Optimization Studies of Microbial Proteases Extracted from Effectual Extremophilic Isolates Screened from Mangrove Swamps

Sarika Chhabria Talreja[a] & Chandra B. Maurya[b]
[a] Research Scholar, Guru Nanak Khalsa College, Matunga, Mumbai 400 019
[b]Research Supervisor & Principal, KLE College of Science and Commerce, Kalamboli, Navi Mumbai 410 218.

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ABSTRACT *Marshy mangrove areas of Goa, India are rich inhabitants of Halophilic bacteria known for their lofty salt tolerant capacity. These halophilic microorganisms are loaded with variety of Proteases having potential applications in numerous biotechnology and industrial fields. The plethora of Proteases affianced in them is largely unknown because of mangrove's enclosed ecosystem. The present study is an attempt to isolate potent microorganisms from mangrove region of Goa for some neoteric proteolytic enzymes. Twenty bacterial isolates B1-B20 were screened for their proteolytic activity; however, B2 and B3 isolates manifested persuasive extracellular Protease activity on skimmed milk agar plates. 16S rRNA sequence analysis further ensued that twenty isolates were related to Bacillus sp. The cell free extract was used to study the effects of temperature and pH on Protease activity. Optimization studies revealed that the optimum temperature and pH for enzyme activity were 60°C and 7.0 respectively. Comparison with other Proteases from different microbial sources indicated that the neutral Protease producing halotolerant cultivable marine isolates in the marshy areas of Mangrove forests of Goa are engraved with Proteases with high thermostability.*

Keywords: *Halotolerant, Mangroves, Neutral Protease, Optimization, Thermostability.*

Introduction

Enzymes as biocatalysts carry out large number of chemical reactions and are commercially exploited in various industries (Kumar and Takagi, 1999). The estimated value of worldwide sales of industrial enzymes was US $ 2.5 billion in 2009 (Rajan, 2004). Among industrial enzymes, Proteases represent one of the three superlative consortia and incapacitate 60% of the overall global sale of enzymes (Rao et al., 1998). Being ubiquitous in nature, Proteases are found in a wide diversity of sources such as plants, animals, microorganisms and are physiologically essential for all living organisms. Microbial Proteases constitute approximately 40% of the global enzyme business (Godfrey and West, 1996). Microbes serve as an ideal enzyme source because of their fast growth, limited space needed for their cultivation and the ease with which they can be genetically manipulated to produce new enzymes with altered features desirable for diverse applications. Direct secretion into the fermentation broth; attributable to the extracellular nature of microbial Proteases simplifies downstream processing of enzyme as compared to that obtained from plants and animals. The inadequacy of Proteases of plant and animal origin to meet the current world supplication has led to increased research attentiveness towards microbial Proteases (Rao et al., 1998).

Microbial extracellular Proteases are of commercial importance and find multiple applications in various industrial sectors like detergent, food, photography, leather and pharmaceutical industries. Although there is good number of available microbial antecedents, only a few are recognized as commercially rewarding producers. Bacterial alkaline Proteases are characterized by their superior activity at elevated pH with optimal temperature of 60°C. With the neoteric emergence of biotechnology, there has been a growing engrossment and demand for enzymes with newfangled properties. Considerable efforts have been directed towards the selection of microorganisms producing commercial Proteases with new physiological properties and their relevance to high end industrial processes. The whole research fraternity is now fanatically pursuing its reverie to increase the microbial Protease production by looking for new strains, optimizing media composition and genetic manipulation.

