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Isolation and Identification of Protease-Producing *Pseudomonas* sp. PD14 in the Gut of Rabbitfish *Siganus guttatus* (Bloch 1787)

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Abstract

Bacterial enzymes associated with the gut of fish are known to aid in digestion and nutrition of the host. Isolation, identification and characterisation of protease-producing bacteria from the gut of rabbitfish *Siganus guttatus* (Bloch 1787) were carried out in the present study. Protease-producing bacteria were isolated in peptone gelatin agar (PGA) plates and the isolated strains were qualitatively and quantitatively screened for enzyme production. Highest protease activity, 25.32±1.06 $U \cdot mg^{-1}$ protein, was observed in bacterial isolate PD14. Biochemical and molecular analysis revealed that the isolate is 99% homologous to *Pseudomonas sp.* The 16S rRNA gene sequence of the isolate was deposited in GenBank with accession number KR779515. Qualitative tests on enzyme production through measurement of the zone of hydrolysis further suggest that optimum protease production was 36 h at 40 ^{o}C, pH 7-8 in a peptone gelatin agar with 2% NaCl. The data gathered from this study could contribute to the utilisation of such bacteria in fish nutrition or other biotechnological applications.

Introduction

The digestive tract of fish, being rich in nutrients, confers a favourable culture environment for microorganisms. These microbiota play an important and diversified enzymatic potential in many marine species (Tambekar et al. 2009). Studies have shown that the gut produces various substances such as vitamins, riboflavin and enzymes that breakdown proteins, carbohydrates and lipids (Ganguly and Prasad 2012). Enzyme-producing bacteria in fish have been demonstrated to breakdown chitin (Itoi et al. 2006), cellulose (Saha et al. 2006), protein (Belchior and Vacca 2006), starch (Ghosh et al. 2002,), phytate (Khan and Ghosh 2012) and tannin (Mandal and Ghosh 2013).

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Microbial proteases, which are mainly produced by bacteria and fungi, are degradative enzymes which break down proteins and are found in various sources such as plants, animals and microorganisms (Ellaiah et al. 2002). These are primarily extracellular and can be secreted in the fermentation medium in a short time with abundant desired enzyme (Muthulakshmi et al. 2011). In recent years, proteases have become one of the most valuable industrial enzymes representing more than 60% of the total enzyme market and microbial proteases specifically, accounting for 40% of the total worldwide sales of enzymes (Uyar et al. 2011). Proteases have diverse biotechnological applications because of their distinct and specificity of action. Several microorganisms are known to produce proteases namely *Penicillium* sp., *Serratia marcescens*, *Streptomyces* sp., *Rhizopus oryzae*, *Pseudomonas*, *Bacillus* sp., *Vibrio*, etc. (Rebah and Miled 2013). Meanwhile, the rabbitfish *Siganus guttatus* (Bloch 1787) was found to contain considerable extracellular enzymes such as amylase, cellulase and protease in its gastrointestinal tract (Simora et al. 2015). Therefore, an attempt was made in the present investigation to isolate protease-producing bacteria from the gut of rabbitfish (*S. guttatus*). The isolated bacterial strains were analysed for qualitative and quantitative extracellular enzyme production and the strain with the highest protease activity was subjected to biochemical and molecular characterisation. Finally, optimum protease production was assessed qualitatively using several factors such as pH, incubation time, temperature and sodium chloride (NaCl) concentration.

Materials and Methods

Sample collection and bacterial isolation

Wild mature rabbitfish (*S. guttatus*) were collected during the months of June and July 2014 at the Southeast Asian Fisheries Development Center Aquaculture Department, Igang Marine Station, Nueva Valencia, Guimaras, Iloilo, Philippines ($10^0$31'44.8"N $122^0$30'44.0"E). The average body weights of the sampled fish were 292±34.12 g, with body length of about 23.42±15.20 cm. Fish were starved for 24 h, gut was aseptically dissected and divided into three portions; proximal (PI), middle (MI) and distal intestine (DI) (Das and Tripathi 1991). Adherent (autochthonous, associated with the gut wall tissue) bacteria of the three gut sections were separated as described in Ringø (1993). Briefly, digesta from the PI, MI and DI were gently squeezed out. Then the three intestinal segments were thoroughly rinsed three times with 3 mL sterile 0.9% saline solution (10:1 v/w) in order to isolate the adherent bacteria. The intestinal segments were transferred to sterile plastic bags and homogenised in a Stomacher (Seward Laboratory, London, UK). Total heterotrophic counts were made on tryptone soy agar (TSA, Difco) plates. Homogenates of the intestinal segments were diluted in sterile 0.9% saline solution and appropriate dilutions (up to 10^{-7}) were pour plated on peptone gelatin agar (PGA, Difco) plates containing peptone, 0.5% w/v; gelatin, 0.4% w/v; beef extract, 0.3% w/v; NaCl, 1.0% w/v and agar, 2.0% w/v at pH 7.5 and incubated at 30 ºC for 24 h. Distinct bacterial colonies were sub-cultured on PGA slants and maintained at 4 °C.

