twice per day. Protocol for feeding and acclimatisation were done following previous works by Trust et al. (1979) and Ghosh et al. (2017). The ranges of limnological parameters of the ambient water in the aquaria were measured as temperature 27.1–28.3 °C, pH 7.1–7.5 (Digital pH and temperature meter, LABINDIA, India) and dissolved oxygen 5.8–6.9 mg.L^{-1} (APHA, 1995).

Processing of gut samples of the specimens and isolation of microbial strains were done following the methods of Ghosh et al. (2017). In brief, after 7 days of acclimatisation, the fish specimens were starved 48 h to clear their GI tracts and eliminate allochthonous microbiota, which might accumulate from the aquarium environment, prior to sacrifice and tricaine methanesulfonate (MS-222, HiMedia, India) was applied as an anaesthetising agent (0.03 %). Then the ventral surfaces of the fish were swabbed with 70 % ethanol and dissected aseptically to remove the intestine. For isolation of gut bacteria, homogenised samples of the gut regions were serially (1:10) diluted up to 10^{-5} and each diluted sample (0.1 mL) was poured aseptically onto sterilised soybean casein digest agar (tryptone soya agar, TSA, HiMedia, India) plates, to determine the aerobic heterotrophic or facultative anaerobic culturable autochthonous bacteria population. The prominent colonies with different morphological appearances (configuration, colour, margin, opacity, and surface) were randomly collected, streaked separately on respective media plates and repeatedly sub-cultured to get pure cultures. Total 42 pure cultures were preserved on slants in a refrigerator (4 °C) for subsequent study.

Characterisation of isolated bacterial strains

Bacterial strains were subject to different biochemical tests (Table 1) following the methods of Cappuccino and Sherman (2005). The bacterial isolates were tested on selected media agar plates and broths to enumerate different extracellular enzyme-producing abilities. Carboxymethyl cellulose, peptone-gelatine, phytase, starch, tributyrin and xylan media were used for cellulase, protease, phytase, amylase, lipase, and xylanase, respectively. Carboxymethyl cellulose, peptone-gelatine, phytase, starch and tributyrin media were prepared following Bairagi et al. (2002), whereas phytase and xylan media were prepared following Howson et al. (1983) and Ninawe et al. (2006), respectively. There were three replicates for each experimental set.

Qualitative extracellular enzyme activities were determined based on measurement halo (diameter in mm) around the colony 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) (Ghosh et al., 2017). Based on the qualitative assay and biochemical tests, two strains were selected for further experiments and named ONKP1 and ONKP2 for ease of understanding. Quantitative determination of exogenous enzymes production (amylase, xylanase, and lipase) was carried out following the standard methods - DNSA method (Miller, 1959), DNS-stopping method (Bailey, 1992) and titrimetric method (Awad et al., 2015) and unit (U) activity have been presented.

Identification of isolated bacterial strains by 16S rRNA gene sequence analysis

After isolation and PCR amplification, the most promising two microbial isolates ONKP1 and ONKP2 were identified through 16S rRNA (bacteria) partial gene sequence analyses. The gene encoding 16S rRNA was amplified using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) universal primers by polymerase chain reaction (PCR) (Ghosh et al., 2017). For the explicit taxonomic position of these strains, obtained 16S rDNA sequences were compared with the 16S rRNA sequences database by the Basic Local Alignment Search Tool in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The genetic distance and phylogenetic tree were estimated in MEGA 7.0 software using the neighbour-joining method (Saitou and Nei, 1987) with bootstrap analysis (Felsenstein, 1985).

Results

The results of the biochemical, qualitative, and quantitative exoenzyme tests of ONKP1 and ONKP2 are presented in Tables 1, 2 and 3, respectively. The two

Table 1. Morphological and biochemical characteristics of *Aeromonas veronii* ONKP1 and *Stenotrophomonas maltophilia* ONKP2 isolates from *Oreochromis niloticus*.

