Isolation and screening of cellulose and organic matter degrading bacteria from aquaculture ponds for improving water quality in aquaculture

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Abstract. Aquaculture is one of the agroindustrial activities that has the highest growth rate in last decades and provides undeniable benefits for humanity such as providing food, generating jobs as well as contributing to economic development. However, intensive aquaculture also is one of the most criticized activities because of their environmental impacts, in which feed is the main source of waste and is responsible for most of the environmental impacts of aquaculture. Besides high concentration of organic and inorganic wastes from uneaten feed and excreta of aquaculture animals, amount of insoluble fiber (e.g., cellulose, hemicellulose, lignin) in aquaculture water is significant due to the majority of aquaculture feed is plant-based feed and contain a high amount of fiber. The polluted aquaculture water results in not only increase of environmental treatment cost but also outbreaks of aquaculture animal diseases. Improving water quality and reducing the accumulation of pollutants in aquaculture crops are therefore constant concerns. In this study, we isolated and screened cellulose and organic matter degrading bacteria from aquaculture ponds for producing probiotics which can improve aquaculture water quality and feed digestibility of aquaculture animals. From sediment and water samples collected at fish ponds in Phu Vang district, Thua Thien Hue provine, we isolated 215 bacterial strains, of which 25 isolates exhibited at least one of cellulolytic, amylolytic and proteolytic activities. Particularly, the strain *Bacillus* sp. W12 produced enzymes that are capable of degrading both cellulose and organic matters (cellulase, protease and amylase). The optimum conditions for enzyme production by *Bacillus* sp. W12 were obtained at pH 7, temperature 30^{o}C, 72 h of incubation and 0.4% of NaCl. Activity of the enzymes reached the highest activity at pH 6-8 and temperature 30 - 40^{o}C. The present study showed the strain *Bacillus* sp. W12 as a potential probiotic candidate for degrading cellulose and organic substrates in aquaculture water as well as improving feed digestibility of aquaculture animals.

1. Introduction

Aquaculture is one of the fastest growing sectors and one of the most important contributors to total global seafood production in last decades. According to the Food and Agriculture Organization of the United Nations (FAO), aquaculture contributed more 44% global seafood production in 2014 [1]. Over more past 30 years (1980 to 2012), global supply from aquaculture has an annual growth rate of 8.6%, whereas the capture fish production has been gradually decreased [2]. In 2014, global aquaculture production has surpassed the production of capture fisheries [1].

In order to keep up with population growth and meet the increasing demand for high protein foods of humanity, the global aquaculture production has rapidly grown and increased pressure on the environment by wastes from aquaculture farms. In aquaculture production, feed and excreta from aquaculture animals are main sources of waste and is responsible for most of the environmental impacts of aquaculture. The use of excessive feeds in intensive aquaculture can result in the high amount of uneaten feed and contribute significantly to pollution loads in aquaculture water [3, 4]. Besides high concentration of organic matters, nitrogen, ammonia, nitrite from uneaten feed and excreta of aquaculture animals, amount of insoluble fiber (e.g., cellulose, hemicellulose, lignin) in aquaculture water is significant due to the majority of aquaculture feed is plant-based feed and contain a high amount of fiber. The polluted aquaculture water results in not only increase of environmental treatment cost but also outbreaks of aquaculture animal diseases [5, 6]. Improving water quality and reducing the accumulation of pollutants in aquaculture crops are therefore constant concerns.

Studies have shown that hydrolytic enzyme producing bacteria can be used as ecological biocontrol or bioremediation agent for the sustainable development of aquaculture. The probiotic bacteria can decrease algae growth, load of pollutants but increase beneficial bacterial population and inhibition of potential pathogens [5, 7]. The bacteria of genera *Bacillus*, *Nitrosomonas*, *Nitrosobacter*, *Rhodopseudomonas*, *Lactobacillus*, and *Saccharomyces* have been used to reduce amount of organic matters, load of pollutants, heavy metals and pathogens in culture ponds [5, 8-11]. Furthermore, enzyme producing bacteria (amylase, protease, and cellulase) have used to improve growth rate and feed digestibility of aquaculture animals as well as improving water quality and remove undesired substrates in different wastewaters, waste sludges (swine wastewater, tannery saline wastewater, sewage sludge) [12-19].

