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Characterisation of Acid Protease-Producing Bacterium *Micrococcus luteus* from the Pitcher Fluid of *Nepenthes distillatoria* L.

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Abstract

Nepenthes distillatoria L., a carnivorous plant endemic to Sri Lanka, produces pitcher fluid, rich in digestive enzymes. Despite its apparently hostile conditions, the pitcher fluid harbours a diverse and unique microbial community. In this study, bacteria were isolated from the open pitcher fluid of *N. distillatoria* collected at two sites in distinct locations in Kalawana, Rathnapura district, in Sri Lanka. From twelve samples, twenty-four bacterial isolates were obtained and subjected to acid-protease enzyme assay using an agar plate method. Relative enzymatic activity was quantified for all the isolates that produced positive results. Notably, one isolate displaying significant acid protease activity was identified as *Micrococcus luteus* based on 16S rRNA sequence analysis. Therefore, this study revealed the pitcher fluid of *N. distillatoria* supports a distinct bacterial community with potential industrial applications, highlighting its value as a promising source for bioprospecting.

Keywords: Bioprospecting, carnivorous plants, enzymes, microcosms, phytotelmata

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Introduction

Carnivorous pitcher plants are characterised by a set of remarkable features collectively termed the carnivorous syndrome (Dkhar et al., 2020). That enables those plants to attract their prey, capture and digest them, and the subsequent absorption of dissolved nutrients to compensate for poor nutrient availability in their native habitat. The carnivorous plant, *Nepenthes distillatoria* L., is endemic to Sri Lanka, and it is the sole representative of the genus *Nepenthes* in Sri Lanka (Senevirathna et al., 2019). This evergreen, climbing shrub usually grows in waterlogged open scrub, along road embankments and other cleared areas, and also in forests from sea level to 700 m (IUCN, 2000).

Nepenthes pitchers arise as modified leaves that can act as passive traps to capture insects (Chan et al., 2016; Gilbert et al., 2020).The pitchers of *Nepenthes* contain a "pitcher fluid" to attract insects and other small invertebrates. It is generally acidic in nature and, depending on the species' pH generally ranges from 2-6 (Takahashi et al., 2005). The unopened pitcher fluid is a colourless liquid. However, after the pitcher becomes open and exposed to the surrounding environment, the liquid's composition changes accordingly due to the presence of trapped insects and other environmental contaminants.

The fluid is composed of a mixture of enzymes generated by the plants themselves, and with the contribution of inhabitant microorganisms. Those include nepenthesins, esterases, phosphatases, ribonucleases, chitinases, and different glycosyl hydrolases, such as β-D-glucosidases and β-D-glucosaminidases (Kanokratana et al., 2016,Takahashi et al., 2005).Those enzymes are responsible for the digestion of captured insect prey. Moreover, *Nepenthes* pitcher fluid also consists of several antimicrobial and anti-fungal compounds (Chan et al., 2016).

Nepenthes pitchers are regarded as a unique microenvironment with extreme conditions characterised by their highly acidic nature and hydrolytic enzymes in the presence of antimicrobial agents (Kanokratana et al., 2016). Therefore, *Nepenthes* pitcher fluid is a habitat for unique microbial taxa with specialised metabolic capabilities. The microbial community of *Nepenthes* pitcher includes bacteria, fungi, and protozoa (Bittleston et al., 2018). This study aimed to characterise *Micrococcus luteus* isolated from the pitcher fluid of *N. disillatoria* collected from Kalawana, Sri Lanka.

Methodology

Sample collection

Pitcher fluid samples were collected into sterile plastic bottles from two different locations in Kalawana, Rathnapura district in Sri Lanka (Site 01- 6°32'48"N 80°20'25"E, Site 02- 6°32'41"N 80°20'59"E). Six samples were collected from one location, labelled, and transported to the laboratory in an ice-filled container. Upon arrival at the laboratory, the pH of the samples was recorded using a pH meter.

