

Bacterial Production of Protease-A Preliminary Study

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Abstract- Proteolytic enzyme, also called protease, proteinase, or peptidase, any of a group of enzymes that break the long chainlike molecules of protein into shorter fragments (peptides) and eventually into their components, amino acids proteolytic enzymes are present in bacteria. Protease are produced from various sources but only little information is available regarding the bacterial population in fish wastes of Chidambaram fish market and their efficiency in synthesizing protease enzyme. Isolated 25 bacterial strains and found that only 5 of them are capable of producing protease enzyme that are *Pseudomonas sp.* and *Bacillus sp.* was the best protease producer among the tested strains. In the present study, all the bacterial species isolated had a better potentiality of degrading market fish wastes.

Keywords- Protease, Enzyme, *Pseudomonas* and *Bacillus*

I. INTRODUCTION

Enzymes are proteins which require a specific substrate on which to work (Okononko et al., 2006). Microorganisms in particular have been regarded as treasure sources of useful enzymes (Shimizu et al., 1997). Biologically active enzymes may be extracted from living organisms. Extracellular enzymes are preferred, because difficult and costly methods of cell disruption are not necessary for their extraction. Microbial enzymes present a wide spectrum of characteristics that make them utilizable for quite specific applications (Hossain et al., 2005). Of the several enzymes being used industrially, over a half is from fungi and yeast and over a third are from bacteria (Ashok Pandey et al., 2000; Meenu et al., 2000).

The demand for industrial enzymes, particularly of microbial origin is ever increasing owing to their applications in a wide variety of processes. Enzyme mediated reactions are attractive alternatives to tedious and expensive chemical methods. Enzyme find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile and cosmetic industries (Saxena et al., 1993). Current developments in biotechnology are yielding new applications for enzymes (Pandey et al., 2000).

Protease is enzymes which cleave or cut or degrade other proteins by hydrolysing peptide bonds. Proteases are one of the most important groups of industrial enzymes and account for nearly 60 % of the total enzyme sale (Dutta and Banerjee, 2006). Most of the available proteases produced commercially are of microbial origin (Oskouie et al., 2007).

Manivannan et al. (2004) studied the production of alkaline protease by *Aspergillus sp.* Shik et al. (2006) studied the production of alkaline protease by *Bacillus pantothenicus*. Soares et al. (2005) isolated extracellular protease from *Bacillus subtilis*. Investigation on extracellular protease production by *Bacillus cereus* which was isolated from the intestine of fish, Mugil cephalus was carried out by Esakkiraj et al. (2008).

II. MATERIALS AND METHODS

Collection of sample

Fish wastes were collected from a fish market at Chidambaram, Cuddalore District and were aseptically transferred to the laboratory for further study.

Isolation of bacterial flora

In the laboratory, the samples serially diluted upto 10^{-5} dilution. From each dilution, 0.1 ml of sample was taken and spread plated on Zobell agar medium. The plates were then incubated at 37°C for 24 to 48 h. The total viable counts (TVC) of the

colonies were finally noted. The isolated cultures were purified individually by streaking on nutrient agar plates and were sub cultured.

Identification of bacteria

The bacterial cultures were identified up the genus level by performing the following biochemical tests:

Gram's staining

The different bacterial cultures (16 to 18 hrs.) were smeared on clean glass slides individually and heat fixed. The smears were flooded with crystal violet for a min. and the stain was washed off using distilled water. The smears were flooded with Gram's stain iodine solution (fixative) for a min. and rinsed with distilled water, decolourized with acetone alcohol, rinsed out and the smears were counter stained with saffranin, air dried and examined under oil immersion microscope. Gram positive bacteria were purple or violet and Gram negative bacteria were in red colour when observed.

Motility test

A depression slide and the cover glass were located well. A small drop of bacterial culture was spreaded around the edges of the cover glass and inverted carefully by lowering it towards the depression slide until it made contact with Vaseline, which was spread around the concavity. The slide was turned upright and drop of culture remained suspended from the cover glass, which was then observed under oil immersion microscope. Thus the motility of bacteria was noted.

Indole production test

Trytone broth tubes were prepared and sterilized at 121°C for 15 min. The cultures were then inoculated and incubated individually at 37°C for 24-48 hours. After the incubation period, a few groups of (0.2 ml) Kovac's reagent was added into the tubes and the result was observed. The development of bright red colour at the interface of reagent and medium indicated the presence of indole and constituted a positive test. The absence of colour at this interface the negative test.

