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Antifouling activity of alkaline protease from halotolerant *Bacillus* sp. isolated from marine source

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In this study, an attempt has been made to explore the antifouling activity of the marine halotolerant bacterium, namely, *Bacillus* sp. About 10 different bacteria were isolated using the Zobell marine agar medium from the marine sediment samples that were collected from the Marina beach, Bay of Bengal, India. The growth pattern tests of the 10 bacteria were documented. Among the 10 different isolates, two exhibited the protease activity in skim milk agar. The antifouling activity was further tested against five different fouling bacteria. The antifouling activity of alkaline protease isolated from *Bacillus* sp. inferred that it exhibited pronounced inhibitory zones against fouling bacterial strains with least minimal inhibitory concentration range between 25 and 100 units/mg protein. Anticrustacean assay was performed using *Artemia salina* of alkaline protease, which showed good activity. The results suggest that *Bacillus* sp. produced an extracellular alkaline protease, which was responsible for the antifouling and algicidal activity of marine fouling organisms. This study tends to indicate that *Bacillus* sp. could be further explored for the development of new antifouling agents.

[**Keywords***:* Halobacteria; Antifouling; Protease; Algicide]

Introduction

In the marine environment, biofouling is formed by a conditioning film consisting of initial colonizer such as bacteria and microalgae, followed by macro algae and invertebrates settlement which cultivate a complex community[1]. Antifouling coatings are widely used on marine vessels and constructions, but unfortunately, they are found to stance a threat to the marine environment, especially owing to metal-based biocides. Many enzymes such as protease and amylases have been proposed as a substitute to traditional antifouling compounds. The idea of using enzymes for antifouling coatings reaches back as far as 1983 and the concept has received increased interest in the recent years[2,3].

The enzymes that claimed to hold an antifouling activity are based on their catalytic functions. The enzyme functions are related with the current knowledge about the chemistry of settlement and adhesion of fouling organisms where specific focus will be on bacteria, microalgae, invertebrate larvae, and microalgal zoospores. Two main conceptions in enzyme-based antifouling are: (i) Breakdown of adhesive components and (ii) catalytic production of repellent compounds *in-situ*. The validity of various modes of action was assessed and the groups of enzymes with the highest potential were identified by earlier studies[4].

The two types of fouling that causes problem are: (i) settlement of individual organisms and (ii) propagation of settled organisms. The former relates to macrofouling larvae (e.g., barnacle cyprids to juvenile and adult barnacles) and spores (e.g., ulva zoospores to plants), while the latter is more alarming, i.e., microfouling (e.g., bacteria and diatoms). Strategies to avert biofouling can thus intrude with the first contact amongst organisms and surfaces or stop settled organisms from developing to problematic levels, although one strategy does not eliminate the other. Enzymes may affect the settlement and adhesion of biofoulants occurs in diverse ways. Initially, they may outbreak the adhesive of settling organisms, thus averting the settlement event. Furthermore, enzymes may damage the polymers in the biofilm matrix formed by proliferating settled organisms. Moreover, enzymes possibly will catalyze the release of antifouling compounds from the exterior. These enzymes may be non-toxic or toxic and subsequently, the enzymes are produced *in-situ*. They can be much less stable than the conventional biocides, which ought to remove the bioaccumulation of detrimental chemicals[5]. The study on the

antifouling potential of the protease enzymes shows the above mechanism. The proteases hold a greater potential for broad antifouling effectiveness, which has fascinating perspectives but less scientific support for marine applications[5].

In the present study, an attempt has been made to screen alkaline protease from marine halotolerant bacteria *Bacillus* sp. for its antifouling activity. The main objectives are as follows:

(i) To determine the antifouling activity of the crude extracts of protease against biofilm-forming bacteria and

(ii) To assess the inhibitory effect against algae and crustaceans.

Such a study will enrich our knowledge in the biological ecosystem of marine halotolerant bacteria producing protease and will be useful for the growth of marine natural antifoulant coatings.

Materials and Methods

About 12 different soil sediment samples were collected from the Marina beach, Bay of Bengal, Chennai, Tamil Nadu. The soil sediment samples were spread on Zobell marine agar (ZMA) and incubated at 37 °C for 48 h. The isolated bacterial colonies were maintained on ZMA slants and used for further study.

Screening of organisms for protease production

Each isolated colony was streaked onto a skim milk agar and incubated at room temperature for 24 h. A clear zone of hydrolysis gave an indication of protease on the plates. Based on the zone of clearance, the promising bacterial colonies were chosen for further study.