Screening for extracellular protease production

The bacterial isolates were analysed on a selective media to determine the intensity of the extracellular enzyme production according to the method of Jacob and Gerstein (1960). Isolates were spot-inoculated on PGA plates and incubated at 30 °C for 48 h. At the end of incubation time, the plates were flooded with mercuric chloride ($HgCl_2$) in hydrochloric acid (HCl) solution. Halo zone was further confirmed using skim milk agar incorporated with 2% NaCl under the same conditions. The appearance of clear zone or halos indicated proteolytic activity of the isolates and presented as scores: 0 (0-3 mm), 1 (low, 4-6 mm), 2 (moderate, 7-9 mm), and 3 (high, >10 mm) (Askarian et al. 2012).

Quantitative protease activity

Isolates that produced the highest zones of diameter (mm) were chosen and sub-cultured in agar-free selective media as described in the previous section, to facilitate the production of crude extracellular protease. Two mL of a 24-h seed culture of the isolates prepared in nutrient broth were added in a 50-mL production medium (peptone-gelatin broth containing ($g \cdot L^{-1}$) peptone, 5; gelatin, 4, beef extract, 5; NaCl, 20) and incubated for 48 h at 30 ºC, with rotational shaking. The cell-free supernatant was collected by centrifugation at 11,000 x g for 10 min at 4 ºC and stored at -20 ºC until it was used for the enzyme assays.

The activities of the enzymes were quantified using caseinolytic method by Anson (1938) wherein one unit (U) of enzyme activity is defined as the amount of enzyme that liberates peptide fragments equivalent to 1 mg of bovine serum albumin (BSA) under the assay conditions. The absorbance was read at 660 nm along with tyrosine standard dilutions. The protein content of the sample was also measured based on the method described by Lowry et al. (1951). Quantitative enzyme activities were expressed as units (U) and specific enzyme activity as $U \cdot mg^{-1}$ protein. All assays were carried out in triplicate.

Morphological, physiological and biochemical characterisation

The most promising isolate based on qualitative and quantitative enzyme assay was chosen and subjected to morphological, physiological and biochemical characterisation according to the methods described in Bergey's Manual of Systematic Bacteriology (1957) and Reynolds (2011).

Molecular identification of the protease-producing bacterial isolate

Molecular identification of the protease-producing bacterial isolate PD14 was made by extracting the genomic DNA using PureLink™ Genomic DNA Kit (Invitrogen) from a 48-h colony grown on a PGA slant. Gene amplification (16S rRNA) was performed using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1429R (5'-GGTTACCTTGTTACGACTT-3').

The PCR reactions were performed using PCR mix containing 0.2 µM of each primer, 2 µL template DNA and 1x Taq Master Mix (Vivantis) which contained Taq DNA polymerase, reaction buffer, dNTPs and $MgCl_2$. Amplification of DNA was performed using a thermocycler (S1000^{TM} Bio-Rad) with the following conditions: denaturation at 95 °C for 3 min followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 3 min to allow any incomplete products to be extended. Polymerase chain reaction products were sequenced using capillary sequencing and sequence similarity search was carried out with Basic Local Alignment Search Tool (BLAST) programme to identify the most closely related database sequences available in Genbank. Based on the BLAST results, the sequences derived were aligned and analysed for finding the closest homology of the microbes. A phylogenetic tree was then constructed incorporating 16S rRNA partial gene sequences and their phylogenetically closest type strains using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software following the neighbour-joining method. Partial sequence of the isolate was deposited in the NCBI GenBank database to obtain accession number.

Effect of physico-chemical factors on protease production

The effect on protease production of some physico-chemical factors such as growth at different incubation time (12, 24, 36, 48, 60 and 72 h), temperature (20, 30, 40, 50 and 60 ^{o}C), NaCl concentration (1, 2, 3, 4 and 5 %) and pH (5, 6, 7, 8, 9 and 10) were investigated on the bacterial isolate. Qualitative protease activity was determined based on the zone of hydrolysis around the colony and expressed in millimetres (mm).