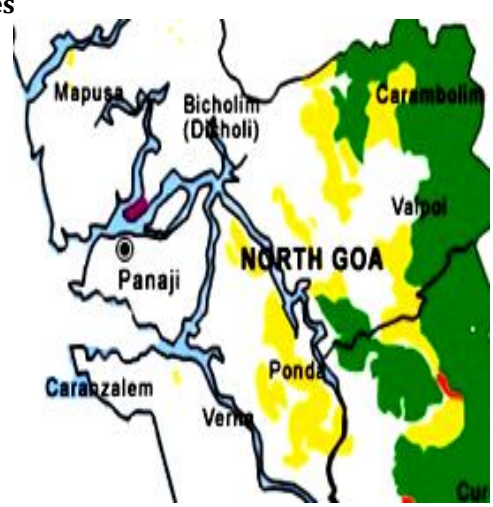
It is noteworthy that the vast biodiversity of microbial world is still unknown considering that barely less than 1% of the total microbiota has been isolated and characterized by standard culturing conditions. As indicated by Staley and Konopka (1985), most of the environmental microbes observed under microscope cannot be cultured under standard laboratory conditions, as some of them may be non-viable while others are viable but nonculturable (VBNC). More than 98% of the microbial biodiversity remains unexploited and underutilized mainly because of the unavailability of suitable culturing conditions and hence their bioresources remain inaccessible (Amann et al., 1995).

Forty two percent of the world's mangrove regions are in Asia, located along the south coast and especially throughout the Indian subcontinent. 91% of the total microbial biomass in the torrid equatorial swampy region of mangroves is constituted by fungi and bacteria, whereas algae and protozoa typify only 7% and 2%, respectively (Alongi, 1988). Major nutrient transformations within a mangrove ecosystem are attributable to the microbial activity. Studies of microbes and their interactions with other habitat components are critical for understanding the mangrove ecosystem, however very little is known about the microbial communities in mangrove sediments (Gray and Herwig, 1996; Ghosh et al., 2010).

Mangrove's marine microorganisms have recently emerged as a rich source for the isolation of industrial Proteases and are staunchly used for varied industrial applications. In the present study, we have screened some marine isolates from sediment samples of mangroves region of Goa for their industrially imperative Proteases. We further performed the optimization studies of enzyme extracted from persuasive bacterial isolates.

Material and Methods

Collection of Sediment Samples



Twenty Mangrove sediment samples (B1-B20) were collected from Panji, Goa, India and screened for their Protease activity. Out of 20, B2 and B3 were found to be competent cultivable source of Proteases. 16S rRNA sequence analysis further ensued that twenty isolates were related to *Bacillus* sp. Phylogenetic analysis confirmed B2 as Bacillus anthracis and B3 as Bacillus safensis (as described in our previous publication by Sarika et. al, November 2017). Latitude and longitude with water depth was measured for the samples. All physicochemical parameters like pH, Temperature, D.O and Conductivity were also analyzed for the sampling site.

Culture Conditions

Isolation and downstream processing of crude Protease involved following steps:

Step I: Bacterial Fermentation for production of Extracellular Protease: Two persuasive cultures B2 and B3 were selected and grown for 48 hours on IGB agar slants. Cells were then inoculated in 20ml IGB broth as seed medium and grown at 37^{o}C for 24hrs. 500ml of production media was prepared containing 0.15% each of Glucose, Potassium dihydrogen phosphate and Disodium potassium phosphate. 1% Gelatin was used as a protein source and all the media were sterilized by autoclaving. The production media was inoculated in sterile conditions in a 1L conical flask and incubated for 48 hours at 37^{o}C and 110 rpm shake flask conditions.

Step II: Cell harvestation by Centrifugation method: Cells were then harvested from the 48 hours grown culture. Cell garnering was done by centrifugation method; cells were centrifuged at 12000 rpm for 5 min. at 4^{o}C. The culture supernatant was accrued separately and residual cell pellet was dispossessed.

Step III: Microfiltration method: In the last step, cell free supernatant was filtered through 0.3µm glass fiber filter using vacuum pressure to remove the suspended particles followed by 0.2µm PES filters. The micro filtered sample was collected in a separate container and processed further.

Enzyme Assay

Protease activity was measured using caseinolytic assay as described by Bergkvist 1963, with slight modifications. Reaction mixture for the extracted enzyme supernatant was prepared as per standard

instructions. 0.65% casein was prepared in the 50mM Sodium phosphate buffer (containing Di-sodium hydrogen phosphate and Hydrochloric acid) of desired pH and absorbance was measured at 660 nm. One unit was ascribed to the amount of enzyme hydrolyzing casein so as to attain color, equivalent to 1.0 µmol of Tyrosine per minute under standard set-up. The absorbance values obtained were used to calculate the micromoles of Tyrosine in the sample. These values were converted to the activity of enzyme in U/ml.

