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Production, isolation and partially purification of alkaline protease from marine macro algae surface associated *Bacillus* spp

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Abstract
Epiphytic bacteria of macroalgae have been reported to have main role in survival by producing inhibitory components against other potential harmful or pathogenic effects. This study was conducted to access the isolation, production and purification of alkaline protease enzyme isolated from the brown algae. These seaweeds collected from Thirumallavaram, Kollam (DT) and Kerala. Among the seaweeds only *Sargassum* spp. and *Padina* spp. were most potent. Bacterial isolation and identification were performed by standard biochemical characterization. Mostly, bacteria were belonging to class Bacillales. Protease enzyme was used in a food and therapeutic enzymes applications. In this study protease was isolated and partially purified. Before the partial purification of alkaline protease enzyme was characterized against different concentrations of *p*H, Temperature, NaCl tolerance of some selective isolates of *Bacillus* spp. Compare than *Padina* spp. associated *Bacillus* only *Sargassum* spp. associated *Bacillus* were showed the screening of higher production activity and eminence characters of partial purified enzyme.

Keywords: Epiphytic, *Bacillus* spp, Alkaline protease, *Sargassum sp.* and *Padina sp*

1. Introduction
Marine bacteria (Epiphytic) associated with nutrient rich algal surfaces and also in the marine invertebrates, have also been shown to produce antibacterial like (Novel drugs) products, which inhibit the settlement of potential competitors (Bernan *et al.*, 1997) [1]. Screening of marine bacteria isolated from the surface of marine algae and invertebrates has shown that a high percentage produce antimicrobial metabolites (Burgess *et al.*, 1999) [2]. The first antibiotic from marine bacterium was identified and characterized in 1966 (Burkholder *et al.*, 1966) [3]. The investigation was to find out screening of alkaline protease of 5 bacterial isolates from the brown seaweeds collected from the west coast of Kollam for suggesting and helpful to the antibiotic production in the pharmaceutical industries against different kinds of diseases.

2. Materials and methods
2.1. Isolation and identification of epiphytic bacteria
The characters of the potent organism were studied following the standard microbiological methods as described in Bergey's Manual of Systematic Microbiology (Holt *et al.*, 1994) [4]. Gram reaction, colony morphology characteristics were observed from 12 h old culture grown on a rotary shaker at 120 rpm, 30°C. The physiological and biochemical characters, included: Indole production, Methyl red, Urease hydrolysis, Citrate utilization, Production of Oxidase, Catalase, Acetoin (Voges proskauer).

2.2. Detection of the proteolytic zones on milk agar
Among the tested isolates for screening the bacteria to produce the alkaline protease enzyme only 5 isolates were isolated on milk agar, with wide range of proteolytic ability. BL1, BL2, BL3 BL4 and BL5 isolates were obtained from two macro algae tested for higher proteolytic activity. The highest proteolysis zones as shown in Table 1 were produced by BL1, BL2, BL3, BL4 and BL5. Only selected isolates were tested for the growth parameters of *Bacillus* spp. for the production of alkaline protease enzyme.

2.3. Effect of *p*H
The alkaline protease was produced by several Bacillus sp. have a variety of *p*H profiles. When the pH is altered below or above the optimum the activity is decreased. The effect of pH on the Bacillus spp. was determined by preparing different pH buffers (*p*H 3.5, 5.5, 7.5, 9.5 and 11.5) and enzyme assay of protease was followed using DNS method under standard conditions at 600nm.

2.4. Effect of Temperature
The bacterial stability is always depends upon the different temperature. The influence of temperature on the activity of Bacillus spp. was determined by incubating the assay reaction mixture at different temperatures (25°C, 35°C, 55°C and 75°C) and further assayed under standard conditions.

2.5. Effect of NaCl Tolerance
To investigate the effect of NaCl on *Bacillus* spp. activity, NaCl at different concentrations against *Bacillus spp.* were used as a tolerance factor. It was added in different concentrations (7%, 8%, 9%, 10% and 11%) to the assay mixture. The isolate BL4 *Bacillus* spp was performed for alkaline protease production among the tested isolates of *Bacillus* spp.

