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Stability Studies of Proteases Engendered From Enticing Microbial Primogenitors Screened From Estuarine Mangrove Detritus

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ABSTRACT

Marshy mangrove areas of Goa, India are opulent incumbents of halophilic bacteria resplendent in variety of Proteases and huge salinity-stress tolerant capacity. Bacteria are the most prevalent consortia of Protease primogenitors serving as quintessential enzyme source; with Bacillus being the premier genus. The colossal heterogeneity of Proteases, coupled with the ease with which they can be genetically manoeuvred with revamped properties desirable for diverse industrial applications, in contradistinction to their modus operandi and explicitness, has captivated worldwide recognition to exploit their commercial utility.

The present study is an attempt to harness bacterial isolates from Goa's mangrove areas for some neoteric proteolytic enzymes. Out of twenty isolates screened, B_2 and B_3 manifested persuasive proteolytic activity. Phylogenetic analysis further ensued that isolates were related to Bacillus Sp. Optimization studies by growth media variation revealed that optimal growth media temperature and pH was 40^{o}C and 7.0 respectively. With respect to the characteristics influencing culture conditions and properties, it was considered sententious to characterize the enzyme through Stability studies. Studies showed that Protease was considerably stable at optimum conditions of 60^{o}C in pH 7.0.

KEY WORDS: Bacillus, Characterization, Mangroves, Proteases, Stability

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1. INTRODUCTION

Proteases form a bulky cluster of enzymes which are ubiquitous in nature and the central category enzymes from industrial view point. Bacteria are the most prevalent consortia of Protease primogenitors serving as the quintessential enzyme source; with Bacillus being the premier genus[1,2]. Their rapid growth, limited space required for cultivation[3] and the ease with which they can be genetically manoeuvred with revamped properties desirable for diverse industrial applications makes microbial Proteases commercially conspicuous [4].

The colossal heterogeneity of Proteases, in contradistinction to their modus operandi and explicitness, has captivated worldwide recognition to exploit their physiological and biotechnological relevance[5,6]. They are one of the most indispensable categories of industrial enzymes employed in brewing, meat and photography industries. They are rampantly exploited in food and pharmaceutical industry for peptide synthesis and leather industry for dehairing. Their application in detergent industry as additives for surfactant formulation[1,7] so as to replace currently used noxious chemicals, is a relatively recent advancement and has conferred added biotechnological significance [6].

Proteases have traditionally detained a predominant share of industrial enzyme market accounting for about 60-65% of total global sale of enzymes[6,8,9,10]. About 35% of the aggregate microbial enzymes utilized in detergent industry are procured from bacterial sources and majority of them are produced by Bacillus Sp. [11,12].

In this paper, we aimed to investigate the stability at varied pH and temperature conditions[13] of neutral Proteases extracted from Bacillus safensis for its possible and potential applications for industrial purposes.

2. EXPERIMENTAL SECTION

Twenty Mangrove sediment samples (B_1 - B_{20}) were collected from Panji, Goa, India and screened for their morphological and Protease production efficiencies. Out of 20, B_2 and B_3 were found to be competent cultivable source of Proteases (as described in our previous publication by Sarika et. al 2017). Protease activity was measured using Caseinolytic assay. Two media containing Casein and Gelatin as the protein substrate were studied for two selected positive cultures B_2 and B_3. Growth curve studies revealed that the culture entered late log phase after 24h and entered stationary phase after 48h. Maximum Protease activity was obtained after 24h of incubation. The results significantly indicated that B_3 exhibited maximum Protease activity in lesser fermentation time which is a major prerequisite for commercial Protease production. The filtered enzyme supernatant was concentrated 10 fold through Tangential Flow Filtration method and used for further studies. Growth Optimization studies showed that the media containing specific protein substrate gave higher

activity than the basic protein mixture media. Growth media variation studies divulged that the optimal pH and temperature for growth medium was 7.0 and 40°C respectively. With respect to the characteristics influencing culture conditions, productivity and properties of Protease, it was considered sententious to purify and characterize the enzyme through its stability studies.

2.1 Stability Studies[14]

Test buffer used was Sodium phosphate buffer for pH 7 and 10.

2.1.1 Stability at optimum conditions: To analyze the pH stability, the Protease that was obtained from TFF concentration method was diluted with the pH 7 buffer and incubated at 60°C for 1h. Enzyme aliquot was taken after every 15 minutes and a reaction was run with Casein.

2.1.2 Stability at extreme conditions: To analyze the pH and temperature stability, TFF concentrated Protease was diluted with the buffer of pH 10 and incubated at 80°C for 15 minutes. Enzyme aliquot was taken after every 5 minutes and a reaction was run with Casein.

2.1.3 Stability at extreme pH and optimum temperature: To analyze the pH stability, TFF concentrated Protease was diluted with the buffer of pH 10 and incubated at 60°C for 1 hour. Enzyme aliquot was taken after every 15 minutes and a reaction was run with Casein.

2.1.4 Stability at extreme temperature and optimum pH: To analyze the temperature stability, TFF concentrated Protease was diluted with the buffer of pH 7 and incubated at 80°C for 15 minutes. Enzyme aliquot was taken after every 5 minutes and a reaction was run with Casein.

