

Original Research Article

Effect of Fish waste based *Bacillus* Protease in Silver recovery from waste X-Ray Films

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A B S T R A C T

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Fish intestine harbors a lot of microflora, which comes under all groups of microorganisms. Some of them include the amylolytic, lipolytic and proteolytic forms. As the fish body is mainly proteins, the proteolytic bacteria have a major role in degrading the fish waste. In the present study, proteolytic organisms associated with fish waste were evaluated; characterized, identified and potential protease producer was screened by spot test. The selected isolates and the reference culture *Bacillus subtilis* 14410 were subjected for protease production in production media, Raw fish Juice, Production media plus raw fish juice and compared for highest protease producing substrates in which raw fish juice gave considerable result. Based on the activity of the protease produced by the isolated *Bacillus sp.*, it was concluded that the strain had potential of producing an alkaline protease under pH 9 and 35^oC and has greater proteolytic activity profile at pH 7 to 8. The protease was partially purified and the molecular weight was determined as 45 Kda and 38Kda. It was used as a major constituent in waste X-ray Silver recovery. Compared to *Bacillus subtilis* 14410, isolated *Bacillus sp.* from fishwaste yield maximum protease and show higher activity in Waste X-ray silver recovery.

Introduction

The catching and processing of fish generates a significant amount of waste, which includes viscera, fins, scales and bones. India alone generates >2metric million tons of waste during fish processing of which 300,000 tons contribute to visceral waste alone

(Mahendrakar, 2000). Due to its high organic content, fish waste is classified s certified waste which is even more costly to dispose. This practice is coming under increased scrutiny to environmental issues (Jespersion *et al.*, 2000) and is becoming an increasing concern and cost burden to the whole sea-food industry (Anon, 2002).

The three most common methods for the utilization of aquatic waste are the manufacture of fish meat/oil, the production of silage or the use of waste in the manufacture of organic fertilizer. Fish silage exploits the endogenous enzymes. Thus, the native proteolytic bacteria associated with fish waste itself would be a better option to hydrolyze compared to other sources (Sugita *et al.*, 1996).

Fish waste is a highly rich source of protein and the role of proteolytic microbes associated with fish or fish by-products becomes all the more important. Furthermore, microbial proteases are an important group of enzymes that can have application in various industries such as leather processing, food processing, pharmaceutical, bioremediation process and in textile industry to remove protein based strains (Anwar and Saleemuddin, 1998; Banerjee *et al.*, 1999; Gupta *et al.*, 2002; Najafi *et al.*, 2005). Non-nutritional uses include cosmetics, fine chemicals, collagen, gelatin and pearl essence (Archer *et al.*, 2001). It was possible to detect proteolytic activities from *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Serratia marcescens* as well as influence that these components displayed in the expression of these enzymes (Vermelho, 1996).

Among the various proteases, bacterial proteases are more significant when compared with animal and fungal proteases. And among bacteria, *Bacillus* species are specific producers of extra-cellular proteases. They find their application in many fields and are important tools in studying the structure of proteins and polypeptides (Bhosale *et al.*, 1995). The expansion of bio-technology has created an increasing demand for new and low-cost protein sources. In the view of their high protein content, fish

represents a potential source of industrial peptones. The fish processing industry generates considerable amounts of by-products as waste that includes viscera, shells, scales, fins and bone frames. They are all often high in protein, which can be processed into useful products. The present investigation aims at isolating proteolytic bacteria from fish waste, finding the effect of pH, temperature on selected isolates, extraction and purification of protease from the selected isolate and determination of the activity profile of extracted protease. Finally it was subjected for waste X-ray silver recovery.

Materials and Methods

Collection and Homogenization of samples

The fish waste (*Neimpterus japonicus*) was collected from the local fish market (Ukkadam, Coimbatore, Tamilnadu) in fresh condition and brought to the laboratory in iced condition. The collected samples were homogenized aseptically in a Philips mixer (homogenizer) and used for analysis.