In this study, we isolated and screened cellulose and organic matter degrading bacteria from aquaculture ponds for producing probiotics which can improve aquaculture water quality and feed digestibility of aquaculture animals.

2. Materials and methods

2.1. Collection of samples and isolation of bacteria

The sediment and water samples in this study were collected from fish ponds in Thuan An, Phu Vang district, Thua Thien Hue province. The samples were contained in sterilized bags and transported immediately to the laboratory.

For isolation of bacteria, 1 g of sediment sample was suspended in 9 ml of sterile distilled water. After serial dilution of the suspension (10^{-4} to 10^{-6}), 100 µl of each dilution was spread on agar plates containing 0.5% peptone, 0.2% beef extract, 03% yeast extract, 0.5% NaCl, 1.8% agar, pH 7). The petries were incubated at 37°C for 24 - 48 h. In case of the water sample, 100 µl of sample was used for isolation with the above described method. The colonies with different morphology were selected for further investigation.

2.2. Screening of cellulose and organic matter degrading bacteria

For screening of cellulose degrading bacteria, the isolates were cultured on CMC agar plates, and then the plates were flooded with the solution of 0.1% Congo red for 15 min and washed with 1 M NaCl. The cellulose degrading bacteria were determined by hydrolysis zone on agar plates.

Organic matter degrading activities of the isolates were examined based on starch and protein degrading activity by bacteria producing enzyme amylase and protease. For screening of starch degrading bacteria, bacterial isolates were streaked on starch agar plates and incubated at 37^{o}C for 24 - 48 h. Then the plates were flooded with Gram's iodine to visualize hydrolysis zone. The starch degrading isolates were determined by hydrolysis zone on agar plates. For screening of protease producing bacteria, bacterial isolates were streaked on skimmed milk agar plates and incubated at 37^{o}C for 24 - 48 h. Protease producing isolates were determined by hydrolysis zone on agar plates.

2.3. Enzyme activity assay

The isolates were cultured in broth medium for 24 - 48 h, and then the cultures were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were collected as crude enzymes for enzyme assay.

Cellulolytic activity was assayed as the described method [20]. Briefly, 0.2 mL of crude enzyme was added to 1.8 mL of 0.5% CMC in 50 mM sodium phosphate buffer (pH 7). The mixture was incubated at 37°C in a shaking water bath for 30 min, followed by adding 3 mL of dinitrosalicylic acid (DNS) reagent and boiling for 5 min. The absorbance/ Optical Density (OD) of samples was measured at 575 nm against the blank sample containing all the reagents without the crude enzyme.

Amylolytic activity was assayed as the described method [21] with some modifications. Briefly, 0.2 mL of crude enzyme solution was added to 1.8 ml of 1% starch in 0.1 M phosphate buffer (pH 6.5). The mixture was incubated for 15 min at 37^{o}C, followed by adding 1 mL of DNS reagent and boiling for 10 min. The absorbance of samples was measured at 540 nm against the blank prepared as above without crude enzyme.

Proteolytic activity was assayed as the described method [22] with some modifications. Briefly, 0.2 mL of crude enzyme solution was added 1.8 mL of 1% casein in 0.1 M phosphate buffer (pH 7.0). The mixture was incubated for 20 min at 40^{o}C, followed by adding 3 mL of 5% TCA. The mixture was kept at room temperature for 15 min, and then centrifuged at 10,000 rpm for 5 min. Next, 0.5 mL of the supernatant was mixed 2.5 mL of 0.5 M sodium carbonate and incubated for 20 min. Then the mixture was added with 0.5 mL of folin phenol reagent and the absorbance of samples was measured at 660 nm against the blank prepared as above without crude enzyme.

2.4. DNA extraction and identification of isolates by 16S rRNA gene sequence

The genomic DNA of the selected isolates was extracted and the 16S rRNA genes were amplified using primer pairs 27F/1525R [23]. The PCR products were sequenced on sequencer ABI PRISM 3100. The sequences were removed poor quality ends using BioEdit software v.2.7.5, then compared with other 16S rRNA gene sequences available in GenBank by using BLASTN program.