Isolation of bacteria

The serial dilution method described by Koch, (1883) was used to isolate bacteria from pitcher fluid. Under aseptic conditions, from each serially diluted sample (10^{-1},10^{-2},10^{-3}), 100 µL was pipetted out and spread evenly on fresh nutrient agar (NA) plates using a sterile spreader. Plates were sealed with parafilm and incubated at room temperature (28± 2 °C) for 48 hours. Bacterial colonies were further purified by repeated streaking on fresh medium.

Acid protease plate assay

Bacterial isolates were inoculated into skimmed milk agar medium with the following composition: 14 g skim milk powder, 1.25 g yeast extract, 0.5 g dextrose, 2.5 g casein, and 5 g of agar in 500 mL of distilled

water, pH=5 (Sharma et al., 2015). After 48 hours of incubation, the presence/absence of a clear zone around bacterial colonies was observed.

The relative enzymatic activity was calculated using the following equation. The index of relative enzyme activity value of 1.0 or greater was classified as having significant enzyme activity.

$$RA = \frac{\text{Total diameter of clear zone} - \text{diameter of colony}}{\text{Diameter of colony}}$$ (Verma & Garg, 2018)

Biochemical characterization of bacteria

A series of biochemical assays, including Catalase, Methyl Red (MR), Voges-Proskauer (VP), gelatinase, KOH, and amylase tests, were conducted following established standard protocols outlined by the American Society for Microbiology.

Molecular characterization

Bacterial genomic DNA extraction was carried out using HiPurA® bacterial genomic DNA Purification Kit, according to the method described in their instruction manual. The polymerase chain reaction for bacteria was carried out according to the method described by More. (2021). The bacterial DNA barcode region, 16SrRNA, was amplified using two universal bacterial primers, 27F forward primer (AGAGTTTGATCCTGGCTCAG) and 1492R reverse primer (GGTTACCTTGTTACGACTT) under the conditions**;** Initial denaturation at 94 ^{0}C for 4 min followed by 30 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 55 ^{0}C for 1 min, extension at 72 ^{0}C for 4 min final extension 72 ^{0}C for 8 minutes. Amplified products were subjected to Sanger DNA sequencing using the same primer pair. The maximum likelihood phylogenetic tree was prepared using the RAxML-HPC BlackBox (8.2.12) tool in the CIPRES online resource.

Results

Isolation of Bacteria

The habit of *N. distillatoria* was documented and is presented in Figure 01. The image shows (a) the specialized pitcher structure responsible for prey capture, (b) the pitcher fluid collected from an open pitcher, which serves as the digestive medium, and (c) the intact *N. distillatoria* plant illustrating the natural growth habit. These observations confirm the presence of functional trapping mechanisms typical of carnivorous plants. A total of 24 bacterial isolates were isolated from both sampling sites.