Characterising tests	ONKP1	ONKP2
Morphology		
Shape	Rod	Rod
Opacity	Opaque	Opaque
Motility	+	+
Biochemical		
Gram stain	-	-
MacConkey agar	+	+
Catalase	-	+
Methyl red	+	-
Voges Proskauer	-	-
Citrate utilisation	+	+
Indole	+	-
H_2S	-	-
Urease	-	+
Carbohydrate fermentation		
Glucose	+	+
Mannitol	+	-
Lactose	+	-
Maltose	+	+
Sucrose	+	-
Gelatine liquefaction	+	+
Species	*Aeromonas veronii*	*Stenotrophomonas maltophilia*

Table 2. Qualitative test scores of extracellular enzymes produced by *Aeromonas veronii* ONKP1 and *Stenotrophomonas maltophilia* ONKP2 isolates from *Oreochromis nilioticus*.

Extracellular enzymes	ONKP1	ONKP2
Amylase	++++	++++
Cellulase	-	-
Protease	-	-
Lipase	+	+
Phytase	-	-
Xylanase	+++++	+++
Tannase	-	-

+++++: very high; ++++: high; +++: good; ++: moderate; +: low; –: nil.

Table 3. Quantitative test values of extracellular enzymes produced by *Aeromonas veronii* ONKP1 and *Stenotrophomonas maltophilia* ONKP2 isolates from *Oreochromis niloticus*.

Extracellular enzymes	ONKP1	ONKP2
Amylase (U)[a]	135.69 ± 1.45	94.68 ± 1.22
Lipase (U)[b]	10.00 ± 0.11	9.93 ± 0.23
Xylanase (U)[c]	700.58 ± 0.22	209.44 ± 0.38

Enzyme activity (unit activity, U) by selected microbial strains, Values expressed as mean ± SE. n = 3.
[a]Microgram (µg) maltose liberated mL^{-1} of enzyme $extract^{-1}$ min^{-1}.
[b]Microgram (µg) free fatty acid liberated mL^{-1} of enzyme $extract^{-1}$ min^{-1}.
[c]Microgram (µg) D-xylose liberated mL^{-1} of enzyme $extract^{-1}$ min^{-1}.

isolates are Gram-negative, motile, and rod-shaped. Both were able to grow in MacConkey agar and capable of utilising starch (Figs. 1.A.I and 1.B.I), maltose, gelatine, xylan (Figs. 1.A.II and 1.B.II), olive oil and citrate (Figs. 1.A.III and 1.B.III). They are unable to produce H_2S gas and Voges-Proskauer negative. In contrast to these common characteristics, the strains differ in many others like catalase, tryptophanase (Indole test) and urease production (Figs. 1.A.IV and 1.B.IV) as well as carbohydrate and mixed-acid fermentation. They utilise starch, xylan and lipid in significant amounts (Tables 2 and 3).

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 (Ribosomal Database Project) databases, strain ONKP1 and ONKP2 were identified as *Aeromonas veronii* and *Stenotrophomonas maltophilia* respectively. The GenBank accession numbers of the reference strain ONKP1 and ONKP2 are MN602971 and MN602972, respectively. Phylogenetic relationships of the identified bacterial isolates with other closely related strains retrieved from NCBI GenBank are presented in the dendrogram (Fig. 2). Horizontal bars in the dendrogram represent the branch length. Bootstrap values have shown similarity and homology of the neighbouring sequences. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004).

Discussion

The GI tract of fish contains either autochthonous (indigenous) microbiota, which is able to colonise epithelial surfaces of the host, or allochthonous (transient) microbiota, which is unable to colonise (Ray et al., 2012). Earlier, the GI tract has been suggested as one of the primary infection routes in fish (Ringø et al., 2016). However, some research on fish gut microbiota hinted at their possible positive effects on the digestive processes and nutrition of fish. Various genera like *Bacillus*, *Pseudomonas*, *Aeromonas*, *Vibrio*, *Staphylococcus*, *Flavobacterium*, *Microbacterium*, *Micrococcus*, *Enterobacteriaceae*, *Acinetobacter*, unidentified anaerobes and yeast have been isolated and identified for their enzyme producing ability and suggested to be possible contributors (Ray et al., 2012; Banerjee and Ray, 2017; de Bruijn et al., 2018; Butt and Volkoff, 2019). Studies on the gut microbiota of fish found that bacteria are the dominant micro-