Methyl red test

The MR-VP broth tubes were prepared and sterilized at 121°C for minutes. The cultures were then inoculated individually into the tubes containing sterilized period, about 5 to 6 drops of methyl red indicator solution was added. The development of stable bright red colour indicated sufficient acid production and constituted a positive reaction. A weekly positive test indicated red orange colour. A yellow orange colour indicated a negative reaction.

Voges-Proskauer test

The MR-VP broth tubes were prepared and sterilized at 121°C for 15 min. The cultures were then inoculated into the tubes containing sterilized MR-VP broth medium and incubated in the tubes at 37°C for 24-48 hours. After the incubation period, about 3 ml of 5%, -naphthol in absolute ethanol and 1 ml of 40% potassium hydroxide (plus creatine) were added. The tubes were allowed to remain undisturbed for 15-20 min. The development of red colour indicated the production of oxidation product acetoin from diacetyl and constituted the positive test. The absence of the red colour indicated negative reaction.

Citrate utilization test

Simmons citrate agar was prepared and dispensed on test tubes and sterilized at 121°C for 15 min and allowed to set as slope. The cultures were then inoculated individually into the tubes containing Simmons citrate agar slants (Stabbed into the bud and streaked on the surface of slants) and incubated at 37°C for 24-48 hours. After the incubation period, the development of intense blue colour from the original green colour of the medium indicated the ability of the organism to utilize citrate as carbon source and constituted the positive reaction. The absence of blue colour indicated negative reaction.

Oxidase test

Strips of Whatmann No. 1 filter paper was soaked in a freshly prepared 1% solution of tetramethyl paraphenyl diamine dihydrochloride and dried. The isolates were streaked individually on the prepared filter paper with a sterile platinum loop. A positive reaction was indicated by an intense deep purple colour appearing within 5-10 seconds and a negative reaction by absence of colouration.

Urease test

Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bonds amide compounds like urea and formalin alkaline end product such as ammonia. The bacterial cultures were inoculated individually in Christensen's agar slant and incubated at 37 °C and the reactions were recorded after 4, 8, 12 and 48 h of incubation. The production of enzyme urease was detected by a change in the colour of the medium from yellow to purple.

Triple sugar iron test (TSI)

TSI test is used to differentiate different group of Enterobacteriaceae from other gram negative Bacilli. The bacterial isolate were inoculated individually in the TSI agar slant by stabbing the butt down to the bottom and then the surface of the slant was streaked incubated at 37 °C for 18 – 24 hours. The following three types of results after inoculation were detected.

- a. Acid butt, alkaline slant (yellow butt, red slant) – glucose has fermented but not lactose nor sucrose.
- b. Acid butt, acid slant (yellow butt, yellow slant) – lactose and/ or sucrose has fermented.
- c. Alkaline butt, alkaline slant (Red butt, red slant) – either glucose, lactose or sucrose has fermented.

Gas production: Indicated by bubbles in the butt or the agar may be broken or pushed upwards.

Hydrogen sulphide production: Indicated by blackening of the butt.

Fermentation of carbohydrate

The bacterial species isolated were checked for their ability to ferment various carbohydrates (lactose, mannitol, sucrose, dextrose).

Oxidation – fermentation basal medium was prepared with 1% of different sugars each and sterilized and inoculated with 2 or 3 loopful of 18 – 24 hrs old broth culture into sterile fermentation medium and incubated for 24 -48 h at 30 °C in an incubator.

A change in the colour of the medium to yellow indicated acid reaction.

Starch hydrolysis test

Starch agar plates containing a loopful of cultures was incubated for 24 hours at 37°C. After incubation, the plates were flooded with iodine solution, clear zone around the growth indicated positive hydrolysis. No change of starch resulted in blue colour and indicated negative hydrolysis.

Casein hydrolysis test

Skim – milk agar plates were prepared and streaked with the bacterial isolates. The plates were then incubated at 37°C for 24 – 28 hrs. The positive organisms formed zones while the casein negative organisms does not form zones.

Gelatin hydrolysis test

The organisms were inoculated individually in the gelatin agar and incubated for 48 hours at 37°C. After incubation, mercuric chloride solution was added and allowed to stand for 5 – 10 minutes. Formation of clear zone around the colony indicated positive result.