3. RESULTS AND DISCUSSION

3.1 Stability Studies

100% activity shown in the data was obtained from enzyme activity measured at 60°C in pH 7.0 and other residual activities were measured accordingly. Activity at extreme conditions reduced to negligible i.e. 1U/ml within 15 minutes hence was not continued for 1h. Protease was considerably stable for 1h at optimum conditions though there was a decrease in the activity observed **(Table 1) (Figures 1, 2, 3, 4)**.

Hideto Takami, 1998, extracted extremely thermostable Protease from an alkalophilic Bacillus sp. No. AH-101 and found that the enzyme production reached its maximum level of 1500 units/ml after about 24 hours in alkaline medium at pH 9.5 [15]. Similarly, Enshasy H., 2008, studied extracellular alkaline Protease of Bacillus licheniformis and said that in optimized medium conditions, maximum Protease production was estimated to be 2400 UmL^{-1} after 96 hours [16]. Joshi et al, 2011, also found that Protease was extracted from Bacillus cereus MTCC 6840 and noted that Protease production was found to be 120U/mL [17]. Ganesh Kumar et al 2004 also found the optimal pH and temperature for alkaline Protease extracted from Bacillus clausii were 11.5 and 80°C [18].

Subba Rao et al, 2009 characterised the thermo and detergent stable serine alkaline protease isolated from Bacillus circulans that exhibited optimum activity at broad temperature range of maximum of 70°C under alkaline pH environment [19,20].

TABLES AND FIGURES

Table 1: Time v/s Enzyme Activity in U/ml at 60°C and pH 7

Duration (minutes)	Blank Absorbance	Test Absorbance	Test - Blank	µmoles/ml	U/ml
0	0.434	0.227	0.207	69.00	207.00
15	0.312	0.231	0.081	27.00	81.00
30	0.31	0.237	0.073	24.33	73.00
45	0.304	0.232	0.072	24.00	72.00
60	0.323	0.252	0.071	23.67	71.00

Table 2: Time v/s Enzyme Activity in U/ml at 60°C and pH 10

Duration (minutes)	Blank Absorbance	Test Absorbance	Test - Blank	µmoles/ml	U/ml
0	0.45	0.202	0.248	82.67	99.20
15	0.311	0.237	0.074	24.67	74.00
30	0.295	0.232	0.063	21.00	63.00
45	0.284	0.242	0.042	14.00	42.00
60	0.257	0.252	0.005	1.67	5.00

Table 3: Time v/s Enzyme Activity in U/ml at 80°C and pH 7

Duration (minutes)	Blank Absorbance	Test Absorbance	Test - Blank	µmoles/ml	U/ml
0	0.4	0.202	0.198	66	79.20
5	0.35	0.21	0.14	46.67	56.00
10	0.276	0.24	0.036	12.00	24.00
15	0.232	0.23	0.002	0.67	1.33

Table 4: Time v/s Enzyme Activity in U/ml at 80°C and pH 10

Duration (minutes)	Blank Absorbance	Test Absorbance	Test - Blank	µmoles/ml	U/ml
0	0.40	0.202	0.198	66	79.20
5	0.344	0.29	0.054	18.00	21.60
10	0.294	0.251	0.043	14.33	17.20
15	0.254	0.252	0.002	0.67	0.80

Table 5: Statistical Analysis (SPSS v. 22) of Enzyme Stability Studies indicated the following results for each set of data:

Correlation: Spearman's Method using SPSS v. 22				
Table 1: Time v/s Enzyme Activity in U/ml				
1. 60°C and pH 7		3. 60°C and pH 10		
2. 80°C and pH 7		4. 80°C and pH 10		
			Time	**U/ml**
Spearman's rho	Time	Correlation Coefficient	1.000	-1.000**
		Sig. (2-tailed)	.	.
		N	5	5
	U/ml	Correlation Coefficient	-1.000**	1.000
		Sig. (2-tailed)	.	.
		N	5	5
**. Correlation is significant at the 0.01 level (2-tailed).				

Statistical Interpretation:

- Time of the test and Enzyme activity have a statistically **significant linear relationship (r = -1.00)** in each of the temperature and pH conditions viz. 60°C and pH 7, 60°C and pH 10, 80°C and pH 7 as well as 80°C and pH 10.
- The direction of relationship, for each condition of temperature and pH, is **negative.** The variables time and enzyme activity move in different directions. As time increases, enzyme activity decreases and vice-versa. It is concluded that Time and enzyme activity are **negatively correlated**.
- The magnitude or strength of the association in each of the temperature and pH condition is **highly strong**.