Isolation of Bacteria from the samples

The part of homogenized samples was serially diluted by mixing 1ml with 99ml distilled water, that gives 10^{-2} dilution. To the first tube of serially arranged 9ml distilled water, 1ml of 10^{-2} dilution was transferred which gives 10^{-3} dilution. Similarly, dilutions were made upto 10^{-6} . Aerobic Plate Count (APC), Total Coliform Count (TCC) and Total Proteolytic Bacterial Count (TPBC) of each sample were determined using standard methods on Plate Count Agar, Eosin Methylene Blue Agar and Skim Milk Agar respectively. 0.1ml of sample

dilution 10^{-4} , 10^{-5} and 10^{-6} were employed for plating.

Computing and recording counts of Bacteria

The formula for finding the colony count is,

$$N = \frac{\sum C}{[(1 \cdot n_1) + (0.1 \cdot n_2)] \cdot d}$$

Where,

N = number of colonies/ml or gram of product.

$\sum C$ = sum of all colonies on all plates counted

n_1 = number of plates in first dilution counted

n_2 = number of plates in second dilution counted

d = dilutions from which first counts were obtained.

Identification of bacterial isolates

Isolates which showed clear zones on Skim Milk Agar were subjected to Gram staining, Endospore Staining and Hanging Drop Technique to determine its motility. The isolates were further characterized based on their reactivity to Indole, Methyl Red, and Voges-Proskauer, citrate utilization, Starch hydrolysis, urea hydrolysis, H_2S production, oxidase and catalase tests.

Selection of proteolytic isolate

Clear zones produced by the isolates on Skim Milk agar plates were measured.

Thus, the proteolytic isolate which exhibited highest proteolytic activity (determined by the extent of clear zone around the colony) was selected for further analysis.

Effect of pH on selected proteolytic isolate

Skim Milk Agar plates with different pH(4, 7, 9, 11, 13) were prepared and inoculated with the cultured isolate for determining the effect of pH on the proteolytic activity of the isolate.

Effect of temperature on selected proteolytic isolate at pH 9

Skim Milk Agar plates with pH 9 were prepared and inoculated with the selected isolate and incubated at different temperatures (15, 25, 35, 45⁰C) for determining the effect of temperature on its proteolytic activity.

Protease Production

The selected proteolytic isolates and the reference culture *Bacillus subtilis* 14410 were separately grown in production media contain 2% NH_2SO_4 (w/v), 1% K_2HPO_4 (w/v), 1% KH_2PO_4 (w/v), 0.4% $MgSO_4 \cdot 7H_2O$ (w/v), 0.01% $MnSO_4$ (w/v), 0.01% $Fe_2SO_4 \cdot 7H_2O$ (w/v), 1% yeast extract(w/v), 10% peptone(w/v) with pH9 maintained at 35⁰C for 24 to 72 hours in a shaking incubator(140rpm). The second production media subjected for protease production was the sterilized remaining part of homogenized fish juice. The third production media subjected for protease production was 90ml production media vortexes mixing thoroughly with 10ml sterilized homogenized fish juice.

Extraction of Enzyme and Dialysis

The cells were harvested by centrifugation at 10000rpm for 10 minutes at 4°C. The precipitate containing the cell debris was discarded and supernatant treated with 70% Ammonium sulphate and allowed to stand at 4°C overnight. Then the precipitate was centrifuged at 10000rpm for 20mins at 4°C. The resulting precipitate was dissolved in 0.9% NaCl and labeled as crude protease extract. The same process was progressed for the remaining above mentioned Medias too. The crude enzyme was partially purified using ammonium sulphate (60% saturation). The precipitated enzyme was used as a crude protease for further studies.

Proteolytic activity profile

The proteolytic activity of crude protease extract was assayed at pH4-10 using the following systems-Citrate phosphate buffer (pH 4 and 5), phosphate buffer (pH 6-8), Glycine-NaOH buffer (pH 9 and 10). The assay mixture consisted of 1.25ml of respective buffers, 0.5ml of casein as substrate and 0.25ml of enzyme extract. The reaction was stopped by adding 3ml of 5% TCA after holding it at 37°C for 30 minutes. The final mixture was filtered and TCA soluble peptides in filtrate were quantified by measuring the absorbance at 660nm using Lowry's method.

