Full Paper

Isolation of cellulase-producing *Microbulbifer* sp. from marine teleost blackfish (*Girella melanichthys*) intestine and the enzyme characterization

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Most animals cannot digest cellulose but have symbiotic microbes that degrade the matrix polysaccharides of plant matter. Herbivorous and omnivorous marine fish are similarly expected to rely on symbiotic microbes, but reports to date on cellulase-producing bacteria in fish intestines are limited. Here, we report the isolation of new cellulase-producing bacteria from the marine omnivorous teleost, blackfish (*Girella melanichthys*), and the characterization of cellulase activity. Three strains of cellulase-producing bacteria sp. were isolated from the hindgut of wild *G. melanichthys*. The strains of cellulase-producing bacteria grew in medium with artificial seawater but not in NaCl alone. Growth was optimum at 20–35°C, but there was no growth at 40°C, suggesting adaptation in a marine environment at a low temperature. Isolates were identified to *Microbulbifer* sp., among which GL-2 strain produced a high enzyme activity. The GL-2 strain was further used for enzyme characterization with carboxymethyl cellulose (CMC) as the substrate. Maximum activity of the cellulase was observed at 60°C, and activity was more than 30% at 20°C, while commercial cellulase Enthiron showed an optimum activity at 50°C and 17% activity at 20°C. Hydrolytic products by GL-2 cellulase were cellobiose but not glucose, suggesting a deficiency of β-glucosidase activity. Active gel electrophoresis containing CMC showed five bands, suggesting several cellulolytic enzymes. The GL-2 strain and its enzyme are potential probiotics for aquaculture fish and the industrial production of cellobiose.

Key Words: bacteria; cellulase; low temperature; marine fish

Abbreviations: MB, marine broth; MBA, marine broth plus agarose; PBS, phosphate buffered saline; AZCL, azurine-crosslinked hydroxyethyl cellulose; CMC, carboxymethyl cellulose; cfu, colony forming units; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Introduction

The aquaculture industry has been increasing worldwide; as of 2013, 60% of fish for food are reared in aquaculture (World Bank, 2013). For efficient production, and to avoid environmental loading by aquaculture, fish feed has been improved. Recent fish feed in Japan is composed of fish meal, soybean flour and fish oil, making fish aquaculture dependent on fish and imported soybeans. Achieving self-sufficiency of feed material sourcing and reducing the amount needed are targets for conducting aquaculture sustainably.

Replacement of fish materials in aquaculture feed with feather meal (Poppi et al., 2011) and plant materials (Drew et al., 2007) has been investigated. For gilt-head bream (*Sparus aurata*), increasing soybean flour to 60% is known to not affect feed efficiency and intestinal microflora (Parma et al., 2016). Thus, testing the addition of vegetable material is promising, and domestic plants may be appropriate resources. However, cellulose cannot be utilized directly by fish, and in order to increase the feed...
efficiency of cellulose, polymer cellulose should be hydrolyzed to oligosaccharides. For cellulose digestion in fish feed production, cellulase is a valuable tool to produce utilisable oligosaccharides. Since the intestinal bacteria of fish act as a symbiotic organism with fish, bacterial cellulases are expected to be appropriate enzymes for fish feed processing.

The β-1,4 glycosidic bond of cellulose can be hydrolyzed by cellulases, including endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). The final product of cellulose hydrolysis is glucose, and oligosaccharides and cellulbiose are formed as intermediates. Cellulases are used for bioethanol production (Carvalho et al., 2014) and for aquaculture feed (Hlopet-Ginindza et al., 2016). In the case of bioethanol production, glucose is essential; however, cellulolytic oligosaccharides are preferred due to their better feed efficiency, their immune system enhancing, and their microflora balancing properties. Oligosaccharide supplement, oligo-arabinoxylan, in fish feed was shown to be effectively utilized by streusen in the presence of lactic bacteria (Geraylou et al., 2013), and fructooligosaccharide enhanced immunity in salmon (Abid et al., 2013). Cellobiose is known to be a growth promoter in ruminant bacteria (Helaszek and White, 1991; Pokusaeva et al., 2011), but its use in fish has not been reported.