Production of protease activity

The production of protease was carried out in Zobell marine broth in a shaker at 150 rpm. After 24 h, the broth was centrifuged at 10,000 rpm for 15 min at 4 °C in a refrigerated centrifuge (Eppendorf). The culture supernatant was taken as crude enzyme source which was used as a source of extra cellular protease.

Determination of total protein and protease activity

The protease activity of the culture supernatant was calculated by following the standard method[6]. Casein is used as a substrate to determine the protease activity. The reaction mixture contains a known amount of protein (50 µg) in 0.5 mL of 1% casein. The mixture was incubated at room temperature and the reaction was ended by adding 0.1% trichloroacetic acid (w/v). The undigested casein was removed by centrifugation at 5000 rpm for 15 min. The aliquots of the supernatant (0.5 mL) were taken in a separate tube and 2.5 mL of reagent was added (2.9% Na_2Co_3 and 0.3 N NaOH). Then 0.75 mL of Folin-Ciocaleau reagent (1:4 diluted with glass-distilled water) was added and incubated at room temperature for color development. The tyrosine equivalents were recorded at 650 nm in a spectrophotometer. A standard curve was constructed using tyrosine as a reference for protease measurement. One unit of protease activity is defined as the amount of enzyme liberating 1 mM of tyrosine equivalents under the assay condition.

Partial purification of protease

The proteins in the supernatant was precipitated with different concentrations (20%, 40%, 60%, 80%, and 100% saturation) of ammonium sulfate and was allowed to stand overnight at 20 °C. The resulting precipitate was collected by centrifugation at 10,000 rpm for 10 min and dialysed further**.**

Antifouling activity

Antibacterial assay: The test fouling bacterial strains, such as *Halomonas aquamarina*, *Bacillus flexus*, *Pseudoalteromonas flavipulchra*, and *Marinobacter salsuginis* were isolated from the marine substrata at fisheries harbor, Ennore, Tamil Nadu, India. The antibacterial activity of the protease isolated from *Bacillus* sp. was tested against fouling bacterial strains through agar well-diffusion method. Briefly, overnight cultures of test fouling bacterial strains were aseptically spread over ZMA plates using sterile cotton swabs. Then, wells of 6 mm diameter were made over ZMA plates and loaded with 100 µL of sample with concentrations of 25, 50, 75, and 100 units/mg protein of protease, which was prepared using sodium phosphate buffer (50 mM, pH 7.5). The plates were then incubated at 37 °C for 24 h and the zone of inhibition (mm) formed around each well was recorded. The assay was carried out in triplicates.

Algicidal activity: The algicidal activity was carried out using *Chlorella vulgaris* where different concentrations containing 25, 50, 75, and 100 units/ mg protein of protease were obtained from *Bacillus* sp. The live and dead cells were detected by using toluidine dye staining method.

Anticrustacean assay: Anticrustacean activity of crude protease extract of *Bacillus* sp. was persuaded

by using brine shrimp, *Artemia salina* larvae as a model organism[8]. Briefly, the cysts of brine shrimp were hatched in vessel containing seawater under mild aeration for 48 h. About 10 active larvae (I instar) were collected using a thin capillary glass tube from the hatching chamber and retained in small test tubes containing 10 mL of sea water with varying concentrations (25, 50, 75, and 100 units mg mL^{-1}) of protease enzyme. The medium without crude protease was used as control and the whole experimental set-up was maintained at room temperature for 24 h under the light. Thereafter, the number of larvae surviving in each test concentration was counted and the LC_{50} values were determined.

*Statistical Analysis:*The data obtained from the present study were collected in triplicate and graphs were drawn using graph pad prism.

Results

About 10 bacterial strains were isolated from the samples collected from the Marina beach (13.0500°N, 80.2824°E), Chennai, Tamil Nadu. Their morphological characteristics were studied.

Screening of protease producing bacterial strain

Among the 10 marine bacteria screened, *Bacillus* sp. was selected as it produced the highest proteolytic activity with a clear zone of hydrolysis around the bacterial strain in skim milk agar. The results of qualitative assay of proteolytic activity revealed that *Bacillus* sp. produced 13 mm clear zone in skim milk agar (Figs 1-3). This zone of hydrolysis appeared after 24 h incubation indicating protease production on the plate and this isolate was selected for further studies.

Qualitative and quantitative estimation of protease

Further, quantitative assay of protease has shown that *Bacillus* sp. produced high protease activity of 4050 units/mL/min in Zobell marine broth on third day (data not shown). It clearly showed the protease producing potential of *Bacillus* sp. Therefore, it is worthwhile in exploring this strain for large-scale protease production.