2.6. Enzyme Production
2.6.1. Production of alkaline protease
Protease production was carried out in medium containing (g/l) yeast extract, 5.0; peptone, 5.0; dextrose, 30.0; K2HPO4,

0.1; CaCl2, 0.01 *p*H was adjusted to 7.5 (Ghani *et al.*, 2013) [5]. The culture incubated overnight at 200 rpm, 37°C and then, sub cultured into a 250 ml Erlenmeyer flask containing 100 ml of the same medium and incubated at 37°C for 24 h. Cell debris and insoluble materials were removed by centrifugation at 10,000 'g for 10 min at 4°C and was used as the source of the crude alkaline protease enzyme.

2.6.2. Partial purification of protease enzyme

The supernatant was further purified to obtain the crude enzyme. The crude enzyme was further purified by ammonium sulfate precipitation and dialysis method.

2.7. Protein estimation

Protein estimation was determined according to the method of Lowry *et al.*, (1951) [6], using crystalline bovine serum albumin as standard. Then the enzyme activity was determined. One ml of enzyme was added to 2 ml of casein (1% w/v in 0.1N Glycine NaOH buffer pH 10) and the mixture was incubated for 15 min at 60°C. The reaction was terminated by adding 3ml of 10% Trichloro acetic acid and then centrifuged for 15 min at 10,000 rpm. Then 1 ml of filtrate was mixed with 5ml of alkaline copper reagent and after 15min, 0.5ml of Folin-Ciocalteau reagent was added, up on standing for 30 min the absorbance was read at 600nm. Similarly blank was carried out by replacing enzyme with distilled water. One unit enzyme activity is defined as the amount of enzyme that releases 1µg of tyrosine per ml per min under the assay conditions.

3. Results

Macro algae samples were collected at Kollam west coast (Fig 1). Two species were identified at [*Sargassum spp* and *Padina spp*] CMFRI, Ramanathapuram. Isolation and identification of colonies were performed by standard biochemical characterization in the laboratory (Table 1 and Fig 2). Proteolytic activity was performed based on the zone of inhibition (Table 2 and Fig 3). Effect of *p*H on *Bacillus* spp. is shown (Table 3). The alkaline protease enzyme was relatively stable and at lower *p*H it was observed that activity was low at *p*H 3.5-5.5. At *p*H 7.5-9.5 there was a sharp and sudden increase in the activity which makes the enzyme more stable at alkaline *p*H. The *p*H stability is shown (Fig 4). At *p*H 11.5 over a time of 1 h the *Bacillus* spp was relatively stable. At temperature ranging between 25-35°C, the activity was high, while the optimum temperature was at 45°C. There was a sharp and steady decrease in activity from 55-75°C. It was observed that, the higher the temperature the lower the activity. The thermo stability of *Bacillus* spp. at various temperatures is shown (Table 4 and Fig 5). The NaCl tolerance was determined the stability and activity on *Bacillus* spp. of marine brown algae. The strong activity was observed at starting stages of 6-7% and stable conditions were observed at 8%. The decreasing conditions were observed at 9-10% of NaCl concentrations shown (Table 5 and Fig 6). Screening for the production of alkaline protease enzyme was performed before all the isolates were screened for the growth parameters, also *Bacillus* spp BL4 was selected for the production of alkaline protease enzyme. Then the alkaline protease enzyme partially purified by ammonium sulphate precipitation and dialysis method. Protein determination was performed by SDS-PAGE. Partial purified protein was analyzed and the protein band occurred as a single band. The estimated molecular weight for purified protease was 45.0 KDa.

Table 1: Morphological, physiological and biochemical characteristics of *Bacillus spp*.