2.5. Optimization of culture conditions for production of enzymes and properties of enzymes

Effects of culture conditions on production of enzymes were performed by cultivation the isolate under different conditions: temperatures (20 - 40^{o}C), pH (5 - 9), incubation times (24 h - 120 h) and salinity (0.2 - 1.0% NaCl).

Effects of temperature and pH on the stability of enzymes were determined by treating the crude enzymes at temperatures (20 - 40^{o}C), pH (4 - 10). The maximum activity of enzymes obtained at temperatures (20 - 40^{o}C), pH (4 - 10) was considered to be 100%.

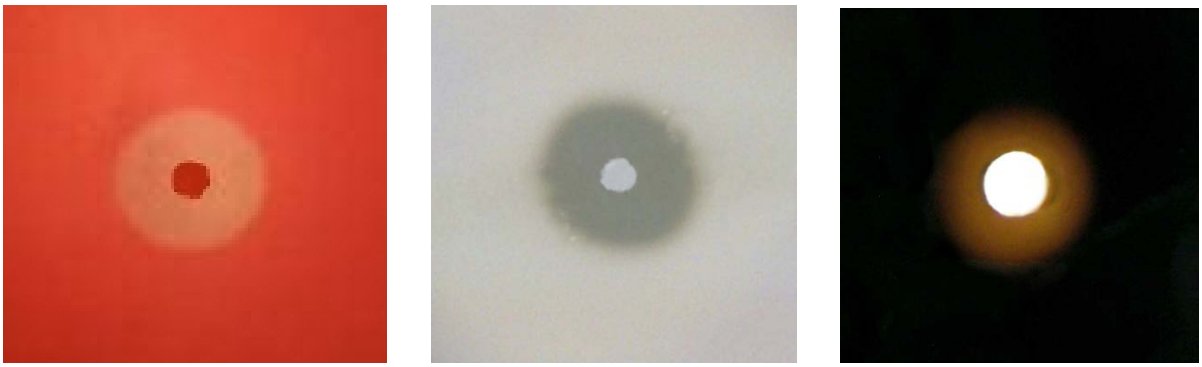
3. Results and discussion

3.1. Isolation and screening of cellulose and organic substance degrading bacteria

A total of 215 bacterial strains were isolated from fish ponds in Thuan An district, Thua Thien Hue province. Out of these strains, 25 isolates exhibited at least one of cellulolytic, amylolytic and proteolytic activities (Table 1). Cellulolytic activity of the isolates was from 3.86 ± 0.15 to 13.21 ± 0.65 U/mL, whereas amylolitic and proteolytic activity of the isolates were from 9.45 ± 0.38 to 19.16 ± 0.74 U/mL and from 9.54 ± 0.82 to 21.67 ± 0.95 U/mL, respectively. Particularly, the isolate W12 exhibited high activity to three cellylolytic, amylolytic and proteolytic activities (Figure 1), the isolate W12 was therefore selected for further investigation.

Table 1. Cellulose, starch and protein degrading strains isolated from fish ponds

Strains	Sources	Cellulolytic activity (U/mL)	Proteolytic activity (U/mL)	Amylolytic activity (U/mL)
S01	Sediment	7.51 ± 0.96	-	-
S03	Sediment	-	-	12.26 ± 0.48
S04	Sediment	6.93 ± 0.45	17.24 ± 0.89	-
S07	Sediment	-	-	19.16 ± 0.74
S10	Sediment	-	10.64 ± 0.94	-
S13	Sediment	12.4 ± 0.84	-	13.25 ± 0.68
S16	Sediment	-	13.62 ± 0.75	-
S21	Sediment	-	-	17.45 ± 0.78
S24	Sediment	5.12 ± 0.65	9.54 ± 0.82	-
S33	Sediment	-	-	15.12 ± 0.76
S36	Sediment	3.86 ± 0.15	-	-
S41	Sediment	10.41 ± 0.58	14.26 ± 0.67	9.45 ± 0.38
W03	Water	-	-	16.23 ± 0.81
W04	Water	-	21.67 ± 0.95	-
W06	Water	9.57 ± 0.87	-	-
W08	Water	-	-	14.56 ± 0.79
W12	Water	13.21 ± 0.65	16.34 ± 0.62	18.27 ± 0.92
W14	Water	8.14 ± 0.74	-	-
W17	Water	-	-	13.26 ± 0.69
W23	Water	9.47 ± 0.69	-	14.36 ± 0.71
W31	Water	-	11.71 ± 0.58	-
W33	Water	-	18.21 ± 0.82	-
W42	Water	11.32 ± 0.71	-	-
W46	Water	-	-	10.84 ± 0.59