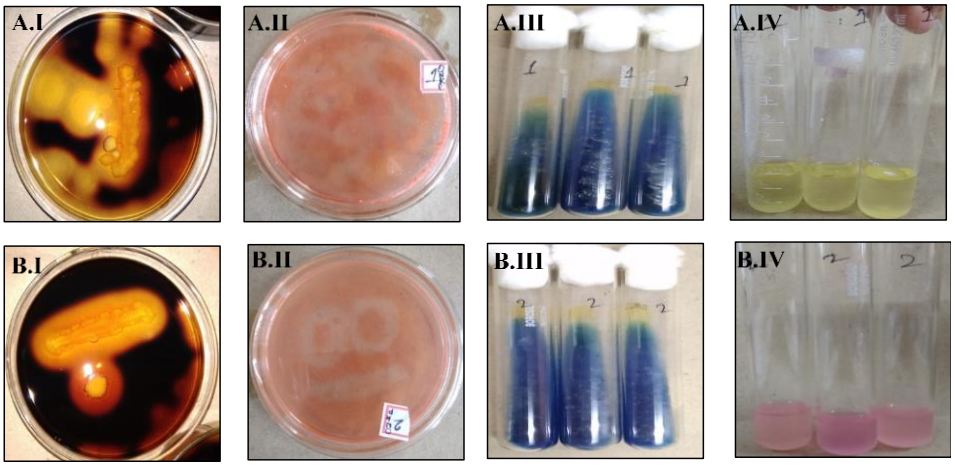

Fig. 1. Biochemical test results of reference strains, A. *Aeromonas veronii* ONKP1 MN602971 (I. Amylase activity: positive; II. Xylanase activity: positive; III. Citrate utilisation: positive; and IV. Urease activity: negative) and B. *Stenotrophomonas maltophilia* ONKP2 MN602972 (I. Amylase activity: positive; II. Xylanase activity: positive; III. Citrate utilisation: positive; and IV. Urease activity: positive).