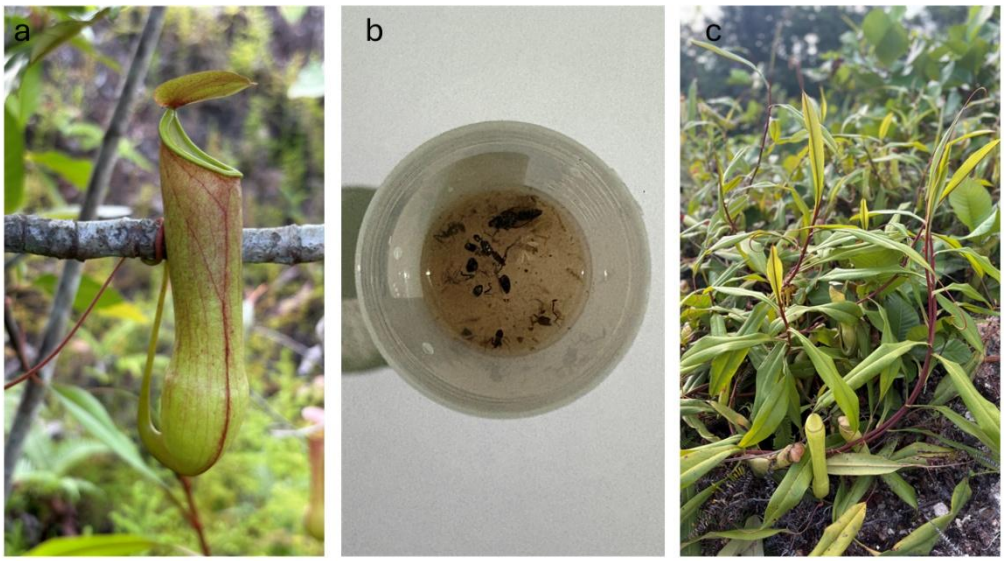

Figure 1: a-*N. distillatoria pitcher b- Pitcher fluid collected from open pitcher c- N. distillatoria plant in natural habitat*

All the samples showed acidic pH ranging from 2.1 to 6.0. (Average = 3.29 SD=1.2). Therefore, it is confirmed that the pitcher fluid is generally acidic in nature, and it is favorable for acidophilic microorganisms.

Acid protease activity

The isolate L1S2B5 tested showed the mean total diameter of 1.65 ± 0.14 of halo zones due to protease activity and mean colony diameter of 0.65 ± 0.1, therefore calculated RA 1.53. (data reported as mean ± std dev., n=4) (Figure 2).

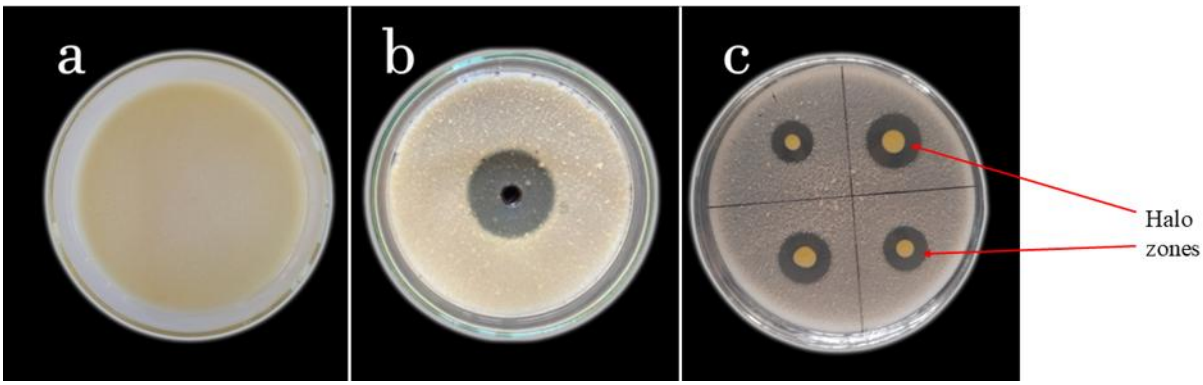

Figure 2: *Formation of clear-zone around bacterial isolate L1S2B5 after 2 days of incubation of skimmed milk*

Biochemical test, molecular and morphological characterisation of L1S2B5

Microscopic observations revealed the presence of Gram-positive, coccus-shaped bacterial colonies, characterized by their spherical morphology and uniform arrangement under the light microscope at 10 × 100 magnification (Figure 03). The cells appeared distinct and clearly delineated against the background, confirming their relatively small size, typical of Gram-positive cocci.