Statistical analysis

Statistical analysis of the experimental data pertaining to quantitative enzyme production was made by analysis of variance (ANOVA) followed by Tukey's test using SPSS 10 (Kinnear and Gray 2000).

Results

Bacterial populations in the gut of rabbitfish revealed that dense heterotrophic bacterial count and proteolytic bacterial count were highest in the DI region (9.0×10^6 bacteria g^{-1} and 2.88×10^5 bacteria g^{-1}, respectively) (Table 1). A total of 14 bacterial isolates from the PI, MI and DI regions of the gut were isolated and maintained as pure cultures (Table 2). The isolates were found to produce extracellular protease based on qualitative enzyme assay but with different intensities on the type of selective media used. High protease activity was found on peptone gelatin agar. Comparing the intensity of protease production on both peptone gelatin agar and skim milk agar, three promising strains namely PM10, PD1 and PD14 were assayed quantitatively to determine their specific enzyme activities. As shown in Table 3, isolate PD14 gave the highest value for specific protease activity ($U \cdot mg\ protein^{-1}$) and was selected for further identification and characterisation.

Table 1. Total heterotrophic and proteolytic count of adherent bacteria isolated from the proximal (PI), middle (MI), and distal (DI) segments of the gut of *Siganus guttatus.*

Intestinal region	Total bacterial counts/ml intestinal tissue*	
	Total heterotrophic count	Proteolytic bacteria
PI	6.03×10^5	2.82×10^4
MI	7.00×10^6	2.40×10^5
DI	9.00×10^6	2.88×10^5

*Data are mean ± SE of three determinations.

Table 2.Qualitative enzyme activity of adherent proteolytic bacteria isolated from the proximal (PI), middle (MI), and distal (DI) segments of the gut using peptone gelatin agar and skim milk agar incorporated with 2% NaCl.

Intestinal region	Bacterial strains	Protease Activity Scores*	
		Peptone gelatin agar	Skim milk agar
PI	PP1	3	0
	PP3	3	0
	PP7	3	0
	PP9	3	0
	PP13	0	0
MI	PM4	2	0
	PM6	3	0
	PM7	3	0
	PM10	3	2
DI	PD1	2	1
	PD5	3	0
	PD7	2	0
	PD12	3	0
	PD14	3	2

*0 (0-3 mm), 1 (low, 4-6 mm), 2(moderate, 7-9 mm), 3 (high, >10 mm). Maximum score is 18 and minimum score is 2.

Table 3. Quantitative enzyme activity of adherent proteolytic bacteria isolated from the proximal (PI), middle (MI), and distal (DI) segments of the gut using peptone gelatin broth incorporated with 2% NaCl.

Intestinal region	Bacterial isolate	Specific protease activity* ($U \cdot mg^{-1}$ protein)
MI	PM10	16.86 ± 0.43^{a}
DI	PD1	21.46 ± 2.25^{ab}
	PD14	25.32 ± 1.06^{b}

*Data are mean ± SE of three determinations. Values with the same superscripts are not significantly different ($p > 0.05$); U = µg of tyrosine liberated/mg protein/ml of culture filtrate.

Table 4. Morphological, physiological and biochemical characteristics of isolate PD14.

Biochemical Tests	Isolate PD14
Cell Morphology:	
Elevation	Convex
Opacity	Dull
Pigment	Green
Gram's reaction	-
Cell shape	Small, rod shaped
Endospore	-
Carbohydrate Fermentation Test:	
Glucose	+
Mannitol	+
Lactose	-
Arabinose	+
Sorbitol	+
Glycerol	+
Hydrolysis of:	
Starch	-
Gelatin	+
Casein	+
Pigment Production:	
Pyocyanin	+
Fluorescein	+
Growth on 55 ºC	-
Catalase Test	+
Oxidase	+
Nitrate Utilisation Test	+
Production of Lecithinase	-
Indole Production	+
Methyl Red Test	-
Motility	Motile
Vogues-Proskaeur Test	+
Citrate Utilisation Test	+
Arginine Dihydrolase Detection	+

+ (Positive); - (Not detected)

On the basis of major morphological, physiological and biochemical characters (Table 4), isolate PD14 has been identified as belonging to the genus *Pseudomonas* following Bergey's Manual of Systematic Bacteriology. The cells appeared as gram negative short rods. The isolate was aerobically motile, oxidase positive, produced diffusible greenish pyocyanin and fluorescein pigments, have denitrification activity but incapable of growing at 55 °C. Moreover, the isolate produced acid from glucose, mannitol, arabinose, sorbitol and glycerol and was positive for nitrate utilisation as sole carbon source for its growth.