(A) **(B)** **(C)**

Figure 1. Hydrolytic zone of cellulase (A), protease (B) and amylase (C) by *Bacillus* sp. W12

3.2. Identification of active isolates

Six isolates showing cellulose, starch and protein degrading activity were selected to sequence the 16S rRNA genes and compared to 16S rRNA sequences on GenBank. The obtained results showed that the sequences of isolates were high similarity (98-100%) to sequences available in GenBank (Table 2). Among them, two isolates belonged to genus *Bacillus*, two isolates belonged to genus *Paenibacillus*, and two remain isolates belonged to genus *Streptomyces* and genus *Pseudomonas*, respectively.

Previous studies have reported that cellulose and organic matter degrading enzymes such as amylase, protease and cellulase have produced by different microorganisms in aquaculture. These enzyme producing bacteria are extremely diverse, including main genera such as *Bacillus*, *Pseudomonas*, *Enterobacter*, *Enterococcus*, *Vibrio*, *Acinetobacter*, *Aeromonas*, *Microbacterium*, *Micrococcus*, *Staphylococcus*, *Streptomyces*, *Stenotrophomonas* as well as other unclassified bacteria [12,18-19, 24-25]. Particularly, many bacteria can produce multiple enzymes such as proteolytic and amylolytic *Bacillus* spp. isolated from shrimp ponds in Karawang, West Java, Indonesia [25], and amylolytic, proteolytic and cellulolytic bacteria isolated from the fish gut of rohu and murrel [26]. The enzyme producing microorganisms are often used as probiotics to improve feed digestibility and growth rate of aquaculture animals as well as environmental quality in aquaculture [12, 17-19]. For example, the use of the amylolytic strain NP5 isolated from intestines of tilapia as a probiotic to feed tilapia resulted in improvement of growth rate and carbohydrate digestibility as well as total digestibility of tilapia [17]. The use of other probiotic strains such as *Bacillus*, *Lactobacillus* spp., *Enterococcus* have has led to positive effects on feed digestibility and growth rate of aquaculture animals [27-31]. In the environmental field, microbial enzymes such amylase, protease, and cellulase have used to improve water quality and remove undesired substrates in different wastewaters (e.g., swine wastewater, tannery saline wastewater) as well as sewage sludge [12-15].

Table 2. Identification of selected activity isolates

Strains	Closest strain in GenBank (Accession number)	% identity	Identification
S04	*Bacillus subtilis* JCM 1465 (AB598736)	100	*Bacillus subtilis*
S13	*Paenibacillus terrae* AM141 (AF391124)	98.5	*Paenibacillus* sp.
S24	*Pseudomonas* sp. CK57 (EU686687)	99.4	*Pseudomonas* sp.
S41	*Paenibacillus amylolyticus* JCM 9906 (D85396)	98.8	*Paenibacillus* sp.
W12	*Bacillus megaterium* NBRC 15308 (AB271751)	99.5	*Bacillus* sp.
W23	*Streptomyces* sp. CHR3 (AF026080)	99.6	*Streptomyces* sp.

3.3. Optimization of culture conditions for production of enzymes and properties of enzymes

Cultivation of the isolates under culture conditions (temperature, pH, incubation time, salinity) showed influences of culture conditions on production of enzymes. Amount of produced enzymes increased when culture temperature increased from 20 to 30^{o}C and reached the highest values at 30^{o}C; however, at higher culture temperatures, amount of produced enzymes decreased. The optimization results also showed that amount of produced enzymes reached at highest values at pH = 7.0, incubation time of 72 h and 0.4% NaCl (Figure 2).