Cellulase has been isolated from bacteria, fungus, protists and arthropoda (Bui and Lee, 2015; Carvalho et al., 2014; Sakatoku et al., 2014; Wheeler et al., 2007). Bacteria isolates have various known origins: soil (Hu et al., 2006), algae (Trivedi et al., 2013; Wakabayashi et al., 2012), marine sediment (Sakatoku et al., 2014) and freshwater fish (Ray et al., 2010; Saha et al., 2006).

For aquaculture feed, herbivorous and omnivorous fish intestines may be hypothesized as a source of cellulyolytic enzymes. At present, two reports on the isolation of cellulyolytic bacteria from marine fish are available: Pseudo- domonas and six other genera from Pacific salmon (Askarian et al., 2012) and Clostridium from pinfish (Stellwag et al., 1995). However, the enzymes have not been characterized. Weiner et al. (2008) reported cellulyase genes from marine bacteria, but the enzyme activity was not examined. Although Wakabayashi et al. (2012) found that marine Microbulbifer produced cellulase, enzymes were not characterized. Here, we aim to isolate cellulyolytic-producing bacteria from marine omnivorous fish and to characterize the enzymes.

Materials and Methods

Sampling. Blackfish (Girella melanichthys) were collected by bait fishing in June 2014 along the Yusu Coast, Ehime Prefecture, Japan. The fish were put on ice and dissected within a few hours. The intestines were separated into three regions, a, b and c, as shown in Fig. S1. Intestinal contents from three individuals were pooled as one specimen. In total, six fish were used and two specimens were prepared. The average body length of the blackfish (n = 6) was 12.3 ± 1.21 cm and the intestine length was 25.4 ± 6.08 cm (Fig. S1).

Isolation of cellulyolytic bacteria. Intestinal contents were diluted 10-fold in PBS and spread on agar plates of 1/10× concentration MB (Becton Dickinson) plus 1.5% agar (MBA), with the salt concentration adjusted with artificial seawater and the addition of fine granules of AZCL (Megazyme) to a final concentration of 0.2%. Duplicate plates were incubated at 25°C for 7 days, and then the colony number was counted. Cellulyolytic-producing bacteria decompose AZCL and produce colonies surrounded by a blue halo and are thus identified as being cellulyolytic-positive (Eida et al., 2012).

Bacterial growth test. Isolation of cellulyolytic-producing bacteria was performed on 1/10× MBA plates, and the growth of isolates was found to be faster on 1× MBA, but growth for subsequent assays was performed in 1× MBA liquid cultures. Isolates were grown at 30°C for 7 h with 120 rpm agitation. Growth was monitored by absorbance at 600 nm (OD₆₀₀) with a Biospectrometer (Eppendorf). Salt dependency was examined at 2%, 4% and 6% NaCl and artificial seawater (17.505 g NaCl, 0.408 g KCl, 3.104 g MgSO₄·7H₂O, 4.517 g MgCl₂·6H₂O and 0.717 g CaSO₄·2H₂O in 1 L).

Selection of cellulyolytic-producing isolates. Bacteria solution (OD₆₀₀ = 1.6, 80 μL) was applied on thick-type 8-mm-diameter antibiotic testing filters (Toyo), which were overlaid on MBA plates containing 0.2% AZCL. The plates were incubated at 30°C for 96 h. The width of the blue halo formed around the filter was measured with a vernier caliper from three directions over a time course. The isolate forming the widest blue halo was selected for further enzymatic study.

Enzyme preparation. Liquid culture of the isolate was centrifuged to remove the cell pellet. Ammonium sulfate was crushed to a fine powder with a mortar and slowly added to the supernatant to 80% saturation, and the solution was gently stirred overnight at 4°C. Precipitated material was collected by centrifugation at 8,400 × g at 4°C for 30 min. The pellet was put into a 5,000 Da cellulose dialysis tube and dialyzed against 50 mM Tris-HCl (pH 8.0). The buffer was changed 4 times, and the final buffer contained 15% glycerol. This was used as the crude enzyme fraction.