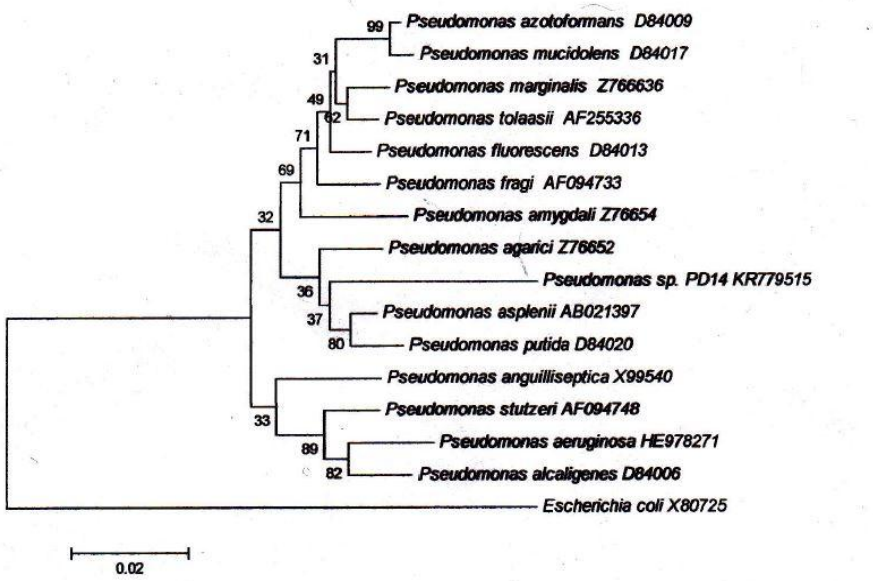

Fig.1.The phylogenetic tree showing the relationship among *Pseudomonas* strains and their phylogenetically closest type strains. The GenBank accession numbers of the type strains and studied strains are shown following species names. Distance matrix was calculated by Kimura's 2-parameter model. The scale bar indicates 0.02 substitutions per nucleotide position. *Escherichia coli* served as an out-group.

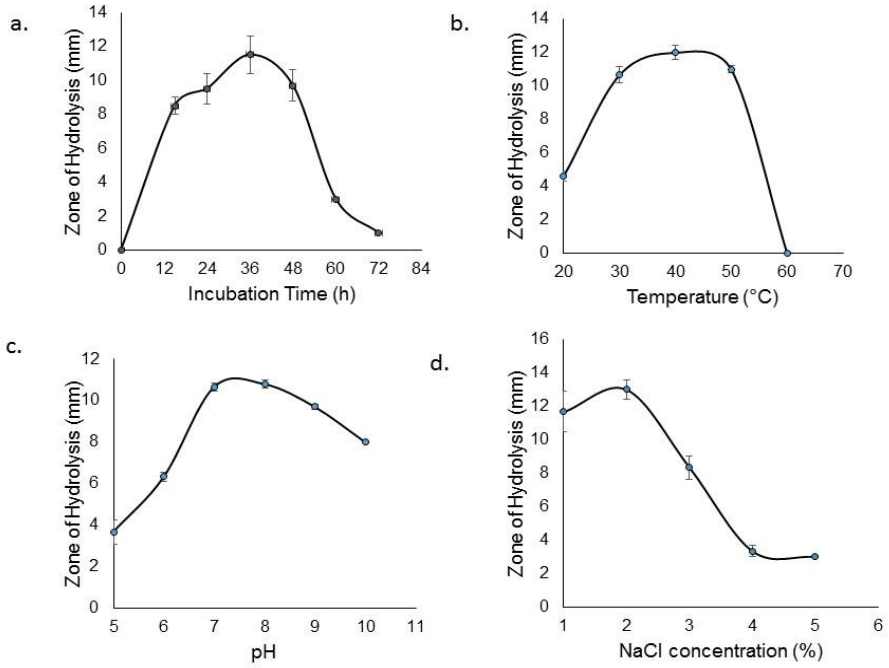

Fig.2. Effect of (a) incubation time (b) temperature (c) pH and (d) NaCl concentration with reference to protease production from isolate PD14. Values are mean ± SE of three determinations.