Lowry's Method for Protein estimation

The blue colour developed by the reaction of phosphomolybdic-phosphotungstic compounds in folin-ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein were measured at 660nm in a spectrophotometer.

Gel Electrophoresis and zymogram

A zymogram of dialyzed enzyme was obtained using polyacrylamide gel electrophoresis followed by treatment with 1% casein as substrate in Tris buffer (pH9). The molecular weight of the protease was determined by comparing with mobility of standard molecular weight marker proteins.

Protease in silver recovery from waste X-rays

The waste X-ray films were washed with distilled water and wiped with cotton impregnated with ethanol, and was cut into 4 x 4 cm² pieces after drying in an oven at 40°C for 30minutes. Each of the films was rinsed in series 100ml of enzyme extract and the pH of the solution were adjusted to 8. The solution and the film were stirred at 50°C in a water bath until the gelatin layer was stripped completely. The obtained slurry was dried and weighed.

Result and Discussion

The results of isolating proteolytic bacteria from fish waste, finding the effect of pH, temperature on selected isolates, extraction and purification of protease from the selected isolate and determination of the activity profile of extracted protease were tabulated (Tables 1,2,3). Thirty bacterial isolates were obtained from fish waste (*Neimpterus japonicas*) of which four isolates was identified as *Bacillus sp.*, *Pseudomonas sp.*, *Alcaligenes sp.*, and *Lactobacillus sp.* *Bacillus sp.* exhibit highest proteolytic activity in spot test. In another study, Waksman (1961) identified 29 isolates as *B. megaterium* and 24 isolates as *B. subtilis*. These agree with the

results of this study that *Bacillus* genera are wide spread among bacteria in fish wastes.

The production of extracellular protease from *Bacillus* sp. was optimum in sterilized raw fish juice and in production medium of pH9 at 35°C under shake culture condition with 2.5×10^9 cells ml⁻¹ as an initial inoculum density at incubation 36 hrs (Table 4; Figure 1,2). It shows that the isolates from same raw source (fish juice) exhibit maximum protease yield when compare to the reference standard *Bacillus subtilis* 14410. The *Bacillus* protease exhibited shows maximum activity in alkaline pH-8, under the maximum temperature 35°C

(160.37 ± 0.18 Uml⁻¹) and beyond this temperature, the enzyme activity decreased. One way ANOVA revealed that the variation in protease activity between the tested temperature was statistically significant ($P < 0.0001$; Fig. 3). This *Bacillus* Protease on waste x-ray silver recovery exhibit greater activity.

So, it is possible to use fish waste, which is considered as a useless property in an effective manner to produce an industrially important enzyme protease from proteolytic organisms. Recycling of these wastes is necessary to prevent pollution and to conserve our planet as well as to improve our industrial perspectives.

Table.1 Aerobic, Coliform and Proteolytic Bacterial load in Fish Waste (*Neimpterus japonicus*)

Sample	Dilution	No of organism (aerobic count)	Coliform count	Proteolytic bacterial count
I	10 ⁻⁴	210	132	17
	10 ⁻⁵	58	35	9
	10 ⁻⁶	6	80	1
II	10 ⁻⁴	156	73	21
	10 ⁻⁵	63	26	13
	10 ⁻⁶	8	6	5
III	10 ⁻⁴	180	93	13
	10 ⁻⁵	75	29	6
	10 ⁻⁶	11	3	-

Table.2 Total Aerobic, Coliform and Proteolytic bacterial count in Fish Waste

Sample	Total plate count (cfu/mg)	Total coliform count (cfu/mg)	Coliform %	Total proteolytic bacterial count (cfu/mg)	Proteolytic bacterial %
I	24.4 x 10 ⁵	15.2 x 10 ⁵	62.29%	24 x 10 ⁴	9.84%
II	19.9 x 10 ⁵	9 x 10 ⁵	45.23%	31 x 10 ⁴	16.23%
III	23.2 x 10 ⁵	11 x 10 ⁵	47.41%	18 x 10 ⁴	7.76%

