

## Screening and Isolation of Protease producing Marine Bacteria

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### Abstract

The study was done with an intention of screening and isolating marine bacterial strains which produce industrially important enzyme, protease. A total of twelve bacterial strains were isolated from three sampling sites along the coast of Arabian Sea. The strains were screened for the production of protease enzyme and six of the twelve strains were showed to be protease producers. Enzyme activities are affected by a number of environmental parameters. Hence, it was attempted to optimize the conditions such as pH, temperature and incubation period for maximum protein production. Strain Bb4 was found to be the best protease producer and showed maximum production at 37°C. Two strains Bb1 and Bb2 showed very high protease production at pH 9. This indicates that these two strains produce alkaline protease which has wide application in industries. The maximum protease production by most of the strains was at the third day of incubation with Bb4 and Tb4 with maximum production.

**Keywords** incubation time, marine bacterial strains, optimization, protease, temperature

### Introduction

Marine ecosystems are rich sources of both chemical and biological diversity. The actual biodiversity is still unknown to us. From relatively small number of microbes, almost 12,000 novel chemicals are isolated [1]. Hence, the potential for the discovery of novel molecules from yet-to-be discovered marine organisms is very high [2-4]. According to the available reports [5-8], marine habitats provide opportunities to discover bioactive molecules such as antibiotics, enzymes, vitamins, drugs, bio-emulsifiers etc. Marine microbes have

evolved in such a manner that they have infinite capacity to thrive in extreme habitats and produce novel metabolites when compared to microbes of other sources [9]. Because of these reasons, marine microbes are becoming an important source of many enzymes, in particular, medically and industrially important ones. Oceans are perhaps the greatest untapped sources of new products. And in oceans, microbial life is least unexplored [10-12].

Now days, there are numerous microbial products used in industries, and the world is developing fast for meeting the needs of food processing, pharma and textile industries. Though enzymes from various sources were being used for this purpose, microbial enzymes are now considered to be the most useful ones due to various reasons. Of the 4000 enzymes that are known to be used in these industries, most of them have either bacterial or fungal origin. This is due to immense diversity and the ease of production of enzymes by microorganisms.

Proteases are one of the major enzymes used in industries. Their catalytic function is to hydrolyze peptide bonds of proteins and break them into free amino acids. The commercial application of these enzymes is very wide, including their usage in detergents, leather, food and pharmaceutical industries [13-14]. There are widespread proteases in nature. But the preferred source of these enzymes to industry is microbial. This is because of their rapid growth, limited space requirement, ease of generating capability and also, they can be genetically manipulated to generate new enzymes with altered properties for various applications.

Hence, this work aims to isolate marine bacterial strains capable of producing protease enzyme, along with optimizing the media conditions in terms of pH, temperature and incubation period for enhanced production.

## Methodology

### *Sample Collection*

Bacterial strains were isolated from marine water collected from three different sites of the Arabian Sea. The sampling sites were Sanghumukham (a beach), Veli (near effluent discharge point of an industry) and Vizhinjam (a fishing harbor). Sampling was done following the standard microbial sampling techniques.

### *Enrichment and Isolation of Bacteria*

The marine water samples collected were subjected to enrichment of bacteria. For this, 1 mL of sample was inoculated in 100 mL Zobell broth. The medium was then incubated at 30°C for 2 days. For better enrichment, the culture was placed in a shaker incubator at 200 rpm. After two days of incubation, a loop full of culture was inoculated in Zobell agar and incubated at 30°C for 2 days. Well separated single colonies were maintained for further studies.

### *Screening of bacterial strains for protease production*

The isolated bacterial strains were screened for the presence of protease enzyme. Two stages of enzymatic screening were done. All the isolated strains were subjected to primary screening, while secondary screening was done for those isolates which showed enzymatic activity in primary screening. For screening, the bacterial strain was inoculated in Skim milk agar and the plates were incubated at 30°C for 7 days. Positive cultures were selected by observing the clearing around the growth.