It was positive for gelatin and casein hydrolysis as well. In order to gain taxonomic information on the selected strain, the 16S rRNA of isolate PD14 was partially sequenced and compared to the local alignment search of the GenBank database using BLAST and showed 99% similarity with 16S rRNA gene sequence of *Pseudomonas putida* (GenBank accession NC_002947.3). The sequence was deposited in GenBank with accession number KR779515. Closest type strains to isolate PD14 were *Pseudomonas asplenii* and *Pseudomonas putida* based on nucleotide homology and phylogenetic analysis (Fig. 1). Qualitative analysis of enzyme production, as determined by measuring the zone of hydrolysis (mm), revealed that isolate PD14 was able to produce optimum protease at 36 h incubation time, 40 ^{o}C temperature, pH range of 7-8 and 2% NaCl concentration (Fig. 2).

Discussion

Considerable proteolytic bacteria were present in the gut of rabbitfish especially in the DI region (Table 1). Being an herbivore fish species, occurrence of protease-producing bacterial population is noteworthy in the digestive tract of rabbitfish. Previous studies on herbivorous fishes such as rohu (Kar and Ghosh 2008) and various carps (Das and Tripathi 1991; Bairagi et al. 2002; Ray et al. 2010) also reported the presence of proteolytic bacteria. The colonisation of proteolytic bacteria in the gut of herbivorous fishes might be due to some bacteria gaining entrance together with the food during ingestion, and adapt themselves in the gastrointestinal tract and form a symbiotic association (Kar and Ghosh 2008). Protease-producing bacteria in the fish gut are involved in the hydrolysis of dietary proteins along with other important digestive enzymes such as aspartic protease pepsin, and serine proteases, trypsin, chymotrypsin, collagenase and elastase (Balti et al. 2009).

Qualitative protease activity based on halo zone formation revealed that 14 isolates were potent protease producers. Isolates that were spot-inoculated on PGA plates gave higher activity scores (Table 2) than when inoculated on skim milk agar. Although both media mainly allow for qualitative determinations of protease activity, the zone of hydrolysis produced may still be dependent on the type of protease-producing microorganism being investigated. In PGA medium, gelatin is incorporated to determine an organism's ability to produce gelatinase, a proteolytic enzyme that hydrolyses gelatin into its constituent amino acids while skim milk agar is commonly used to demonstrate proteolysis by organisms capable of hydrolysing casein (Hurst et al. 2005).

The intensity of protease production by the microorganism is related to the zone of hydrolysis observed on skim milk agar (Vermelho et al. 1996). Protease produced by *Bacillus licheniformis* is an exception; in spite of large volume of enzyme produced by submerged culture, very narrow zones of hydrolysis on milk agar plates were observed (Aunstrup 1974). The top three strains with high protease activity were further assayed quantitatively and results showed that isolate PD14 exhibited the highest proteolytic activity.

The specific protease activity (U·mg protein^{-1}) of isolate PD14 was considerably high (25.32±1.06 U·mg protein^{-1}) as compared to the specific protease activities of the bacterial enzymes from the gut of other herbivorous species such as grass carp *Ctenopharyngodon idella* (Valenciennes 1844) (1.3 U·mg protein^{-1}) and common carp *Cyprinus carpio* Linnaeus 1758 (1.1 U·mg protein^{-1}) in the study of Bairagi et al. (2002). Bacterial strain PD14 was identified as *Pseudomonas* sp. based on biochemical and molecular characteristics. Biochemically, the organism was capable of hydrolysing proteins such as casein and gelatin linking to strong proteolytic activities.

In addition, the isolate was able to fermentatively utilise a wide variety of carbohydrates and able to produce pigments, pyocyanin and fluorescein which are characteristics of *Pseudomonas* species. Arginine dihydrolase enzyme was also detected in the isolate which means that it can metabolise the amino acid arginine for carbon and energy. The ability of some intestinal isolates to metabolise amino acids may be indicative of their growth and colonisation in the digestive tract (Kar et al. 2008).