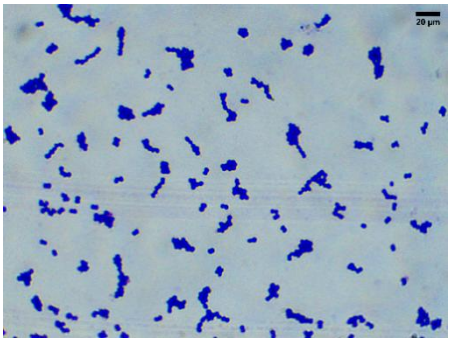

Figure 3: *Gram-positive coccus-shaped bacterial colonies observed under light microscope (10 ×100, scale bar 20µm)*

Among the biochemical assays performed, only the bacterial isolate showed a positive response for the Catalase test, indicating that the isolate produces the enzyme catalase. All other tests, including Methyl Red (MR), Voges-Proskauer (VP), gelatinase, KOH, and amylase, were negative, suggesting the absence of the corresponding enzymatic activities in this isolate. According to Bergey's manual of bacteriology, *Micrococcus luteus* is a Gram-positive, non-motile, coccoid bacterium, usually arranged in tetrads. It is catalase-positive, oxidase-positive, and generally non-pathogenic.

Molecular characterization of L1S2B5

The phylogram generated for the isolate L1S2B5, with closely related taxa, is shown in Figure 04. According to the phylogram (Figure 04), L1S2B5 can be identified as *Micrococcus luteus*.

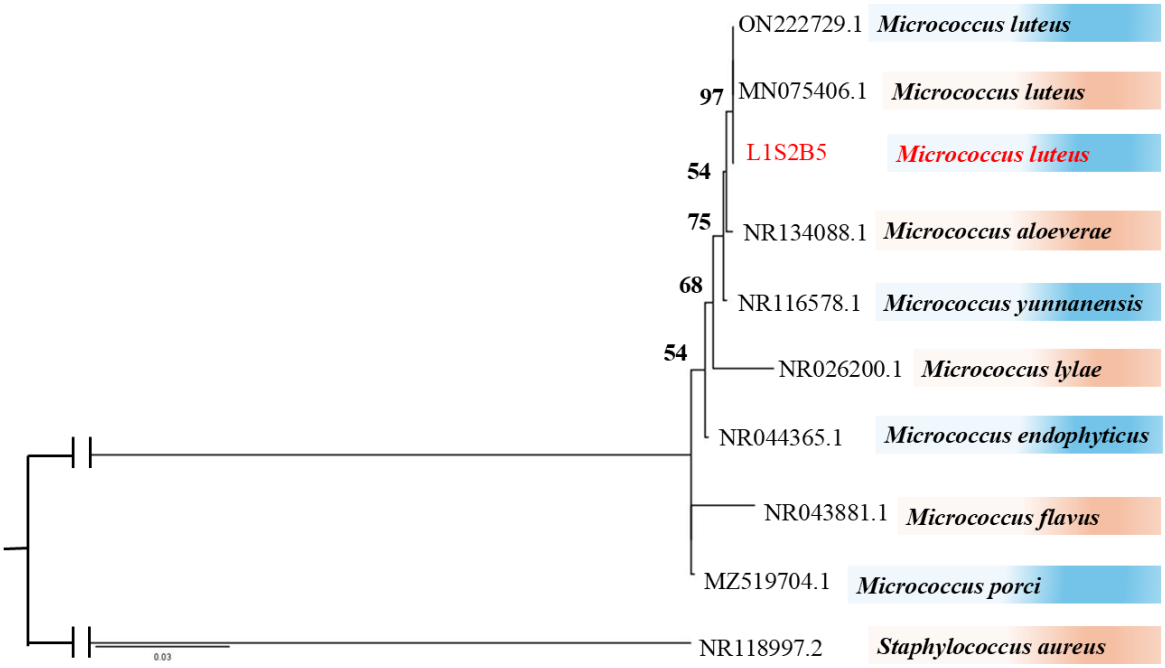

Figure 4: *Phylogram constructed for L1S2B5 (highlighted in red) from maximum likelihood analysis based on 16SrRNA sequences. The tree is rooted with Staphylococcus aureus. Bootstrap values (1000 replicates) which are ≥ 50% are noted on the branches.*

Discussion

The pitcher fluid of *N. distillatoria* is an example of understudied phytotelmata, i.e., the small bodies of liquid contained within the pitcher traps, which have unique micro-environmental conditions and are inhabited by organisms such as bacteria, fungi, and algae encountered in tropical habitats.