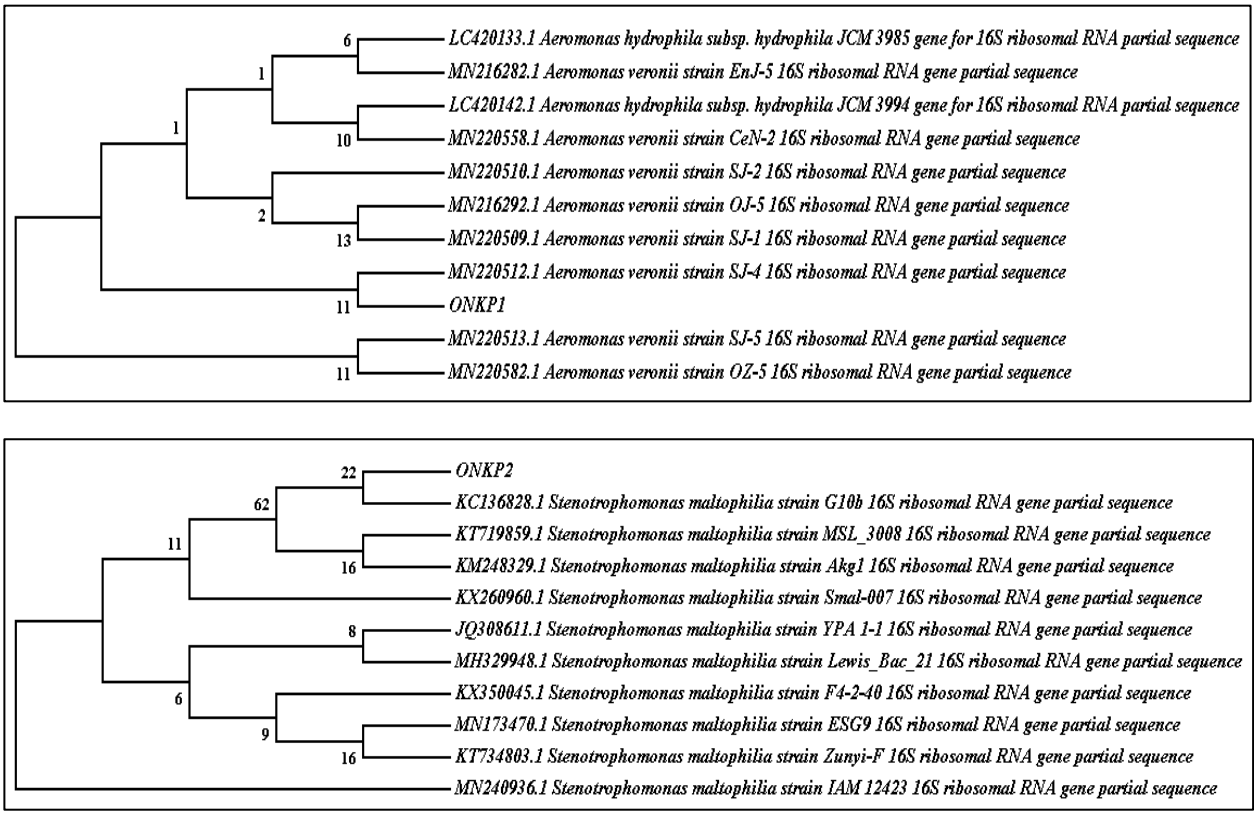

Fig. 2. Dendrogram showing phylogenetic relations of the bacterial strain, *Aeromonas veronii* ONKP1 (Top) and *Stenotrophomonas maltophilia* ONKP2 (Bottom) isolates from *Oreochromis niloticus*, with other closely related strains retrieved from NCBI GenBank and RDP.

biota of the fish intestine, and factors like life stage, diet, trophic level, season, captive-state, habitat, sex, and phylogeny influence the intra and inter-species level diversity (Egerton et al., 2018; Kokou et al., 2019; Yukgehnaish et al., 2020).

Previously, enzymes producing *Aeromonas* spp. have been identified from the intestine of various fish. For example, among one of the first studies, Trust et al. (1979) isolated amylase, lipase and protease producing *Aeromonas hydrophila* from the gut of grass carp (*Ctenopharyngodon idella* (Valenciennes, 1844)). Later, amylolytic, cellulolytic and proteolytic *Aeromonas* spp. were isolated from grey mullet (*Mugil cephalus* Linnaeus, 1758) (Hamid et al., 1979), some freshwater fishes (Sugita et al., 1997), roach (*Rutilus rutilus* (Linnaeus, 1758)) (Skrodenyte˙-Arbac˘iauskiene˙, 2007), grass carp (Jiang et al., 2011) and African catfish (*Clarias gariepinus* (Burchell, 1822)) (Ariole et al., 2014).

Aeromonas spp. is generally predominant in most freshwater fishes' intestinal tract, especially in omnivorous tilapia and herbivorous grass carp (Ray et al., 2012). The composition of GI tract microbial community is directly dependent on the food habit of the host and environment (Ringø et al., 2016). The results of the present study are also in agreement with the previous studies, which found high amylolytic activity by *A. veronii* ONKP1 strain, indicating the plant-based diet of tested tilapia. *Aeromonas veronii* ONKP1 strain also possesses glucose, sucrose, mannitol, maltose, and lactose utilising ability but lacks cellulolytic activity. *Aeromonas veronii* ONKP1 strain showed good lipolytic activity, indicating their probable contribution to fish lipid metabolism.

Interestingly, *A. veronii* ONKP1 strain can produce xylanase, an enzyme of industrial importance. It is known to be produced by the microbial population (bacteria, fungi, and actinomycetes) only. Xylanase hydrolyses xylan (hemicellulose) component of agro-industrial residues and thus is advantageous for the recovery of sugars for biotechnological applications (Ray et al., 2012). However, reports on the production of xylanase by fish gut endosymbionts are scanty. Moreover, *A. veronii* ONKP1 strain has gelatinolytic activity, i.e., able to liquefy gelatine but lacks caseinolytic activity. The gelatinolytic activity of bacteria might help in collagen digestion in fish. Proteases like gelatinase are also known to help bacteria in biofilm formation and mobility (Thurlow et al., 2010). However, gut microbiota utilising different substrates *in vitro* does not necessarily have any *in vivo* effect (Ray et al., 2012) and thus the topic needs further investigations. Strains of *A. veronii* are rarely reported for use as probiotics in aquaculture due to their widely known various pathogenic strains. However, studies indicate that administration of safe intestinal autochthonous *A. veronii* strains can improve growth, immune response, disease resistance and modulate