Enzyme assay. Reaction mixtures (600 μL) containing 50 mM acetate-NaOH buffer (pH 6.0), final concentration of 10 mg/mL of CMC (Nacalai Tesque) and the appropriate amount of enzyme were incubated at 50°C for 60 min and stopped by heating at 100°C for 10 min. The reaction product and the reducing sugar were quantified by the 2-cyanoacetoamide method (Gross, 1982). Enzyme reaction solution (300 μL) was mixed with 500 μL of 500 mM borate-NaOH buffer (pH 10.0), and then 100 μL of 1% 2-cyanoacetoamide was added, and the reaction was incubated at 100°C for 10 min. Absorbance at 276 nm (OD₂₇₆) was measured with the Biospectrometer. One unit (1 U) of cellulase was defined as the activity producing 1 μmol of reducing sugar equivalent to glucose and expressed as units per mg protein. For comparison, commercial cellulase Enthiron MCH derived from fungus Trichoderma (Rakuto Chemical) was used. Protein was quantified by the Bradford method (Bradford et al., 1976) us-
Enzyme characterization. To examine temperature and pH dependency, reaction mixtures containing 9.6 U/mg of GL-2, or 23.8 U/mg of Enthiron, were prepared. Incubation was performed at 20, 30, 40, 50, 60 and 70°C for 60 min for the temperature assay. The pH dependency test was performed at pH 3.0, 4.0, 5.0 and 6.0 with 50 mM acetate buffer, pH 6.0, 7.0 and 8.0 with 50 mM phosphate buffer and pH 8.0, 9.0 and 10.0 with 50 mM Tris-HCl. Relative enzyme activities were expressed as the percent of the maximum value for each enzyme.

Active gel electrophoresis. Crude enzyme was applied to SDS-PAGE. Dialysed enzyme fraction was mixed with 2× SDS PAGE sample buffer (0.125 M Tris, 10% 2-mercapptetanol, 4% SDS, 6% glycerol, 0.004% bromophenol blue) and heated at 100°C for 5 min. A sample was applied onto polyacrylamide gel containing 0.15% CMC. The polyacrylamide concentration was 9% in running gel and 4.75% in stacking gel. The sample was applied to two lanes in order to apply protein stain and CMC stain. Gel was electrophoresed with 20 mA at room temperature. After the electrophoresis, a half portion was cut and stained with Coomassie blue, and the other half portion was washed with 50 mM Tris-HCl (pH 7.5) three times at room temperature for 1 h. The gel was then stained with Congo red and washed with 1 M NaCl. Residual CMC on the gel was stained in red, and the surrounding of active polypeptide showed clearance bands. The molecular size marker was sp-0110 (APRO science).

Degradation product from carboxymethyl cellulose. Identification and quantification of reaction products of CMC were carried out using HPLC with a Multi-Station LC-8020 model II system (TOSOH). Samples (100 µL) were applied at 30°C to an Aminex HPX-87H Organic Analysis column (300 x 7.8 mm, Bio-Rad) linked to an RID-8020 refractive index detector (TOSOH) and eluted with 5 mM H₂SO₄ at a flow rate of 0.6 mL/min.

Statistics. Most bacterial and enzymatic experiments were performed in triplicate, and the significance of differences between pairs of data points were examined by the Student’s t-test.

Results

Isolation and classification of cellulase-producing bacteria

Hind gut (Region c in Fig. S1) of one specimen had bacteria cfu of 6.4 x 10⁵ cfu/g and 3.0 x 10⁴ cfu/g cellulase-producing colonies, which were average numbers from duplicate plates. Samples from another specimen and other regions (Regions a and b in Fig. S1) showed swarming motility, and cfu could not be determined. Eventually, we established three strains, GL-1, -2 and -3 from Region c of the specimen for which distinct colonies formed during culturing.

Based on 16S rRNA gene sequences, the GL-1, 2 and 3 strains were very close to each other (>99.8% similarity) and identified to be Microbulbifer sp. The most closed species was M. epialgicus (LN812998.1). The phylogenetic relationship of the GL strains to the reported isolates is shown in Fig. 1.

Bacterial growth characteristics

All three strains showed good growth within the temperature range of 20–35°C with the optimum being 35°C, but none grew at 40°C (Fig. 2). Salt requirements indicated that the three strains needed artificial seawater. Strain GL-3 showed a low growth in media with 4% NaCl, but strains GL-1 and GL-2 did not grow within the range of 2–6% NaCl (Fig. 3A). The pH dependency indicated that...
the strains showed growth within pH 7-9, with the optimum being pH 7 for GL-1 and pH 8 for GL-2 (Figs. 3B–D). In order to further study the bacteria with consideration of their use in industry, the strain showing the highest degradation ability was selected.