Enzyme Concentration by Tangential Flow Filtration (TFF)

Enzyme extracted from B3 was processed further because it showed considerable activity as compared to B2. The filtered enzyme supernatant was concentrated through Tangential Flow Filtration (TFF) apparatus of PALL (Minimate TFF system 200-240V AC OAPMP220) with a 10KDa cassette. The TFF cassette of 10 KDa was connected with the required tubing and vessel. The filtered sterilized water was allowed to run in order to wash the system. Sample was then added to the vessel having graduations. Concentration was judged by reduction in volume of enzyme sample in the vessel. Samples once reached to the required volume were collected in a sterile tube. The crude enzyme was concentrated 10 fold and used for further studies.

Growth Media Variation Studies

Inoculum was prepared from IGB medium agar slants to IGB medium broth. The growth was seen to be faster with second condition. Six different media were studied. Substrate variation and other studies were performed using these media. Enzyme obtained from the gelatin media was used for further studies.

Growth Media pH and Temperature Optimization Studies

Gelatin media from the above study was used with varying pH. The pH of 500ml media was adjusted before sterilization by autoclaving. Four different pH conditions, 6, 7, 8 and 10 were used for the essay. The media was inoculated and incubated as before. After 72 hours of incubation at 37^{o}C, the supernatant was checked for enzyme activity. Similarly, another set of media at pH 7 was incubated at different temperatures of 30, 40, 50 and 60^{o}C.

Enzyme pH and Temperature Optimization Studies

Effect of pH on Enzyme Stability

Effect of change in pH on the activity of enzyme was studied as per the method described by Jadhav, et al., 2014. Briefly different buffers (0.2M) of pH 5, 6, 7, 8 and 10 were prepared and 1% casein substrate of varying pH was also prepared in the respective buffers. For pH 5 and 6, Sodium acetate - Acetic acid (NaOAc - AcOH) buffer was used while pH 7, 8, and 10 buffers were prepared using Sodium phosphate - Hydrochloric acid [$Na(PO_4)_3$ - HCl]. Enzymatic reaction mixture was prepared in these buffers of varying pH and exposed for 30 minutes at optimum temperature of 60^{o}C. Tyrosine released was estimated by Folin method.

Effect of Temperature on Enzyme Stability

Cell free extract was exposed to various temperatures ranging from 30-80°C with a variation of 10^{o}C to study the effect of increase in temperature on enzyme stability as described by Srividya Shivakumar, 2012. The samples were removed at different time intervals ranging from 30 minutes to 8 hours and Protease activity was determined.

Statistical Analysis

All the experiments were performed in triplicate. For statistical analysis, the standard error of the mean values was calculated and the means were tested according to ANOVA-one way for significant differences among the samples. A statistical significance was accepted at $P<0.05$.

Results and Discussion

Bacterial Sampling and Isolation

20 bacterial samples were collected from marshy sediments in Panji, Goa and screened for their morphological and Protease production efficiencies (reported in our early publication). Out of 20, two bacterial isolates, B2 and B3 were laced with stupendous Protease production properties and were thus carried further for optimization studies. Latitude and longitude with water depth were measured for the sampling site. All physicochemical parameters like pH, temperature, D.O and conductivity were also measured. The sampling parameters are summarized in **Table 1.**

Table 1: Sampling Parameters

Sample	Isolates	Latitude and Longitude	Depth (m)	pH	Temp (°C)	Salinity (ppm)	Conductivity (ms)	D.O. (mg/l)
1	**B2**	**15°, 30', 27" N; 73°, 51', 36" E**	**14.3**	**8.5**	**31.24**	**79.24**	**687.25**	**5.32**
2	**B3**	**15°, 30', 27" N; 73°, 51', 36" E**	**9.5**	**7.9**	**32.54**	**31.27**	**536.54**	**4.91**

Bacterial Growth Curve Studies

Two media containing Casein and Gelatin as the protein substrate were studied for the two selected positive cultures B2 and B3. It was observed that B3 showed considerably higher activity for both the substrates than B2; hence B3 was considered for further studies. Growth curve studies revealed that the culture entered late log phase after 24 hours and entered stationary phase after 48 hours. Maximum Protease activity was obtained after 24 hours of incubation **(Table. 2).**

