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Screening and isolation of protease producing bacteria from *Leiochrinus nilgirianus*

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Abstract
Protease enzymes are ubiquitous in nature and usually found in all living organisms. The present study was conducted for screening bacteria with the ability to produce extracellular proteases. This study was conducted to screening and isolation of protease from bacteria present in the gut of *Leiochrinus nilgirianus*. Based on experiments, three bacterial strains produced clear transparent zone into casein agar plate were identified as protease producing bacteria. Different culture parameters such as pH and temperature were optimized for enzyme production. According to the morphological and biochemical tests, the isolated strains were identified as *Enterobacter sp.*, *Bacillus sp.* and *Aeromonas* sp. The first strain *Enterobacter sp.* showed high potentiality for maximum protease production (0.29 U/ml) at pH 7.0 after 24 h of incubation at 40°C in a medium containing 1.0% casein. The scavenging action of *Leiochrinus nilgirianus* on sooty mould deposition on coconut palms is due to the presence of protease from *Enterobacter sp.* The present information of protease activity from bacteria present in the gut of *Leiochrinus nilgirianus* could assist the future attempts to unravel the scavenging action of insect and will provide the much needed impetus to agricultural development.

Keywords: protease, bacteria, enterobacter sp, screening, scavenging and characterization

Introduction

Leiochrinus nilgirianus, as a sooty mould scavenger, the specially aided adaptive leg features of *L. nilgirianus* with bristle-like hairs on the undersurface of tarsomeres probably aid in proper adhesion on sooty mould laden plant surface and well developed tarsal claws that perhaps assist swift movement on fungus-laden leaflets. *In situ* habitat conservation of *Leiochrinus nilgirianus* would help to reduce the sooty mould at no cost in the most natural and eco-friendly manner avoiding chemical management options and other expensive methods. Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 Billion by 2012 (Deng *et al*., 2010). They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al*., 2007 and Jellouli *et al*., 2009) [1, 2] Sources of proteases include all forms of life, that is, plants, animals, insects and microorganisms. Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by fungi. Proteases having pH optima in the range of 7.0 or around are called neutral proteases. Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kocher and Mishra, 2009) [3]. Screening of proteases producing bacterial species from different ecological environments can result in isolation of new alkaline proteases with unique physiochemical characteristics (Singh *et al*., 1999). One of the most important characteristics that determine the environment suitability of proteases is their requirement of high pH for optimum enzyme activity. In this paper, aimed to isolate newer source of extracellular protease from the bacteria present in the gut of *Leiochrinus nilgirianus* to potential application of the proteases for fungal degradation in palm tree.

Materials and Methods

Sample collection and preparation of crude extract

Samples were collected from CPCRI, Kayamkulam and species was identified as *Leiochrinus nilgirianus***.** The whole organism was grind with Tris - HCl buffer (pH 7.4, 0.1 M) using a pestle and mortar. The sample was centrifuged for 10 min at 10000 *g* at 4°C. The resultant supernatant was separated and stored in 2.0 ml vials at -20°C.

Isolation of protease producing bacteria

The techniques used for isolation of bacteria were serial dilution and spread plate method. The serial dilution (10 -1 to 10 -6) of crude extract were carried out. 0.1 ml of each aliquot was spread on skim milk agar (1%) plate at temperature 37ºC for 48 hr. The zone of hydrolysis was noted for each sample. The colony showing highest zone of inhibition was selected for further study. The colony was grown on nutrient agar plate repeatedly and preserved on nutrient agar slant at 4ºC. Based on the morphological and biochemical tests the bacterial isolate was identified (Sneath *et al*, 1986).

Identification of bacteria

The identification of bacteria was carried out by morphological studies i.e. staining including Gram staining, motility test, acid fast test, endospore staining. Cultural characterization on agar plates like colony morphology that is shape, size, margin, elevation, opacity, texture and pigmentation and also growth in broth and biochemical test includes catalase test, oxidase test, carbohydrate fermentation test, indole, methyl red, citrate utilization test, Voges Proskauer test, H_2S production test, Starch hydrolysis test, urease production test, nitrate reduction test (Aneja K R).