Catalase test

A nutrient agar slant was inoculated with the test culture and was incubated at 37°C for 24 hours. Then, 1 ml of 3% hydrogen peroxide was trickled down the slant and was examined immediately and after 5 minutes for the evolution of bubbles, which indicated a positive test,

Nitrate reduction test

Nitrate broth was prepared and 5 ml was dispensed in test tubes. The isolates were inoculated individually into nitrate broth. The tubes were then incubated at 37° for 96 h. After incubation, 0.1 ml of sulphinilic acid and alpha naphthalamine were added and mixed well. Nitrate positive organisms showed a red colour while the negative organisms did not show any red colouration.

Screening for protease production

Skim milk agar (Components (g/l): Skim milk powder: 100 g; Peptone: 5.0 g; Agar: 15.0 g; pH: 7.2) plates were prepared and streaked with test organisms. They were then incubated at 37°C for 24 – 48 h. After incubation, the plates were flooded with HgCl₂ solution and were observed for zone formation (Plates 1, 2, & 3).

III. RESULTS

Isolation of bacteria

The total viable count of bacterial colonies recorded in fish waste sample was $181 \times 10^2 \pm 0.23$ CFU/g in 10⁻¹ dilution and $50 \times 10^6 \pm 0.13$ CFU/g in 10⁻⁵ dilution (Table.1).

Table.1 Total viable counts of bacterial colonies at different dilutions.

Sl.No	Dilution	Number of colonies (CFU/g)
1.	10 ⁻¹	181×10 ²
2.	10 ⁻²	145×10 ³
3.	10 ⁻³	102×10 ⁴
4.	10 ⁻⁴	83×10 ⁵
5.	10 ⁻⁵	50×10 ⁶

Identification of bacteria

Based on the morphological, physiological and biochemical characteristics, *Bacillus sp.* and *Pseudomonas sp.* of bacteria were identified (Table.2).

Table 2. Morphological, physiological, Carbohydrate fermentation and biochemical characteristics of identified bacterial strains.

S.No	Culture number	Gram staining	Spare staining	H ₂ S	Motility	Citrate test	Methyl red	VP test	Urease	Gelatinase	Indole	Oxidase	Catalase	Nitrate	TSI	Identified organisms
1.	S. 1	G(+) rods	+	-	-	-	-	+	-	-	-	-	+	+	K/A	<i>Bacillus sp.</i>
2.	S.2	G(+) rods	+	-	+	-	+	-	-	+	+	-	+	+	K/A	<i>Bacillus sp.</i>
3.	S.3	G(+) rods	+	-	-	-	+	+	-	+	-	-	+	+	K/K	<i>Bacillus sp.</i>
4.	S.4	G(-) rods	-	-	+	+	-	-	-	-	+	+	+	-	K/A	<i>Pseudomonas sp.</i>
5.	S.5	G(-) rods	-	-	-	+	-	-	-	-	+	+	+	-	K/A	<i>Pseudomonas sp.</i>

+ = Positive; - = negative ; K/A= Alkaline slant/ acid butt; and K/K Alkaline slant/ alkaline butt

All the *Bacillus sp.* of bacteria of the present study are Gram positive whereas, all the *Pseudomonas sp.* is Gram negative (Table 2).

Screening for protease production

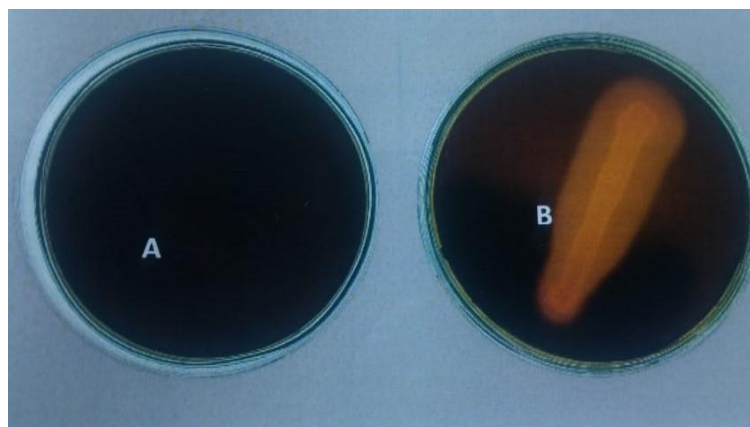
All the identified bacterial species showed positive proteolytic activities (Table.3).