Nucleotide homology and phylogenetic analysis of the 16S rRNA gene confirmed that the partial sequence of isolate PD14 belonged to *Pseudomonas* species. Related studies reported that species belonging to *Pseudomonas* are commonly dominant among culturable bacteria in the gastrointestinal tract of fish. In a study conducted by Ariole and Kanu (2014), *Pseudomonas* spp. were present in the qualitative composition of bacteria in the digestive tract of tilapia together with *Aeromonas, Bacillus, Staphylococcus, Salmonella, Vibrio, Escherichia, Flavobacterium, Lactobacillus, Micrococcus* and *Enterobacter*. Furthermore, Askarian et al. (2012) also reported two strains of *Pseudomonas* sp., isolated from the gut bacteria of Atlantic salmon *Salmo salar* Linnaeus 1758 fed with chitin and identified through 16S rRNA gene sequencing to be enzyme-producing including protease. Limited studies were reported on extracellular enzyme-producing bacteria particularly protease-producing associated with the gut of herbivorous fish.

Media components and physical factors such as incubation time, temperature and pH highly influence microbial enzyme production. The results indicated that the optimum incubation period for protease production was 36 h. Similar finding was observed by Gupta and Khare (2007) for solvent tolerant strain (PseA) of *P. aeruginosa* where optimum protease production was also 36 h. Also, Myhara and Skura (1990) optimised the culture conditions affecting the production of extracellular proteinases by *Pseudomonas fragi* ATCC 5973, and reported that the optimum incubation period for proteinase production by *P. fragi* was 38 h. There was a decline in the enzyme production after the optimum period (Fig. 2a), which might be due to the depletion of nutrients available for microorganisms. Temperature and pH are other two important factors affecting enzyme production. Temperature strongly affects the rate of biochemical reactions thereby inducing or repressing enzyme production (Uyar et al. 2011).

The optimum protease production by isolate PD14 was observed at 40 ºC. A comparison of the literature on the characteristics of alkaline proteases of microbial origin revealed that most strains were mesophilic type with temperature optima of 30-40 ºC (Uyar and Baysal 2004; Prakash et al. 2014). With this regard, isolate PD14 was in agreement with the literature (Wery et al. 2003; Secades et al. 2001; Genckal and Tari 2006). The optimum protease production was observed at pH 7 to 8 (Fig. 2c) which indicates that protease activities are higher at near alkaline conditions. Similarly, Jobin and Grenier (2003) investigated the production of four proteases by *Streptococcus suis* and found that the optimum pH for all four proteases was between pH 6 and 8. At higher pH level (over pH 10), protease production decreased as a consequence of suppressed metabolic action of the bacterium. Microbial alkaline protease production is largely dependent upon extracellular pH because pH in the culture medium greatly affects various enzymatic processes and transport of cell components across membranes, which in turn maintain cell growth and product production (Ellaiah et al. 2002). The effect of NaCl concentration was investigated in the present study because rabbitfish is primarily a marine species and autochthonous bacteria in its gut might require a saline environment for growth and metabolism.

Results indicate that a 2% NaCl concentration enhanced the protease production of isolate PD14 (Fig. 2d). Scant reports were found on the beneficial effect of sodium on microbial alkaline protease production. Lakhsmi et al. (2014) reported that the presence of 1% NaCl in the medium enhanced protease production of *Bacillus licheniformis* (MTCC No. 7053). The enhancing effect of sodium chloride on alkaline protease production was also observed by Chandrasekaran and Dhar (1983) in their study on the production of extracellular alkaline proteinase using tapioca starch. An increase in salt concentration (greater than 2%) greatly reduced the protease production implying that an increased salt concentration creates changes in the lipid composition of the bacterial cell membrane. Hence, the growth rate decreases causing reduced enzyme production (Chandrasekaran and Dhar 1983). Based on the optimum temperature and pH, the possible types of proteases present in isolate PD14 are serine proteases. These proteases are generally active at optimum pH between 6-11 and optimum temperature between 50-70 ^{0}C (Ellaiah et al. 2002). The presence of alkaline serine proteases especially trypsin in the viscera of various fish species has been reported (Kishimura et al. 2005; Kishimura et al. 2006; Bougatef et al. 2007). Trypsin is a ubiquitous serine protease in animal digestive glands and specifically hydrolyses proteins and peptides at the carboxyl group of arginine and lysine residues (Balti et al. 2009).

Conclusion

Though many studies are focused on protease production, literature on the screening of microbial proteases with potent activity is limited. The results from this study indicated that the extracellular protease produced by a novel *Pseudomonas* sp. PD14 isolated from the gut of rabbitfish will serve as an effective microbial protease enzyme.

This protease-producing bacteria can be beneficially used as probiotics in the formulation of commercial feeds or in other biotechnological applications. However, further studies are needed to investigate its full potential as a protease-producer.

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