Preparation of casein solution

Casein was used as substrate. It was prepared from alkali soluble casein which was dissolved in 10 ml distilled water. The insoluble portion was dissolved by addition of the alkali. The pH was adjusted to 8.0 with 0.1 M sodium hydroxide.

Crude enzyme preparation

The protease producing bacterial colony was inoculated in casein broth medium. It was incubated at 37ºC for 48 hrs. Using Whatmann No.1 filter paper cultured medium was ++filtered aseptically in laminar air flow. The filtrate was subjected to centrifugation at 10,000rpm for 10 minutes to remove unwanted particles. The supernatant was used as crude enzyme preparation for further studies.

Protease activity assay

To study proteoloytic activity, supernatant was used as enzyme source. 1% casein in 0.1 M phosphate buffer and pH 7.0) was used as substrate.1ml enzyme and substrate was incubated at 50ºC for 60 min. To stop the reaction 3ml Trichloroacetic acid was used. One unit of protease activity was defined as the increase of 0.1-unit optical density at 1 hr incubation period. Then it was centrifuged at 5000 rpm for 15 min. From this, 0.5ml of supernatant was taken, to this 2.5ml of 0.5 M sodium carbonate was added, mixed well and incubated 20 min. Then it was added with 0.5ml of folin phenol reagent and the absorbance was read at 275 nm using Spectrophotometer (Bharat Pokhrel *et al*, 2014).

Effect of pH on enzyme activity

To study the effect of pH culture media pH was adjusted using different pH buffer ranging from 5.8 -8.0. It was incubated at 37oC for 48 hrs. Enzyme activities was determined by standard enzyme assay.

Effect of temperature on enzyme activity

To study the optimum temperature where an enzyme shows its maximum activity the substrate with crude enzyme were exposed to different temperatures between 10ºC to 100ºC. Enzyme activities were determined by standard enzyme assay.

Statistical analysis

The data were analyzed and expressed as means ± SD. The variance was analyzed using oneway ANOVA (SPSS.ver.22).

Results

Isolation of protease producing bacteria from *Leiochrinus nilgirianus*

Protease producing bacteria were isolated from *Leiochrinus nilgirianus* and cultured between 1-7 days on different growth media. Each isolate was assigned a code depending on the culture media. Total three protease producing bacteria (LGB1 to LGB3) were isolated from *Leiochrinus nilgirianus* and sub-cultured to obtain pure cultures.

Screening of Proteolytic activity

Protease activity was observed from the zone of hydrolysis observed on agar surface mentioned in Table 1. The first strain *Enterobacter sp* showed high potentiality for maximum protease activity (21mm) at pH 7.0 after 24 h of incubation at 40°C (Figure 1). For further study the *Enterobacter sp* was considered and maintained by repeated sub culturing.

Table 1: Showing zone of inhibition (mm)

Sl. No	Isolates	Zone of Inhibition(mm)
1	LGB1	21±0.01
2	LGB2	10±0.02
3	LGB3	8±0.01

Fig 1: Primary screening of protease producing bacteria

Identification of bacteria

According to the morphological (Table 2, 3) and biochemical tests (Table 4), the isolated strains were identified as *Enterobacter sp.*, *Bacillus sp.* and *Aeromonas sp*. The LGB1 from *L. nilgirianus* were showed the clear zone formation by producing amylase which indicates the positive result (Figure 2).