The previous studies have revealed that different microorganisms can produce maximum enzymes under different culture conditions. The strain *Bacillus licheniformis* TD4 produced maximum protease (141.46 U/mg) in 24 h incubation at pH 8 and 1 M NaCl [32]. The optimum production of protease by *Bacillus ceresus* FT1 was obtained at 35^{o}C, pH 9.5 after 48 h of incubation [33], whereas protease production of the strain *Bacillus* sp. reached the highest activity at 30^{o}C and pH 8 [34]. In case of cellulase production, the optimum conditions for cellulase production by the strain ME27-1 were observed at pH 8, temperature of 28^{o}C and 60 h of incubation [35]. In another study, maximum cellulase prodution of the strain *Bacillus* sp. Y3 was obtained at 37^{o}C, pH 7 and 96 h incubation [36]. For amylase production, the optimum conditions for amylase production by *Bacillus* sp. WangLB were observed at

35ºC, pH 10 and 48 h of incubation [37], whereas cellulase production by a thermophilic bacteria isolated from hot spring (S2.3) reached the highest activity at 50ºC and pH 7.5 [38].

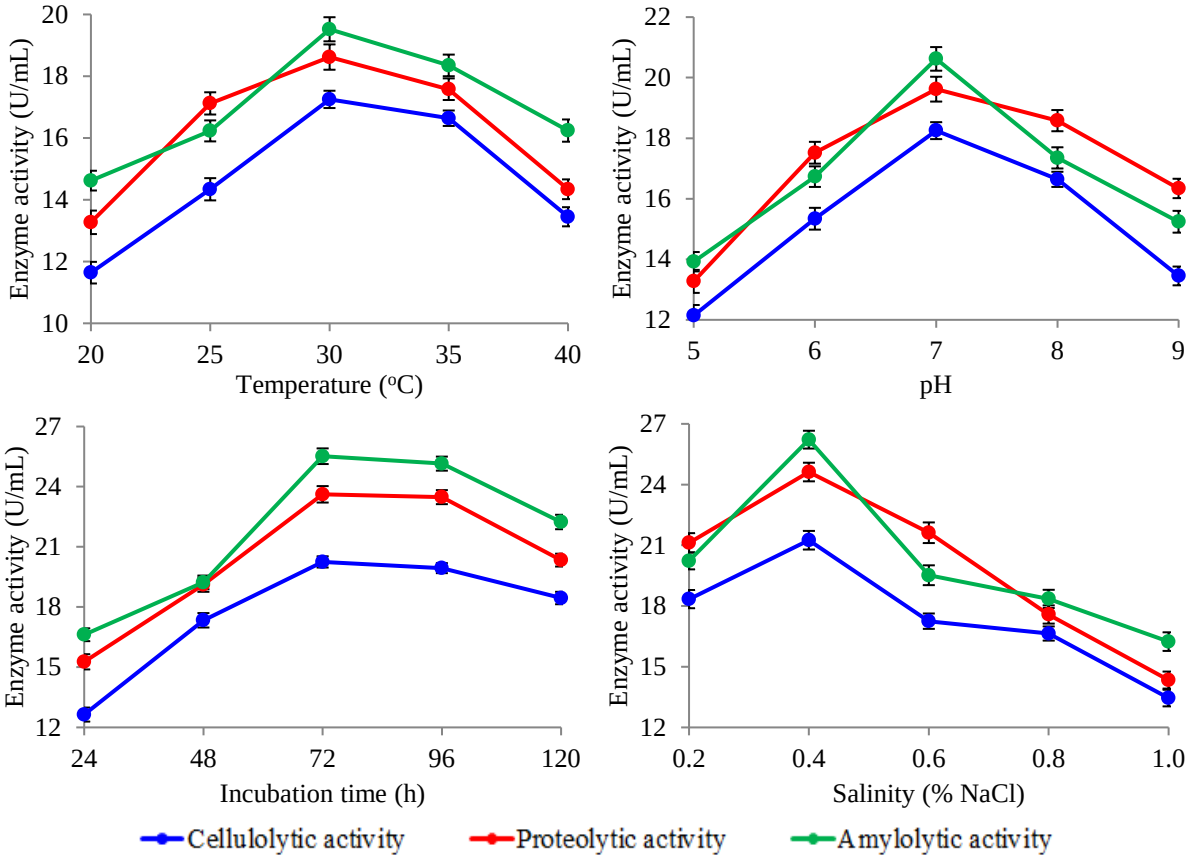


Figure 2. Influence of culture conditions on enzyme production of *Bacillus* sp. W12

Furthermore, temperature and pH were influenced on stability of the enzymes. The enzymes reached the highest activity within pH 6 - 8 and temperature 30 - 40ºC. The activity of the enzymes was still stable within pH 5 - 9, with more than 80% activities being retained. In addition, the enzymes maintained more 90% activity after heated at 20 - 40ºC for 1 h. At pH was lower than 5 and temperature was higher 45ºC, the activity of enzymes was significantly reduced (Figure 2).