Thus, GL-2 had the highest cellulase activity on AZCL plates (Fig. S2), showing a greater blue halo width at 96 h incubation than GL-1 and GL-3 ($p < 0.05$). GL-2 was used for the subsequent enzyme study.

Enzyme activity

GL-2 showed the highest enzyme activity at 60°C and Enthiron showed the highest activity at 50°C (Fig. 4A). The relative activity of the two enzymes was identical in the range 30–70°C, but activities of GL-2 (32.8%) and Enthiron (17.1%) were significantly different ($p < 0.05$) at 20°C. The effect of pH on enzyme activity was quite different between the two enzymes with GL-2 being active at pH 6.0–8.0 and Enthiron being active at pH 4.0–5.0 (Fig. 4B).

Degradation products of CMC

By using CMC as the substrate, enzyme reactions were examined at 50°C and pH 6.0, which are within the appropriate ranges of the GL-2 enzymes as shown in Fig. 4. HPLC profiles (Fig. 5) show the separation of products, cellobiose and glucose. Cellobiose and glucose were eluted at 7.5 and 8.8 min, respectively. The peak of glucose in reacted samples were found at 0.3 min earlier than the standard due to buffer solution in samples. The small peak
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of glucose in Fig. 5A (found at 90 min reaction) was a contamination in CMC, which slightly increased at 30 min but was not significantly different. Time course changes in degradation product amounts are shown in Fig. 6. Cellobiose increased with time in GL-2 enzyme, but glucose did not (Fig. 6A), whereas Enthiron produced both cellobiose and glucose (Fig. 6B). Product formation was dependent on the enzyme amount used (Figs. 6C and D). These experiments indicated that GL-2 enzyme did not produce glucose. Contamination glucose in CMC did not increase time and enzyme dependently (Figs. 6A and C).

Polypeptide size of active enzymes

Crude enzyme was analyzed by SDS-PAGE and active gel (Fig. 7). Enzyme activity was found at molecular weights 69.8, 66.3, 53.7, 41.4 and 36.6 kDa (Fig. 7, lane 1). Among the five active bands, the 53.7 kDa band showed the strongest activity. The crude enzyme fraction contained many other polypeptides (Fig. 7, lane 2).

Discussion

We isolated three strains of cellulase-producing Microbulbifer sp. from blackfish intestine, and characterized the cellulase. Although the species of genus Microbulbifer is reported to produce cellulase (Wakabayashi et al., 2012), the present study is the first report of cellulase-producing bacteria from marine herbivores or omnivores. Since Microbulbifer species 6532A was isolated from algae (Wakabayashi et al., 2012), our isolates could be derived from food algae of the fish. Species listed in Fig. 1, Teredinibacter turnerae T7901 (Distel et al., 2002), Saccharophagus degradans 2–40 (Ekborg et al., 2005), Saccharophagus sp. Myt-1 (Sakatoku et al., 2012, 2014) and Pseudomonas sp. MM15 (Yang and Dang, 2011) were reported to have cellulase production and cellulase genes.

The three strains in the present study grew over the range of 20–35°C. In other cellulase producing bacteria, P. aeruginosa associated with termites showed optimum growth at 30°C (Bholay et al., 2014), the soil-derived Streptomyces griseorubens (Prasad et al., 2013) and porcine gut-derived Bacillus subtilis (Yang et al., 2014) grew at 45 and 50°C, respectively. Our strains grew at a lower temperature than these, and needed not only NaCl but also other salts for growth. Additionally, the optimum pH was 7.0–8.0, which is a range similar to that of seawater. Taken together, our strains are well adapted to a seawater environment.