Table 2: Colony characters on nutrient agar plate

Size	Shape	Edge	Elevation	Opacity	Texture	Pigmentation
Big	irregular	lobate	flat	opaque	Rough	white

Table 3: Result of Staining of LGB1

Sl. No	Staining	Result
1	Gram Staining	Positive
2	Endospore Staining	Positive
3	Motility	Motile

Table 4: Biochemical characterizations: various biochemical tests

Sl. No	Biochemical Test	Result
1	Indole Production Test	Negative
2	Methyl red Test	Negative
3	Voges Proskauer Test	Positive
4	Citrate utilization Test	Positive
5	H_2S Production Test	Negative
6	Urease Test	Positive
7	Catalase Test	Positive
8	Oxidase Test	Negative
9	Starch Hydrolysis Test	Positive
10	Nitrate reduction Test	Positive
11	Gas Production from glucose	Negative

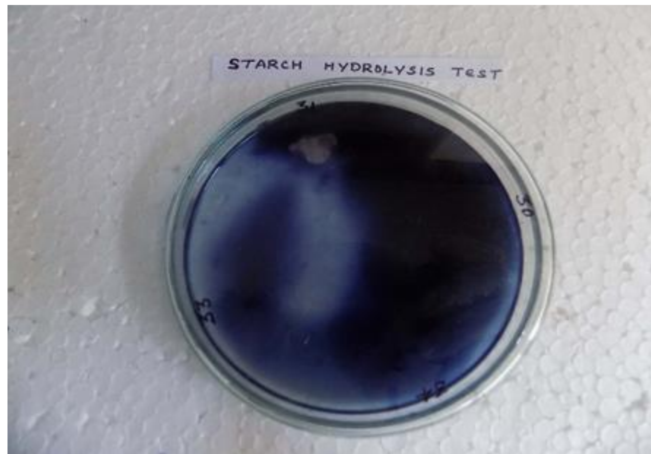

Fig 2: Starch Hydrolysis Test

Protease activity assay

Tyrosine standard curve was plotted to measure the enzyme activities of protease. The 0.845 µg/ml of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity (Figure 3).

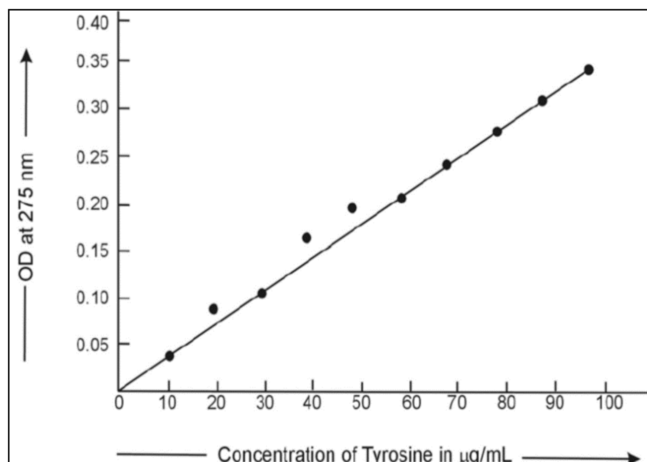

Fig 3: Tyrosine standard curve

Effect of pH on enzyme activity

To study the effect of pH culture media pH was adjusted using different pH buffer ranging from 5.8 -8.0. It was incubated at 37°C for 48 hrs. Enzyme activities was determined by standard enzyme assay. The maximum activity of enzyme was found at pH 7.4.

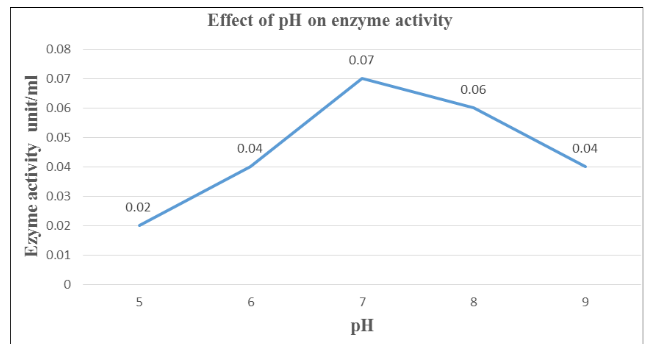

Fig 4: Effect of pH on Protease activity

Effect of temperature on enzyme activity

The protease activity is relatively stable in the temperature range 60-70°C and retains 85.2% of its activity at 60°C. In present study the maximum activity was found at 60°C.