Partial purification of protease enzyme

The 48 h old culture *Bacillus* sp. was harvested by centrifugation and the supernatant was collected. The supernatant or the crude enzyme is subjected to fractionation by precipitation with 80% ammonium sulfate saturations. The fraction of proteins obtained on precipitation with 80% saturation of ammonium sulfate has shown proteolytic activity with total protein 2.5 mg/mL with specific activity of 1570 U/mg protein. Further, the dialysed sample was estimated for its protease activity which showed proteolytic activity with total protein 1.35 mg/mL with specific activity of 2250 U/mg protein.

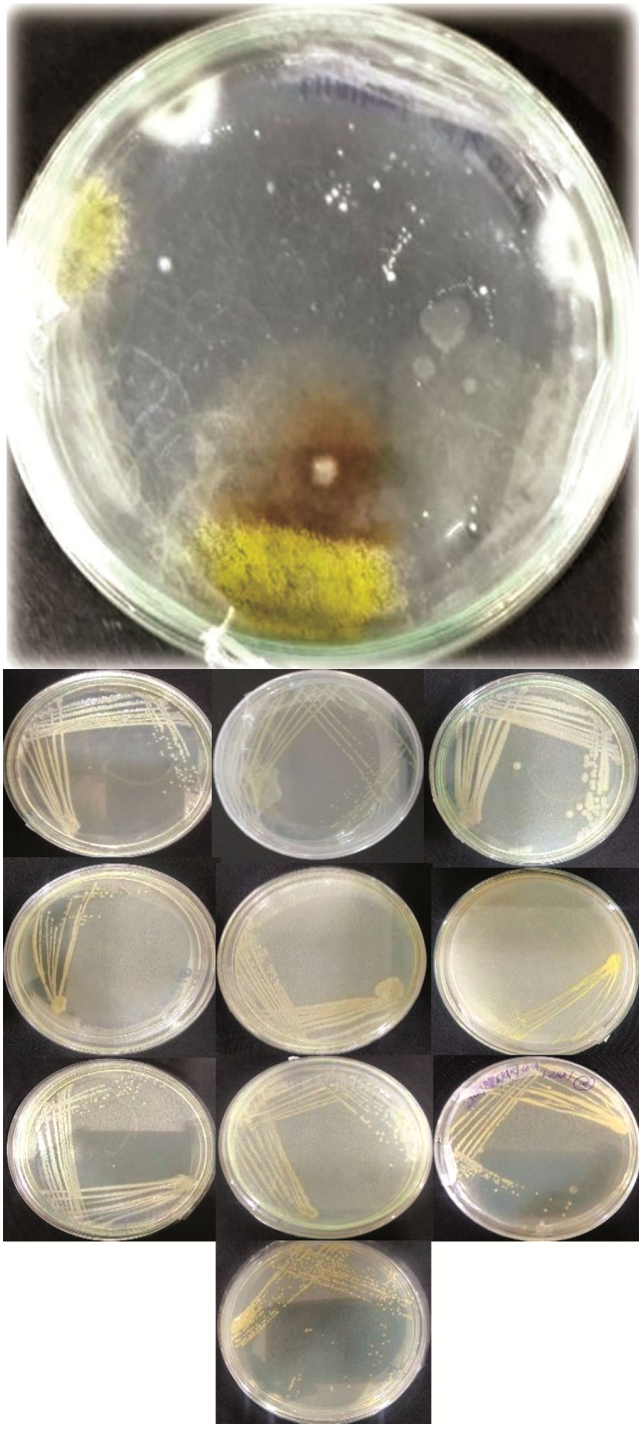

Fig. 1 — (a) Isolation of bacteria from marine sediment and (b) Isolation of 10 different bacteria isolate from marine sediment

Fig. 2 — Isolated culture of *Bacillus* sp.