Fish intestinal Bacillus circulans from freshwater tilapia Oreochromis mossambica is known to produce cellulase, which has a symbiotic effect on host fish in terms of being a nutrient donor (Saha et al., 2006). Many herbivorous fish have been identified in freshwater but not in seawater, and this might be due to the greater inflow of plant materials from land to rivers and lakes, and less inflow to marine environments. Thus, terrestrial plant input and the beneficial symbiotic cellulase-producing bacteria are likely to be less available to marine herbivorous, and omnivorous, fish. The marine bacteria obtained in the present study suggest that they play a symbiotic role in blackfish to digest cellulose derived from seaweed. Agarases are frequently found in marine bacteria (Hu et al., 2009; Wakabayashi et al., 2012) because agar is a major matrix polysaccharide in marine algae that is utilized by bacteria.
We chose strain GL-2 based on it having a higher cellulase activity than the other two strains. Enzymatic characteristics of GL-2 were different from Enthiron in terms of the temperature and pH ranges over which it was active. GL-2 enzyme had a significantly higher activity (32.8%) at 20°C than Enthiron (17.1%) \((p < 0.05)\), although the temperature of maximum activity was similar. It is known that marine bacterial enzymes are active at low temperatures (Huston et al., 2004; Odagami et al., 1993; Watanabe et al., 2005), including cellulase Cel5M from marine sediment bacterium *Pseudomonas* sp. MM15 (Yang and Dang, 2011). Marine sediment-derived *Saccharophaga* sp. Myt-1 produced cellulase CelMylB, which had an optimum temperature at 55°C and activity at 25°C of 30% (Sakatoku et al., 2014). Our GL-2 cellulase was similar, suggesting that adaptation to the marine condition occurred at the enzyme level. Theoretically, a high catalytic efficiency of enzymes at low temperatures is due to their structural flexibility and higher turnover number (Mageswari et al., 2017). However, these properties cannot preserve the protein structure, resulting in declining activity at high temperatures (Gerday, 2013). The GL-2 enzyme shows a high optimum temperature but remains active at low temperatures, suggesting that multiple enzymes are involved in the cellulolytic activity. This is supported by the SDS-PAGE-active gel result, showing that at least five polypeptides could degrade CMC. The Cel5M enzyme was at its optimum at pH 4.5 while only 20% activity was detected at pH 8.0 (Yang and Dang, 2011). GL-2 enzyme was at its optimum at pH 8.0, which is suitable for the marine seawater environment.

The kinetics of cellulose hydrolytic products showed time- and dose-dependent increases in cellobiose, but not glucose. This suggests that GL-2 does not produce \(\beta\)-glucosidase, which produces glucose as a catabolite. Enthiron and other fungal enzymes produce glucose (Igarashi et al., 2008). Until now, genetically mutated bacteria *Neurospora crassa* have been reported to produce cello-oligosaccharide (Wu et al., 2013), but production of cellobiose by bacteria isolated from the natural environments and fish intestine is not known. In spite that GL-2 enzymes showed a mixture of enzymes, the product was cellobiose, not glucose. This suggests that the GL-2 does not have an enzyme producing glucose from cellulose. Cellulolytic enzymes contain endoglucanases, cellobiohydrolase and \(\beta\)-glucosidase (Koeck et al., 2014), by which glucose is produced from cellulose. This suggests that GL-2 does not have \(\beta\)-glucosidase. Marine *Saccharophaga* strain genome analysis revealed it to have \(\beta\)-glucosidase (Weiner et al., 2008). The present study is the first report of marine bacteria that produces cellobiose, but not glucose.

Utilization of saccharides by intestinal bacteria is well known, showing that cellobiose can be more efficiently utilized by *Bifidobacterium breve* than glucose (Pokusaeva et al., 2011). *Enterococcus munditii* utilizes cellobiose as a substrate for lactic acid production (Abdel-Rahman et al., 2011). Furthermore, cellobiose is a growth promoting factor in rumen bacterium *Ruminococcus flavefaciens* (Helaszek and White, 1991), indicating that cellobiose should provide a positive effect on animal intestinal microbes. However, no direct evidence has been obtained for fish to date, and the effect of cellobiose on fish physiology should be studied further. *Pseudomonas* sp. is reported as a probiotic in aquaculture of rainbow trout *Oncorhyncus mykiss*, in which the suppression of *Vibrio anguillarum* infection was observed (Gram et al., 1999). However, there are no reports on cellulase and its applications for aquaculture. The present study showed cellobiose-producing bacteria in fish intestine. We expect that applying GL-2 and its enzyme to aquaculture feed processing and probiotics will be beneficial for the aquaculture of marine fish.

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**Compliance with Ethical Standards**

Conflict of interest Daiki Tanaka declares that he has no conflict of interest. Ken-ichiro Ohnish declares that he has no conflict of interest. Seiya Watanabe declares that he has no conflict of interest. Satoru Suzuki declares that he has no conflict of interest.

**Ethical Approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

**Supplementary Materials**

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/gjam).

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