During this study, 12 open pitcher fluid samples were collected for the isolation of microorganisms. The unopened pitcher fluid is a colourless liquid, while the colour and composition of the open pitcher fluid vary greatly depending on the insects trapped in it. Considering the samples collected from both sites, most of the trapped insects are ants. As expected, all the samples showed acidic pH values ranging from 2.1 to 6.0. The variation of pH values between open pitcher fluid samples is due to many reasons, such as the presence of acidic/basic compounds on the trapped insect bodies, production of acidic/basic compounds during the digestion of insect body parts, production of acidic/basic compounds due to microbial activity on insect debris, evaporation of pitcher fluid and due to the dilution of pitcher fluid from rainwater.

Before inoculation, pitcher fluid samples were subjected to serial dilution. Even at a 10^{-1} dilution, the plates did not exhibit overcrowded growth, suggesting that the pitcher fluid is generally unfavorable for microbial proliferation. From 12 open-pitcher fluid samples, a total of 24 distinct bacterial isolates were successfully obtained.

Those isolated bacteria were subjected to enzymatic plate assays. This qualitative method helps the primary identification of enzyme producers before expensive quantitative methods come into action. Those qualitative methods enable the screening of large numbers of microorganisms within a short period using minimal resources.

The protease plate assay was carried out using the skimmed milk agar medium to identify the acid protease-producing microorganisms. During the study, 3 bacterial isolates were identified as acid protease-producing microorganisms. Among all, one bacterial isolate showed significant enzymatic activity. During the skimmed milk agar plate assay, protease-producing microorganisms secrete extracellular proteases into the medium and degrade the protein substrate skimmed milk, creating a clear zone around the microbial colony. The formation of a clear zone around microbial colonies was used to distinguish protease-producing microorganisms from non-protease producers.

Other than that, Bacterial isolates are also subjected to acid amylase and acid chitinase plate assay. During the amylase plate assay, 2 bacterial isolates were identified as acid amylase producers. But none of their enzymatic activity was significant. None of the bacterial isolates showed positive results for the acid chitinase plate assay.

Most of the bacterial isolates that showed positive results for enzymatic plate assays did not show significant enzymatic activity. One possible reason is that cultural conditions may not optimize for effective enzyme production. During the enzyme plate assay, the pH value of the medium was maintained at pH 5. This is quite high when compared with the *Nepenthes* pitcher's pH value. However, the decrease in pH in the media did not allow agar to set and form a solid medium. This is one limitation encountered during the acid enzymatic plate assay. To overcome the above problem and optimize the cultural conditions, a method like a spectrophotometry-based assay is suggested for the enzymes identified above, which microorganisms produce. A broth culture can be used without a solidifying agent-agar during a spectrophotometry-based assay.

Biochemical testing of bacteria provides valuable insights into the biochemical reactions of bacteria. During the study, bacteria were subjected to catalase, MR, VP, Gelatinase, and KOH tests. They also aid in the identification process.

According to the phylogram (figure 4), L1S2B5 tested positive for acid protease activity and was identified as *Micrococcus luteus*. This bacterium is a well-known protease-producing bacterium found in various habitats, such as air and soil. (Kumari, 2014, Wieser et al., 2002). Therefore, air and insects can be considered as possible sources of *Micrococcus luteus* isolated from *Nepenthes* pitcher fluid. Since it showed a relative enzymatic activity of 1.5 after 2 days of incubation, it can be considered as a possible candidate for the industrial production of acid protease.

Conclusion

This study reveals that the *N. distillatoria* microbial community consists of bacteria with the potential to produce industrially important enzymes such as acid proteases. Most of the bacterial communities associated with *N. distillatoria* are commensals, while the identified enzyme producers may have a mutualistic relationship with the plant. Molecular characterisation is always recommended for all bacterial isolates isolated from environmental samples. This study suggests the significance of studying *N. distillatoria* microcosm to discover the diversity and potential applications of unique microflora present in these poorly known habitats.

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