### *Protease assay*

Casein was used as a substrate for protease assay. The broth culture of bacteria was centrifuged and the supernatant crude enzyme was collected. Half mL of this crude enzyme was combined with 2% casein in 0.1M Tris-HCl buffer with a pH of 7 at 37°C. Further, 5 mL of 5% trichloroacetic acid was added to stop the reaction. The filtrate was taken and was treated with 4.0 mL of 0.1 N NaOH and 0.5 mL diluted Folin-Cocalteau reagent. After 30 minutes of incubation, the amount of tyrosine was estimated spectrophotometrically at 670 nm. Protein was estimated using BSA as the standard [15]. One unit of protease activity is expressed as the amount

of enzyme which converts 1 $\mu$ g of tyrosine per 1min at 37°C [16].

### *Optimization of enzyme activity*

Optimization of enzymatic activity was done by using shake flask method, at various temperature, pH and incubation period for the bacterial isolates which showed positive activity for protease. The enzyme activity was checked at 3rd, 6th 9th and 12th day. The various parameters like pH (4, 7 and 9) and temperature (20°C, 37°C and 50°C) were also determined.

## Results and Discussion

Isolation and characterization of bacteria from the marine samples was done in this study. Enrichment plates showed heavy growth of bacteria. In total twelve bacterial strains were isolated, 4 from each site. The strains were named as Ab1, Ab2, Ab3 and Ab4 (Sanghumukham); Bb1, Bb2, Bb3 and Bb4 (Vizhinjam) and Tb1, Tb2, Tb3 and Tb4 (Veli).

### *Screening for protease activity*

All the isolated strains were screened for protease activity. Six of the bacterial strains showed positive results for the protease production (Figure 1).

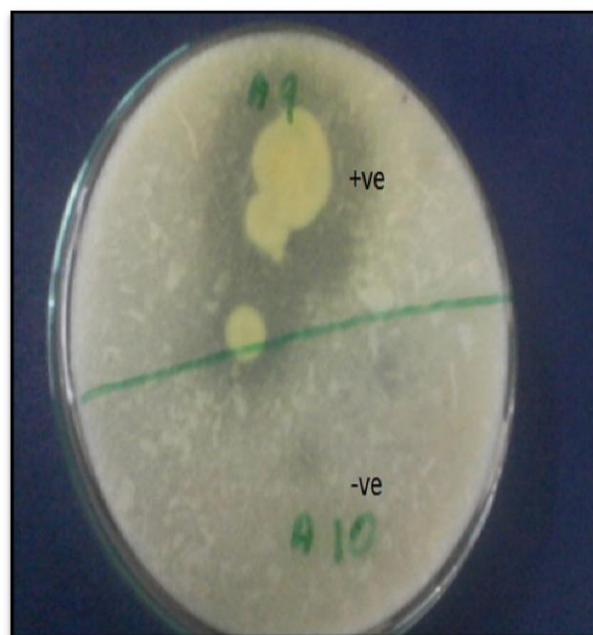


Figure 1. Clearance zone showing protease activity

### Optimization

#### pH

The microbial isolates were incubated in 3 different media with pH 4, 7 and 9; and protein production in these pH conditions was measured. Generally, the production of protease enzyme was found to be highest at neutral pH. Except in Bb2, which produced highest production of protease at pH 9, all others samples showed minimum activity at neutral pH. Bb2 showed maximum amylase activity at alkaline pH, which indicated its potential to grow and metabolize at alkaline conditions (Table 1). Since, the protease activity of strain Bb2 was found to be highest at pH 9.0, it can be identified as alkaline protease, which could be posed as a candidate enzyme for industrial applications. Ibrahim et al. [17] identified and isolated microbial alkaline protease at pH 10.