Table 2: Colony Count and Enzyme Activity Change (U/ml) during Fermentation

Duration (Hours)	Culture Code	Colony count (CFU/ml)	Activity (U/ml)
24	B2	1524 x 10^4	9.6
48		119 x 10^4	14.7
24	**B3**	1760 x 10^4	10.9
48		**350 x 10^4**	**20.2**

The results significantly indicate (*p>0.05.0.089*) that B3 exhibited maximum Protease activity in lesser fermentation time which is a major prerequisite for commercial Protease production. B2 and B3 were also grown in Casein and Gelatin broths to optimize their culture conditions. B2 showed significantly higher ((*p>0.05, 0.231, 3 folds*) Protease activity in CB compared to GB. Similar trend was showed by B3 in both type of broths i.e. GB and CB. The Protease activity was significant higher (*p>0.05.0.66, 2 folds*) in GB compared to CB (**Table 3**).

Table 3: Crude Protease Activity in CB - Casein Broth; GB - Gelatin Broth

Culture Code	Reaction Conditions	Media	OD (660 nm)	Activity (U/ml)
B2	37^0 C / pH 7.0	CB	0.013	12.13
		GB	0.005	4.40
B3		CB	0.027	26.13
		GB	**0.055**	**53.33**

Growth Optimization Studies

Media containing specific protein substrate gave higher activity than the basic protein mixture media. The media which gave highest activity of 50.2 U/ml was found to be containing Gelatin as the substrate. The results for media variation studies are summarized in **Table 4**.

Table 4: Media Variation Studies

Media	Composition	Enzyme Activity (U/ml)
1	0.5% Yeast Extract + 0.5% Peptone + 0.5% Malt Extract	14
2	1.5% Casein + 0.2% Di-potassium phosphate + 0.1% Glucose + 0.5% Peptone	36
3	1.5% Gelatin, 0.2% Di-potassium phosphate + 0.1% Glucose + 0.5% Peptone	20
4	1.5% Casein + 0.2% Di-potassium phosphate + 0.1% Glucose	26.7
5	**1.5% Gelatin + 0.2% Di-potassium phosphate + 0.1% Glucose**	**50.2**
6	Soyabean Casein Digest Medium	10

Growth Media pH and Temperature Optimization Studies

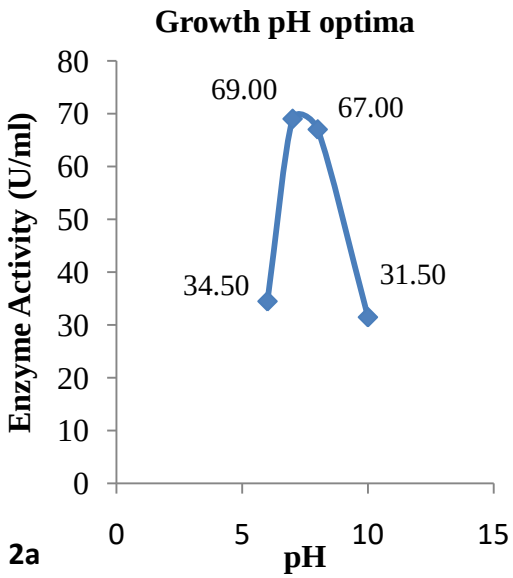
The results for growth media pH and temperature optimization studies are summarized in **Tables 5 and 6.** Optimum pH for growth medium was observed to be 7.0 and the optimum temperature as 40°C **(Fig. 2a and Fig. 2b)**.