S. No	Parameters	Characters
1.	Gram staining	Positive
2.	Motility	Positive
3.	Oxidase	Positive
4.	Catalase	Positive
5.	Urease	Positive
6.	Hemolysis	Variable
7.	Indole	Positive
8.	MR	Positive
9.	VP	Positive
10.	Citrate	Positive
11.	Proteolysis	Positive

Table 2: Proteolytic activity of *Bacillus* spp on Skim milk Agar

S. No	Zone of Inhibition on Skim Milk Agar
BL1	20±0.2
BL2	22±0.4
BL3	23±0.4
BL4	25±0.3
BL5	21±0.1

Table 3: Effect of *p*H on *Bacillus* spp

S. No	3.5	5.5	7.5	9.5	11.5
BL1	0.5	0.4	0.4	0.2	0.1
BL2	0.8	0.8	0.3	0.2	0.0
BL3	1.0	0.8	0.5	0.1	0.1
BL4	1.0	0.9	0.7	0.3	0.1
BL5	1.0	0.7	0.3	0.1	0.0

Table 4: Effect of Temperature on *Bacillus* spp

S. No	25°C	35°C	55°C	75°C
BL1	1.0	0.8	0.5	0.3
BL2	0.9	0.9	0.3	0.2
BL3	0.9	0.7	0.4	0.2
BL4	1.1	1.0	0.6	0.4
BL5	0.8	0.5	0.3	0.3

Table 5: Effect of NaCl on *Bacillus* spp

S. No	7%	8%	9%	10%	11%
BL1	0.5	0.4	0.2	0.1	0.1
BL2	0.2	0.2	0.1	0.1	0.0
BL3	0.5	0.4	0.3	0.1	0.0
BL4	0.8	0.6	0.5	0.2	0.2
BL5	0.3	0.3	0.1	0.1	0.1