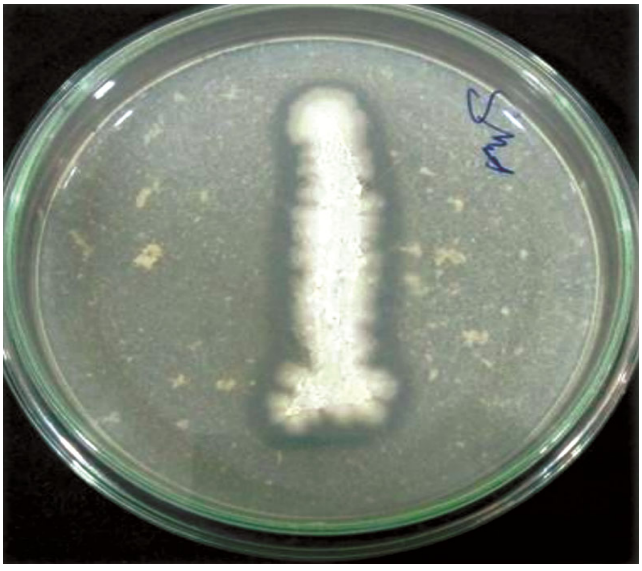

Fig. 3 — Protease activity of *Bacillus* sp. in skim milk agar after 24 h

Antibacterial assay

The antibacterial activity of partial purified protease was tested against four different fouling bacteria, namely, *H. aquamarina*, *B. flexus*, *P. flavipulchra*, and *M. salsuginis*. The zone of inhibition was found to be 17 and 18 mm against *P. flavipulchra* and *B. flexus*, whereas 12 and 8 mm against *H. aquamarina* and *M. salsuginis*, respectively (Fig. 4).

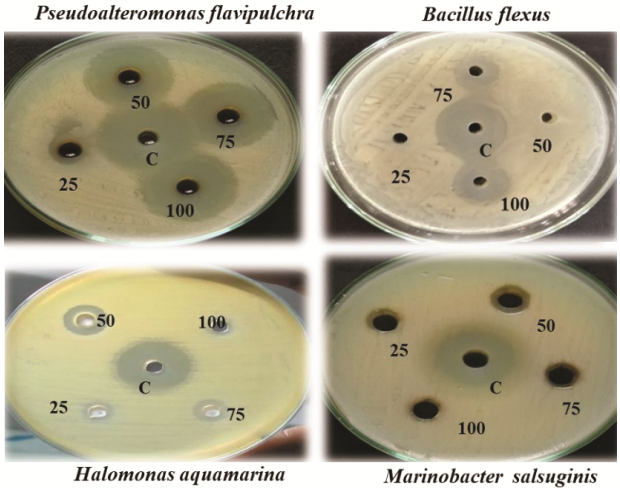

Fig. 4 — Antifouling activity against marine fouling bacteria. (a) *Pseudoalteromonas flavipulchra,* (b) *Bacillus flexus*, (c) *Halomonas aquamarina,* and (d) *Marinobacter salsuginis*

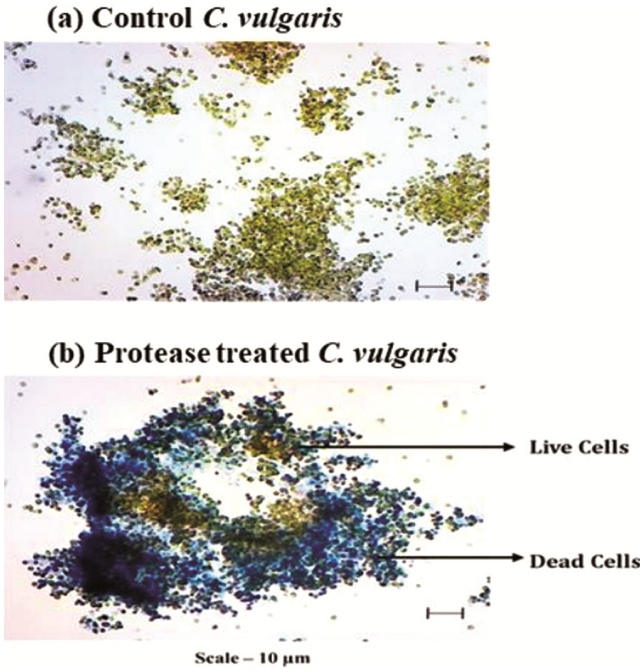

Fig. 5 — Algicidal activity of alkaline protease isolated from *Bacillus* sp. after 24 h. (a) Control *Chlorella vulgaris* and (b) protease treated *Chlorella vulgaris*

Algicidal activity

The algicidal activity of the protease of *Bacillus* sp. was determined using *C. vulgaris* in 96-well polystyrene plates. Among the different concentrations of protease tested against fouling bacterial cultures, 75 units/mg protein concentration of protease showed the best activity. The live and dead cells were detected by toluidine dye staining (Fig. 5).