Table 3. Different enzymatic activities of identified bacterial strains.

Culture. No	Bacterial strains	Proteolytic activity
S1	<i>Bacillus sp.</i>	+
S2	<i>Bacillus sp.</i>	+
S3	<i>Bacillus sp.</i>	+
S4	<i>Pseudomonas sp.</i>	+
S5	<i>Pseudomonas sp.</i>	+

+ = positive enzyme activity

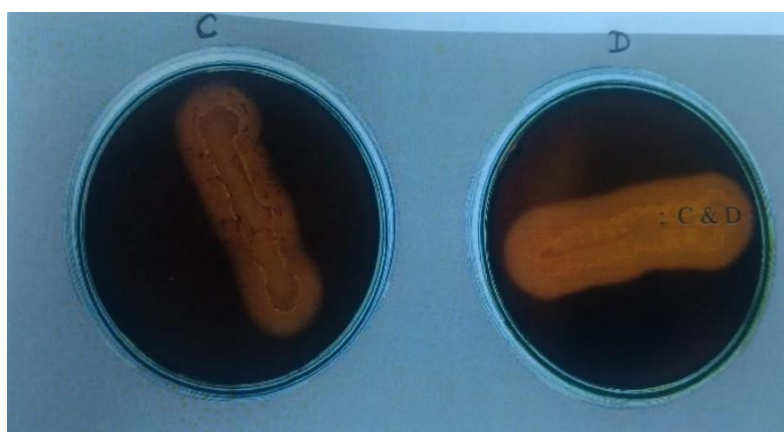
Fig1. Protease activity of isolated bacteria in skim milk agar medium



A: Control

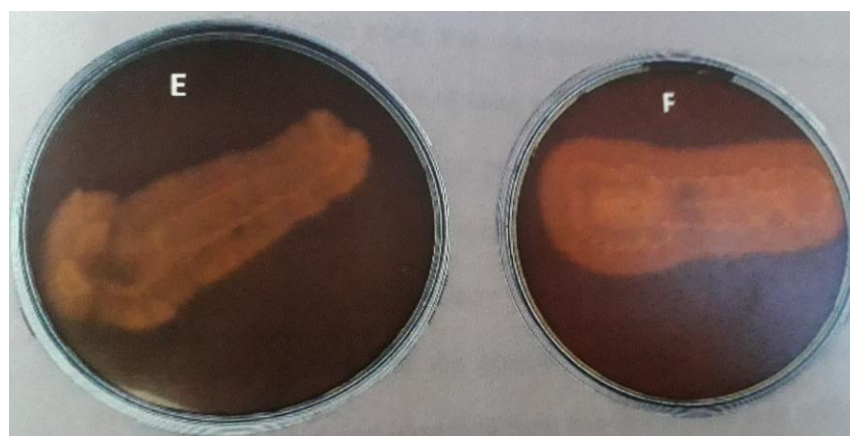
B: *Bacillus sp.*

Fig 2. Protease activity of isolated bacteria in Skim milk agar medium



C and D: *Bacillus. Sp*

Fig 3. Protease activity of isolated bacteria Skim milk agar medium



E & F: *Pseudomonas sp.*

IV. DISCUSSION

Fish waste is one of the common spillage in every fish market area. (ukami et al. 2002). Proteolytic bacterial of fish wastes and their enzyme production abilities are very interesting and hence the present study was undertaken. (Cheong et al., 2014). The isolated 25 bacterial strains and found that only 19 of them are capable of producing protease enzyme and revealed that, those strains with highest protease activity will have a better potential of degrading the proteins presenting shrimp shell wastes. In the present study, all the five isolated bacteria produced protease. Moriarty (1999) stated that, *Bacillus sp.* are generally present in the marine sediments. Similarly, Hoshino et al. (1997) isolated a *Pseudomonas sp.* from fish which was capable of producing protease enzyme. Sabir et al. (2009) reported that, *B.subtilis* was the best protease producer among the tested strains. In the present study also, different *Bacillus sp.* and *Pseudomonas sp.* were isolated and identified from fish wastes and their ability to produce pretease enzyme was qualitatively studied. Similarly, Hoshino et al. (1997) isolated a *Pseudomonas sp.* from fish which was capable of producing protease enzyme. Sabir et al. (2009) reported that, *B.subtilis* was the best protease producer among the tested strains. In the present study, all the bacterial species isolated had a better potentiality of degrading market fish wastes.

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