Fig 1: *Sargassum* spp and *Padina* spp

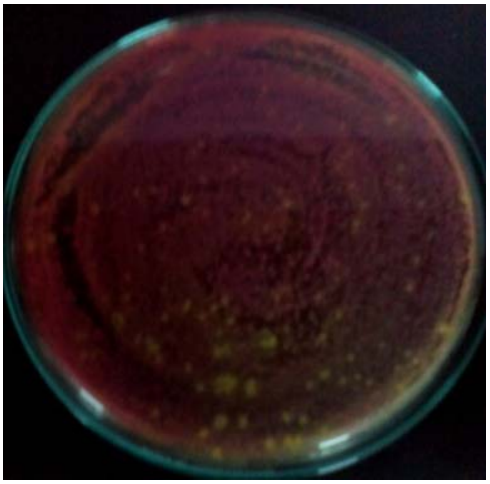

Fig 2: Colonies of *Bacillus* spp on MYP agar

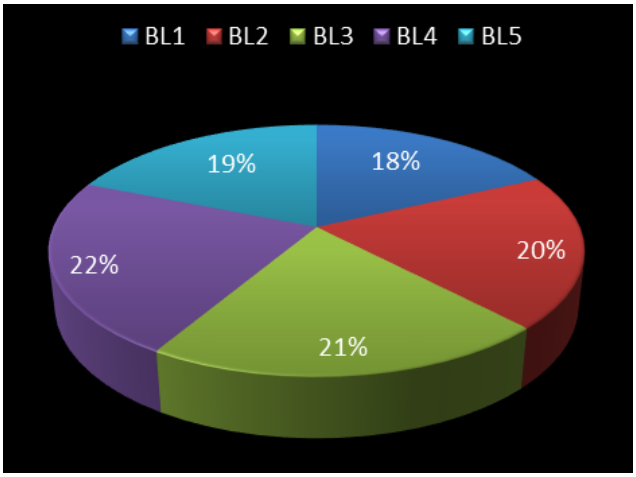

Fig 3: Zone of Inhibition produced by *Bacillus* spp on Skim milk agar

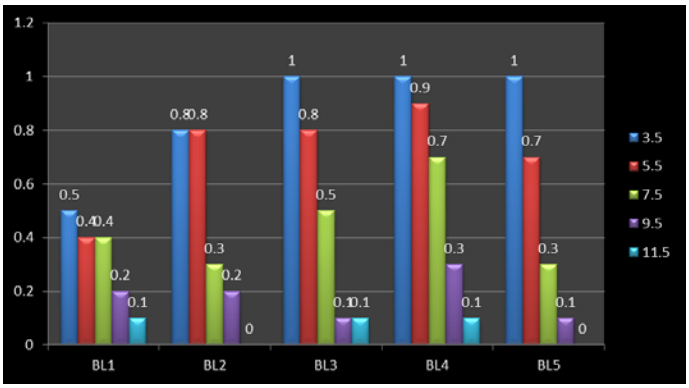

Fig 4: Effect of *p*H on *Bacillus* spp

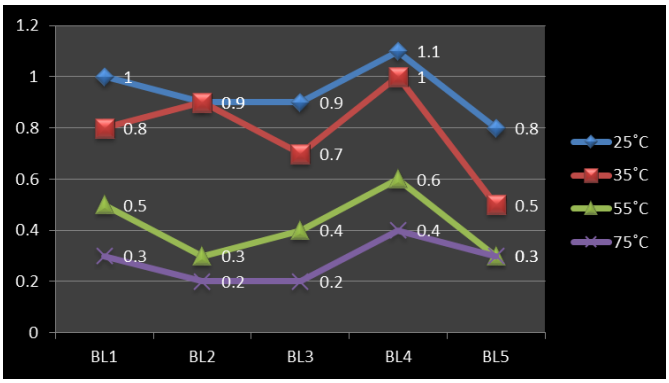

Fig 5: Effect of Temperature on *Bacillus* spp

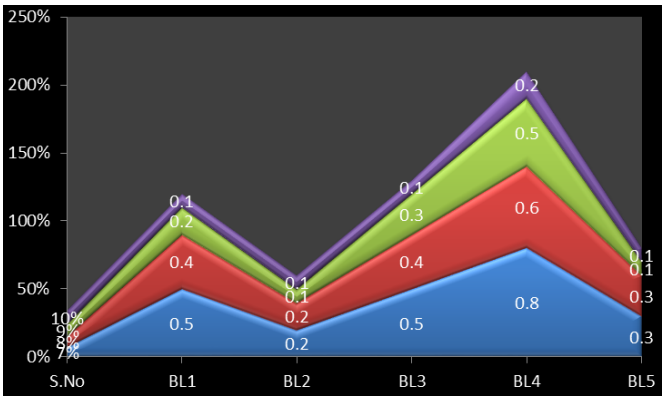

Fig 6: Effect of NaCl on *Bacillus* spp

4. Discussion

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from microbes, many based on their use in traditional medicine. However, an increasing role has been played by microbes in the production of antibiotics and other drugs for the treatment of some serious diseases.

The similar results of marine bacterial isolation from the macro algae by Ramalingam and Amutha (2013) [7] also reported that screening and antibacterial possessive bacteria were isolated from the marine red seaweeds against poultry and cattle associated pathogenic microorganisms.

Same results were observed on the growth effect of *p*H on *Bacillus licheniformis* that all the isolates have immense ability to grow at wide *p*H range therefore, proteases produced by *B. licheniformis* isolates (Ghani *et al.*, 2013) [5].

In the present study *Bacillus* spp Gram positive bacteria showed proteolytic activity compare than other bacterial strains of *Bacillus* isolates. Okazaki and Okami (1972) [8] also reported a similar screening the fungal spp from marine sediments and these observation indicated that the antibiotic component of Gram positive bacteria produce as bioactive compounds.

5. Conclusion

Macro algae such as *Sargassum* and *Padina* spp., among all those isolates, the epiphytic bacterial isolates BL3 and BL4 had higher activity compare than other bacterial strains in proteolytic screening (23mm and 25mm) test. The epiphytic bacterial strain had moderate activity among the all growth characterization. From the present study, it is cleared that the bacteria associated with the seaweeds are having the antibiotic

compounds and they may be helpful and used to medicine (antibiotic) production in pharmaceutical industries for treating the various diseases.

6. References

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