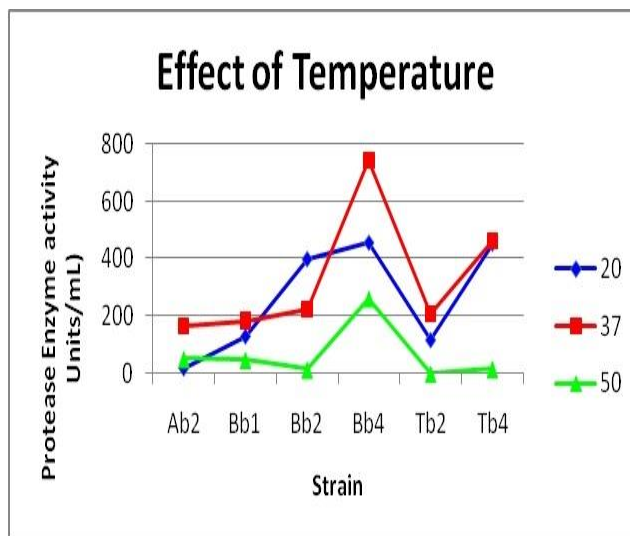
#### Temperature

In case of incubation temperature, except Bb2 (showed maximum activity at 20°C), all other isolates showed highest activity at 37°C (Table 1). Though the quantity was lower, the pattern of

**Table 1 Effect of pH and temperature on protease production**

Bacterial strains	Protease Enzyme activity (Units/ml)					
	pH			Temperature (°C)		
	4	7	9	20	37	50
Ab2	99	167	42	18	167	51
Bb1	533	185	173	129	185	48
Bb2	75	224	236	398	224	14
Bb4	344	742	2	455	742	260
Tb2	138	209	41	116	209	0.2
Tb4	449	464	21	452	464	17

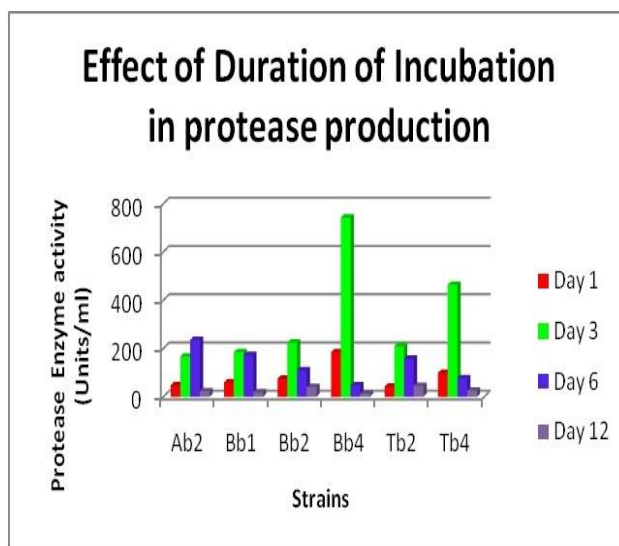
secretion of protease at 50°C was similar to that at 37°C (Figure 2). At higher temperatures, that is at 50°C, the production of all the isolates decreased when compared to the overall production. Bb4 showed maximum protease activity at 37°C which comes about 742 Unites/mL. Since protease activity was maximum at 50°C, it could be a thermoprotease. It was a very significant observation as far as industrial applications are considered. Nascimento et al. [18] isolated a thermophilic *Bacillus* species which showed maximum protease activity at 60°C.



**Figure 2. Effect of temperature on protease production**

#### Incubation time

All isolates except Ab2, showed higher enzymes production on third day of incubation. Of all the isolates, exceptionally good activity was shown by Bb4 and then Tb4 (Figure 3). This may be because of their logistic growth during this period of incubation.



**Figure 3. Effect of incubation time on protease production**

### Conclusion

In this study, bacterial strains were isolated from three different sites of Arabian Sea (Sanghumukham, Veli and Vizhinjam). These sites

are under human pressure due to various reasons. The first one was a touristic beach area, where so many people used to visit. The second sampling site was near the outlet of an industry and the third site was a fishing harbor. Because the activities in these sites were different, the microbial diversity of these areas is also different. As diversity differs their potentials will also be different. A total of 12 bacterial strains were isolated from these three sites. Of these, six strains were screened positive for protease activity. The protease activity of the bacterial strains was optimized by giving three different pH, three different temperatures and four different periods of incubation. Of all the strains, strain Bb4, which was isolated from Vizhinjam followed by Tb4, strain isolated from Veli near the outlet of industrial outlet showed exceptionally good activity.

#### Acknowledgements

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