The effects of pH and temperature on stability of the enzymes have previously reported. Amylase enzyme produced by *Cronobacter sakazakii* Jor52 was stable within pH 6 - 8 and temperature 30 - 40ºC [39], whereas amylase enzymes produced by *Bacillus* spp. exhibited the maximum activity at pH 3 - 10 and 40 - 80ºC [37]. The highest activity of cellulase from *Cellulomonas* sp. ASN2 is reported at 60ºC and pH 7.5 [40]. In case of cellulase produced by *Scophthalmus maximus*, the highest activity was obtained at 50ºC and pH 5 [41]. For protease enzymes, the pH and temperature for maximum enzyme activity produced by *Pseudomonas aeruginosa* PT121 were 8.0 and 60ºC, respectively; however, the protease exhibited good stability and retained more than 90% activity within pH 7 - 9 and temperature 30-50ºC [42]. Another protease produced by a thermophilic *Bacillus* sp. exhibited good activity within a broad range of pH and temperature, and reached the highest activity at pH 8 and temperature 70ºC [43].

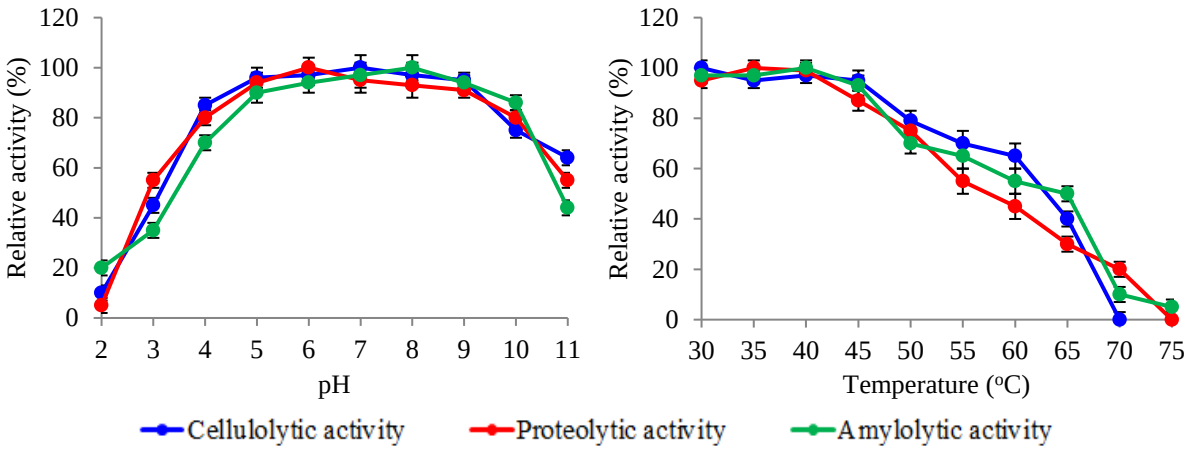


Figure 3. Influence of pH and temperature on properties of enzymes produced by *Bacillus* sp. W12

4. Conclusion

The current study isolated and screened 25 cellulolytic, amylolytic and proteolytic bacteria from fish ponds in Thuan An, Phu Vang district, Thua Thien Hue province. The optimum culture conditions for hydrolytic enzyme production of the strain *Bacillus* sp. W12 were at pH 7, temperature 30°C, 0.4% NaCl and 72 h of incubation. The activity of enzymes reached the highest activity at pH 6-8 and temperature 30 - 40°C. This study showed the strain *Bacillus* sp. W12 as a promising probiotic candidate for improvement of aquaculture water quality and feed digestibility of aquaculture animals.

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