Table 5: Growth Media pH Optimization Studies

pH	Blank Absorbance	Test Absorbance	Test -Blank	µmole s/ml	Enzyme Activity (U/ml/min)
6	0.434	0.227	0.207	69.00	34.50
7	**0.749**	**0.335**	**0.414**	**138.00**	**69.00**
8	0.694	0.292	0.402	134.00	67.00
10	0.401	0.212	0.189	63.00	31.50

Table 6: Growth Medium Tempearture Optimization Studies

Temperature (°C)	Blank Absorbance	Test Absorbance	Test -Blank	µmoles /ml	Enzyme Activity (U/ml/min)
30	0.2	0.204	68.00	0.404	34.00
40	**0.235**	**0.414**	**138.00**	**0.649**	**69.00**
50	0.2	0.16	53.33	0.36	26.67
60	0.21	0.091	30.33	0.301	15.17



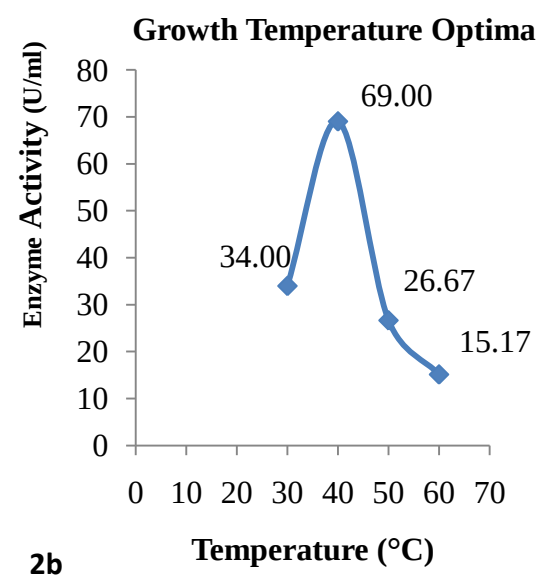


Fig.2a. Growth pH Optima Studies for B3 *Bacillus safensis* strain.
Optimum pH was recorded as 7.0.
Fig. 2b. Growth Temperature Optima Studies for B3 *Bacillus safensis* strain.
Optimum temperature was recorded as 40°C.

Enzyme pH and Temperature Optimization Studies

Effect of pH on Enzyme Stability

pH optima studies revealed that maximum Protease activity was observed at pH 7.0 indicating neutral nature of the enzyme **(Table 7)**. The Protease activity decreased with increase in pH **(Fig. 3a).** Similar results were obtained by Debananda S. et al, 2010, who found that their enzyme alkaline Protease from *Bacillus Subtilis* Strain SH1 and *Bacillus* sp. strain CR-179 respectively showed pH optima of 8.0. Similarly, Kiranmayee Rao et al, 2013, found that alkaline Protease from *Bacillus firmus 7728* exhibited maximum activity at pH 9.0. Joshi et al, 2010 observed optimum pH 8.0 for his Protease extracted from *Bacillus firmus*

Tap5. Similarly Alkaline Proteases from *Aspergillus niger* showed optimum pH of 10.0 when studied by Devi et al, 2008. Chi et al, 2009, purified alkaline Protease from *Aureobasidium pullulans*, which showed highest activity at pH 9.0. Thus there is a huge difference between the pH stability of variety of Protease of bacterial origin.

Table 7: Enzyme pH Optimization Studies

pH	Blank Absorbance	Test Absorbance	Test -Blank	µmoles/ml	Enzyme Activity (U/ml)
5	0.084	0.098	0.014	4.67	6.22
6	0.086	0.147	0.061	20.33	27.11
7	**0.076**	**0.243**	**0.167**	**55.67**	**74.22**
8	0.071	0.232	0.161	53.67	70.56
10	0.069	0.224	0.155	51.67	58.20

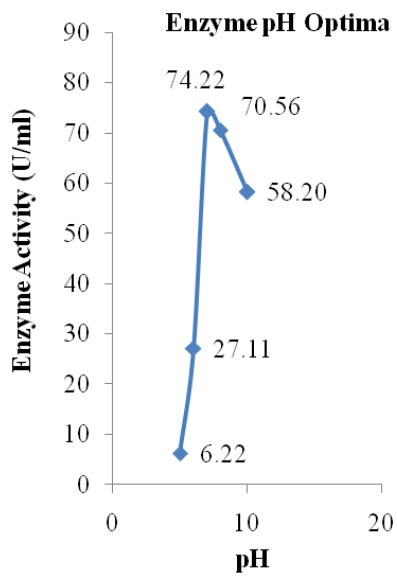


Fig.3a. pH Optima Studies of Protease extracted from B3 *Bacillus safensis* strain. Optimum pH was recorded as 7.