Discussion

With an increasing emphasis on environmental protection, the use of enzymes particularly from extremophiles has gained significant attention[7]. Amongst the several species of bacteria, the genus *Bacillus* has always embraced distinct significance in the research field for the past 60 years, as the members of this genus are recognized to yield a huge display of compounds with varied biological assets. Exploration on marine *Bacillus* took out a huge sum of strains with assorted prospective counting on enzyme production. Nevertheless, the examination of marine microbes especially *Bacillus*'s protease production is in infancy and still they have not been fully explored in the field of antifouling. There is an incredible scope and potentiality in the identification of rare and novel organisms with various biological properties, including enzyme production from this harsh marine environment. The role of proteases is vital in numerous industrial applications as the demand for these enzymes is growing day-by-day. Hence, it is significant to identify protease producing microbial strains to counter the rising industrial demand[8].

Marine biofouling poses a serious problem that causes huge economic loss to the shipping industries. The environmental impact of using harmful biocides currently used in antifouling coatings under review process is that it often results in new restrictions and limitations. Biofouling on marine structures, especially ship hulls, is characterized by two critical events: (i) The irreversible, secondary adhesion of biofouling species and (ii) the proliferation of the microorganisms in biofilm. The most widely suggested enzyme-based approach to effective prevention of both of these events is the hydrolytic breakdown of adhesive or structural polymers based on protein and polysaccharide components. Proteases are concluded to bear the highest potential in this respect, although some protein structures may be highly resistant to proteolytic breakdown[5]. Because of this, the need for innovative and environmental-friendly solutions to prevent marine biofouling is becoming an area of primary importance.

Therefore, the present study was aimed to isolate marine bacteria *Bacillus* sp. from the Bay of Bengal and screen them for protease activity. Qualitative assay of protease has revealed that the 10 marine bacterial isolates produced protease, caseinase, and gelatinase. After screening, *B. subtilis* had high proteolytic activity, with a clear zone of 22 mm, whereas this test strain produces about 18 mm when caesin was used as the matrix with a maximum level of 10.8 U/mL. The partial purification process has resulted in 59% recovery which is similar to the earlier study. Similar to our study, a recovery of 32% was reported for alkaline protease produced by *Aspergillus niger* with a molecular weight of 38 KDa. Further Charles *et al*. in 2008 reported an alkaline protease of 42 KDa with a recovery of 39% from *A. nidulans* HA-10.[9–11]

The antibacterial assay results unveiled that the protease of *Bacillus* sp. strongly inhibited the growth of all the test fouling bacterial strains with significant ($P < 0.001$) inhibitory zones (8–18 mm) and recorded the least minimal inhibitory concentration (MIC) values (25–100µg mL^{-1}). The promising growth inhibitory activity indicated the presence of protease and correlates very well with our previous findings; wherein the extracellular polysaccharide of *B. flexusdis* played striking inhibitory activity against fouling bacterial strain[12,13].

The antifouling activity of protease enzyme against the microalgae *Chlorella* and *Artemia* was stronger even at a concentration of 75 units/mg protein. At present, the industries control biofouling by using highly toxic antifouling compounds[14,15]. Suppressing biofouling without the use of biocides that kill propagules can be done by controlling propagules adhesion, either by impeding the production of adhesive or by curtailing the strength of that adhesive once secreted. In the natural environment, microorganisms can easily biodegrade enzymes to amino acids and finally to carbon dioxide and water; therefore, it is generally accepted that enzymes are environment-friendly agents[16]. The stability of enzymes in seawater and solvent-based paints limits commercial application of enzymes as antifouling agents. It has been shown that some enzymes (like halophilic proteases) are quite stable in organic solvents[17].

The critical doses for proteases are relatively lower than those for other common antifoulants. In our experiments, the minimal effective protease concentration was about 75 U/mg protein, whereas for biocides, such as Tributyltin, the effective concentration is six-fold higher[18]. It is also reported that the inhibition capability of a serine protease (subtilisin) on the growth of a marine bacterial biofilm produced a wide antifouling activity of subtilisin and α-amylase on 16 different bacterial strains which were isolated from the food-industry processing lines[19].

In both cases, however, the antifouling effectiveness of the enzymes was explored on pure bacterial cultures and for a very limited incubation period (from 3 to 72 h), too short for assessing the actual antifouling potential of the studied enzymes for an environmental application[20]. Therefore, proteases may be useful in the development of future environment-friendly antifouling coatings.

Conclusion

This study demonstrated that the protease from *Bacillus* sp. effectively inhibited the growth of marine biofouling organisms. In the development of fouling agents, the use of protease was able to act on the ratio between proteins in the EPS components of the biofilm. Thus, it is proposed as a promising environment-friendly strategy to prevent marine biofouling. The protease enzyme is alkaline and stable in seawater and hence the chemical composition impregnated in antifouling coatings might be stable in the microenvironment where antifouling enzymes will be active. Thus, the integration of advanced coating design and enzyme technology provides a commercially viable antifouling solution.

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