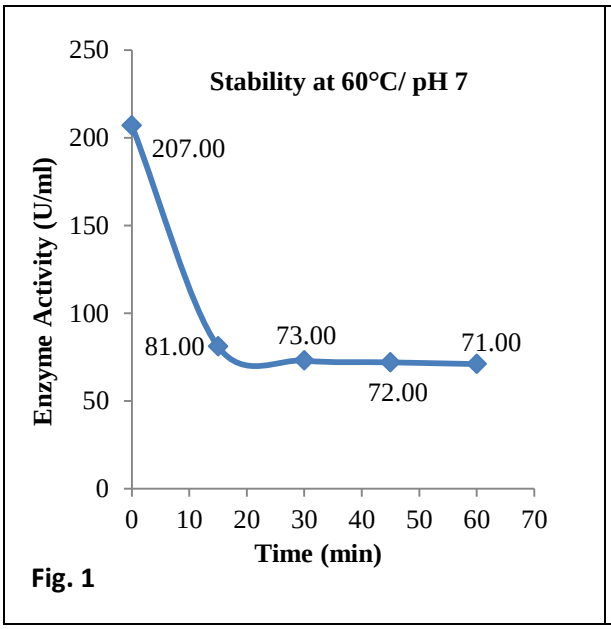

Fig. 1

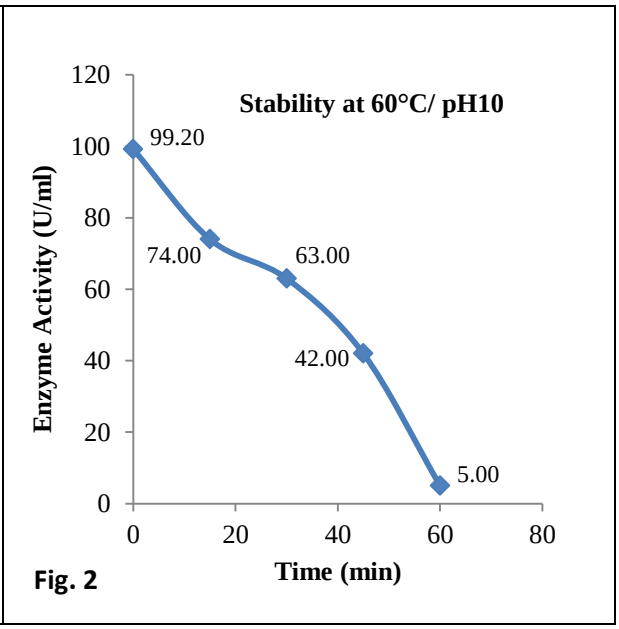

Fig. 2

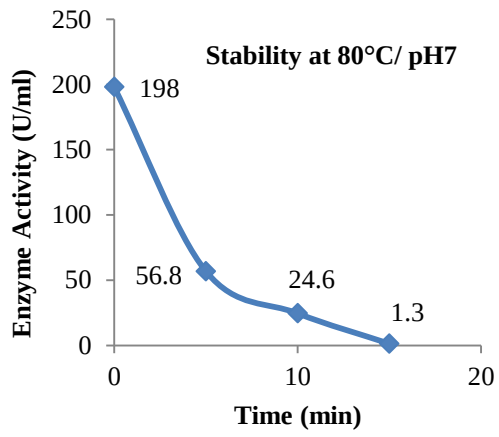

Fig. 3

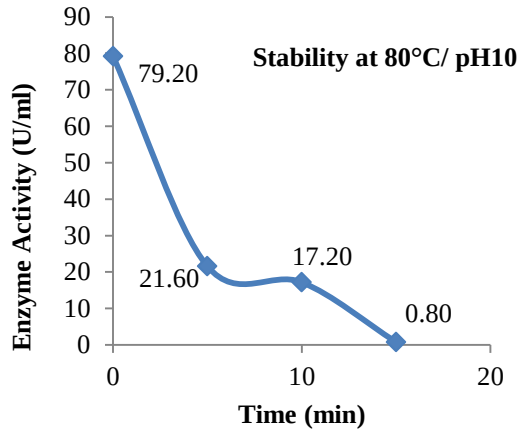

Fig. 4

Fig. 1 depicts stability of Protease from B_3 strain at 60°C / pH 7 incubated for 1h.

Fig. 2 depicts stability of Protease from B_3 strain at 60°C / pH 10 incubated for 1h.

Fig. 3 depicts stability of Protease from B_3 strain at 80°C / pH 7 incubated for 1h.

Fig. 4 depicts stability of Protease from B_3 strain at 80°C / pH 10 incubated for 1h.

4. CONCLUSION

The escalated cognizance of biocatalytic potentialities of enzymes has made feasible the progression of novel biological products in diverse industrial spheres. Exploration of new cost effective process for enzyme production by exploiting various novel sources is the basic need of today in the current competitive enzyme market. Additionally, the enzyme thermostability confers numerous advantages viz. enhanced reaction velocity, prolonged half life, diminished contamination menace and decrease in viscidity permitting higher concentration of less dissolvable materials.

In conclusion, the present work describes Protease production by moderately halophilic Bacillus safensis. The utilization of cheap substrate for enzyme production and higher purification fold with easy protocols could make this enzyme economically feasible. Collectively, the results justify the suitability of Bacillus safensis sp. for commercial production of Protease, using inexpensive materials. Some of the features of the secreted Proteases by this bacterial specie, such as optimum neutral pH (7.0), reasonable activity at high temperature up to 60°C and compatibility characteristics are credentials to future studies.

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