Table.3 Morphological and Biochemical Characteristics of Isolates

Tests	Bacterial Isolates number			
	EKPi1	EKPi2	EKPi3	EKPi4
Morphological Characteristics				
Shape	rods	rods	rods	Rods
Gram's stain	-	+	-	+
Endospore stain	-	-	-	+
Motility	+	+	+	+
Biochemical Characteristics				
Indole	+	+	-	-
Methyl red	+	-	-	-
Voges-proskauer	+	+	-	-
Citrate utilization	+	-	+	-
Starch hydrolysis	+	+	+	+
Urea hydrolysis	+	+	-	+
H ₂ S production	-	-	-	-
Oxidase	+	+	+	+
Catalase	+	-	+	+
	<i>Pseudomonas</i>	<i>Lactobacillus</i>	<i>Alcaligenes</i>	<i>Bacillus</i>

Table.4 Proteolytic Zones Produced By Isolates on Skim Milk Agar

Isolate number	Organism	Zone diameter (cms)
1	<i>Pseudomonas</i> sp.	2
2	<i>Lactobacillus</i> sp.	1.3
3	<i>Alcaligenes</i> sp.	1.7
4	<i>Bacillus</i> sp.	2.3

Figure.1 Effect of pH on Proteolytic Activity of *Bacillus* sp.

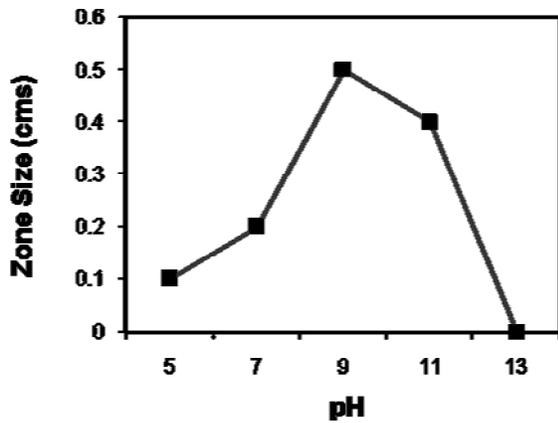


Figure.2 Effect of Temperature on Proteolytic Activity of *Bacillus* sp. at pH 9

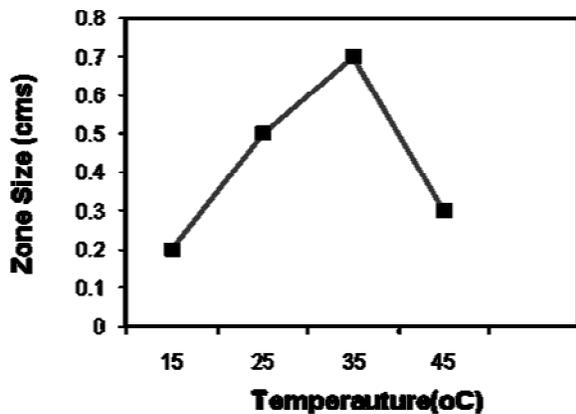
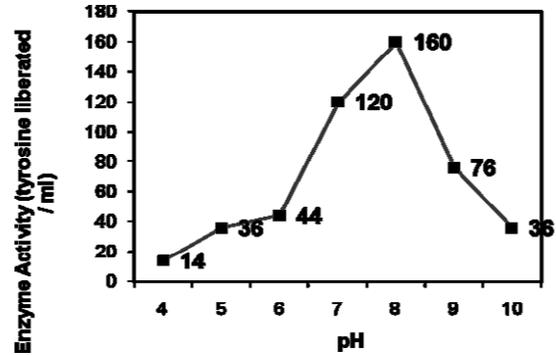


Figure.3 Activity Profile of Protease Produced by *Bacillus* sp. at Different pH



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