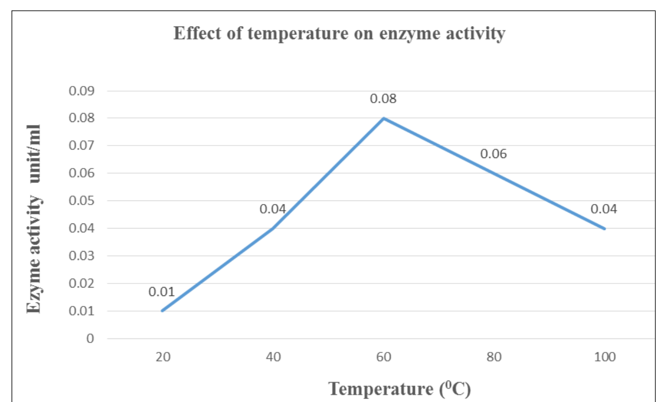

Fig 5: Effect of temperature on protease activity

Discussion

In the present study three bacterial isolates (*Enterobacter sp.*, *Bacillus sp.* and *Aeromonas sp*) shown proteolytic activity. Out of this, bacterial colony showing maximum proteolytic activity was studied further. The most active protease producer was identified as *Enterobacter sp.* Various physiological factors were studied like effect of pH and temperature. It is Gram positive, motile, catalase positive, spore former indole negative, VP positive, methyl red negative. The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Increase in pH shows increase in enzyme activity. In current study, the maximum activity of protease enzyme in *Leiochrinus nilgirianus*, was found at pH 7.4 (0.07 unit/ml). Maximum activity at alkaline pH 9 was reported by Odu *et al.* (2012) [5]. In literature it was shown that the enzyme also gave high activity in the alkaline pH range 6.0-9.0 (Sevine *et al.* 2011) [6] Abebe *et al* (2014) [7] reported optimum pH for protease producer was pH 8-10.

The maximum activity of enzyme shows as thermostable and this property can be exploited in detergent application. It also shows better result of washing with detergent (Sidra *et al.* (2006) [8]. In present study the maximum activity of enzyme in *Leiochrinus nilgirianus,* was found at 60°C (0.08 unit/ml). The optimum temperature was 60°C as also reported by Sidra *et al.* (2006) [8]. It was found that enzyme activity increases with rise in temperature reaches to maximum at 60°C. Enzyme activity was stable with temperature within range of range of 40°C to 70°C. Enzymes was also still active at 80°C. In literature, optima

temperature has been reported between 30-70ºC for *Bacillus sp.* protease in Sevine *et al*. (2011) [6]. Optimum temperature for protease of *B. lichenformis* UV-9 was found to be 60ºC by Muhammad *et.al* (2013) [9]. Abebe *et al* (2014) [7] reported optimum temperature for proteolytic activity of protease producing bacteria was 37ºC - 50ºC. Abebe *et al* (2014) [7] reported optimum temperature for proteolytic activity of protease producing bacteria was 37ºC -50ºC.

Conclusion

In this present study *Leiochrinus nilgirianus* shows presence of protease producers. The most effective protease bacteria were screened and identified as *Enterobacter sp*. Various physiological factors were studied like pH and temperature. Keeping in view about use of protease enzyme it can be harnessed for biotechnological processes. The scavenging action of *Leiochrinus nilgirianus* on sooty mould deposition on coconut palms is due to the presence of protease from *Enterobacter sp.* The present information of protease activity from bacteria present in the gut of *Leiochrinus nilgirianus* could assist the future attempts to unravel the scavenging action of insect and will provide the much needed impetus to agricultural development.

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