Effect of Temperature on Enzyme Stability

Maximum Protease activity was obtained at 60°C **(Table 8)** which was significantly higher (*P<0.05, 0.00214)* among all temperatures used **(Fig. 3b).**

Table 8: Enzyme Tempearture Optimization Studies

Temperature (°C)	Blank Absorbance	Test Absorbance	Test -Blank	µmoles/ml	Enzyme Activity (U/ml)
30	0.081	0.213	0.132	44.00	35.20
40	0.099	0.277	0.178	59.33	47.50
50	0.084	0.437	0.353	117.67	94.13
60	**0.094**	**0.652**	**0.558**	**186.00**	**148.80**
70	0.077	0.473	0.396	132.00	105.60
80	0.133	0.162	0.029	9.67	7.73

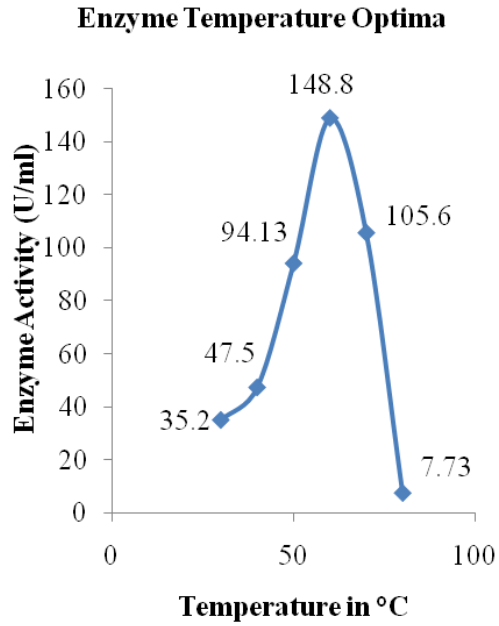


Fig. 3b. Temperature Optima Studies of Protease extracted from B3 *Bacillus safensis* strain. Optimum temperature was recorded as 60°C.

It was concluded that extracted Protease from *Bacillus safensis* is potentially thermostable. Similar results were obtained by M. A. Ferrero et al, 1996, for serine alkaline Protease from *Bacillus licheniformis* MIR 29. They found maximum enzyme activity at 60°C and enzyme was stable up to 60°C in the absence of stabilizers. Gençkal, H., 2006, extracted, purified and studied alkaline Protease from *Bacillus* sp L21 which exhibited maximum Protease activity at 60°C and retained 90% of its activity at 80°C. They also found that the alkaline Protease was found to be stable in a temperature range of 30°C to 50°C but lost about 30% of its activity at 60°C, after 30 minutes and 1 hour incubations both in the presence and absence of Ca^{2+}. Manavalan Arulmani, 2015, studied an extracellular thermostable alkaline Protease from *Bacillus laterosporus*-AK1 which exhibited highest activity at 75°C. Mane R. and Bapat M., 2001, also established that maximum activity of their bacterial originated Protease was at 70°C. In the view of these studies, it is perceived that Proteases from bacterial origin are thermostable and thus commercially important.

Conclusion

Increased awareness about the biocatalytic capabilities of microbial enzymes has made possible the development of new biological products in various industrial sectors. Interest in thermostable enzymes has grown, mainly due to the fact that these can be run at high temperatures and can be extracted from natural sources. There are certain advantages in using thermostable enzymes in industrial processes such as high reaction rate, longer half life, lesser risk of contamination and decrease in viscosity allowing higher concentrations of less soluble materials. Results of the present study indicate that the neutral Protease from B3, a novel thermostable enzyme from mesophilic organism, appears to have considerable potential for industrial applications. Advances in the field of genetic and enzyme engineering coupled with the novel properties of this enzyme from halotolerant microorganism will make it possible to move the desired gene coding for this novel enzyme from prokaryote to eukaryote and to amplify its expression. Thus our studies corroborate with the earlier findings and suggest that our bacterial isolates are novel with an unconventional and atypical primogenitor for industrial production of Protease.

Conflicts of Interests

Author shows no conflicts of interests.

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