gut microbes of grass carp (Chi et al., 2014) and common carp (*Cyprinus carpio*, Linnaeus, 1758) (Hao et al., 2017; Jinendiran et al., 2021). As *A. veronii* ONKP1 is also an autochthonous intestinal microbe isolated from healthy fish, it is possible to use it as a probiotic.

Stenotrophomonas maltophilia has emerged as an important opportunistic pathogen in hospitalised and immunocompromised patients (Geng et al., 2010). It is rarely reported in cultured fishes. As yet, few studies have isolated *S. maltophilia* from diseased fish, for example, snubnose dart (*Trachinotus blochii*, (Lacépède, 1801)) (Zhou et al., 2001), Japanese amberjack (*Seriola quinqueradiata* Temminck & Schlegel, 1845) (Furushita et al., 2005), sea bream (*Sparus aurata* Linnaeus, 1758) (Kapetanović et al., 2006), giant gourami (*Osphronemus goramy* Lacépède, 1801) (Musa et al., 2008), channel catfish (*Ictalurus punctatus* (Rafinesque, 1818)) (Geng et al., 2010) and African catfish (Abraham et al., 2016). Abraham et al. (2016) found *S. maltophilia* (isolated from the kidney) positive for gelatinase, lipase and haemolytic activity. As *S. maltophilia* species once belonged to the genus *Pseudomonas* and *Xanthomonas* (Carmody et al., 2011), there is a chance of misidentification in past studies. *Pseudomonas* spp. is a common bacteria found in diseased fish and reported from the healthy fish gut (Ray et al., 2012). There are significant difficulties identifying and clearly distinguishing between the *Pseudomonas* and *Stenotrophomonas* genera without using molecular methods like 16s rRNA analysis (Aksentijević et al., 2016). In the present study, isolated *S. maltophilia* ONKP2 strain is able to produce amylase, lipase, xylanase and gelatinase enzymes like *A. veronii* ONKP1. This is the first report of *S. maltophilia* as extracellular enzyme-producing gut bacteria from tilapia, *O. niloticus*.

Currently, the application of exogenous dietary enzymes in aquaculture is limited. Some studies have shown that the dietary supplement of enzymes including lipase (Fei et al., 2018; Zhang et al., 2020b), amylase (Ghosh et al., 2001; Kumar et al., 2006) and xylanase (Ranjan et al., 2018; Jin et al., 2020) in aquafeed can improve the growth, lipid utilisation, antioxidant capacity and immune response in fish. Moreover, the nutritional value and digestibility of feed could be increased by applying amylase (Qazi et al., 2012) and xylanase (Tsai et al., 2019; Dahiya et al., 2020). Moreover, xylanase is also used to produce prebiotic xylooligosaccharide, which provides health benefits to aquatic species (Zhang et al., 2020a; Poolsawat et al., 2021). Thus, further research and more attention may be encouraged in beneficial gut bacteria and their application in aquafeed to improve aquatic species' health and nutrition.

Conclusion

In this study, *Aeromonas veronii* and *Stenotrophomonas maltophilia* isolated from the intestine of tilapia, *Oreochromis niloticus*, produced amylase, xylanase, and lipase enzymes. They may play roles in the digestion of food in the host fish. Although, assumptions based on *in vitro* experiments might not conform precisely to *in vivo* conditions. However, they could be used for the fermentation of plant-based feed to increase their digestibility, reduce the cost of feed and recovery of raw materials (e.g., sugars, polypeptides) for the sustainable production of biofuels, biopower and nutraceuticals. Further studies on the bacteria can validate their possible use directly as probiotics or their products as prebiotic to boost host health. This is the first report of isolation of enzyme-producing *S. maltophilia* from the gut of healthy fish, *O. niloticus*.

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Conflict of interest: The authors declare that they have no conflict of interest.

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