

Research Article

Metagenomics Profile of Bacterial Community and Its Biocatalytic Activities of The Cultured Bacteria in Liquid of Pitcher Plant (*Nepenthes Adrianii*)

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ABSTRACT

Nepenthes, commonly known as pitcher plants, are carnivorous plants that catch insects. One species, *Nepenthes adrianii*, is endemic to Mount Slamet in Central Java. The bacterial community present in the fluid of *Nepenthes* pitchers is supposed to play a crucial role in digesting trapped insects, converting them into nutrients for the plant. This study investigated the metagenomics profile of the bacterial community found in the fluid of *N. adrianii*, collected from two different altitudes, and evaluated the biocatalytic activity of cultured bacteria. Next-generation sequencing (NGS) was performed on the V3-V4 hypervariable region of the 16S rRNA gene. Additionally, biocatalytic screening—including cellulolytic, amylolytic, proteolytic, and chitinolytic tests—was conducted by growing bacteria on basal media, each supplemented with 1 % of carboxymethyl cellulose (CMC), starch, skim milk, and colloidal chitin. The formation of a clear zone in the media indicated bacteria with biocatalytic activity, which were subsequently identified molecularly using the 16S rRNA gene. The results indicated that the four most abundant bacterial phyla were Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteria, with Proteobacteria having the highest abundance in all altitudes. Bacterial diversity in the fluid of *N. adrianii* showed high with a Shannon diversity index exceeding three, along with several dominant bacterial species. Bacterial isolates confirmed to have proteolytic activity included *Leifsonia aquatica*, *Bacillus tequilensis*, and *Bosea lupini*. Cellulolytic activity was attributed to *Pseudomonas* sp. Amylolytic activity was linked to *Pseudomonas* sp. and a Bacillaceae bacterium, while *Pseudomonas aeruginosa* exhibited chitinolytic activity.

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INTRODUCTION

Nepenthes is a group of genera from the Nepenthaceae family commonly known as pitcher plants. Nepenthes are famous for their unique pitcher-shaped structures at the tip of their leaves, used to catch insects and other small animals and as a nutrient source. Nepenthes had a high species diversity; in 2021, 168 species were successfully identified. Nepenthes can be found in Indonesia, some of which are *Nepenthes adrianae*, *Nepenthes gymnamphora*, *Nepenthes mirabilis*, *Nepenthes ampullaria*, *Nepenthes rafflesiana*, and *Nepenthes spathulata*. *N. adrianae* is particularly notable as it is considered rare, only growing in specific locations in the highlands, and is endemic to Mount Slamet (Batoro & Wartono 2017).

Nepenthes species can thrive as an epiphyte, which often leads to nutrient deficiencies, prompting the plant to adapt by modifying its leaves into a specialized structure known as a digestive pitcher. This pitcher, often referred to as a pitcher, not only serves as a trap but is also equipped with complex digestive fluids. The structure of the Nepenthes pitcher has five distinct leaf zones, including the base of the leaf, tendrils, digestive zone, wax zone, and leaf cap or lip. When insects or small animals enter the pitcher, they first encounter the wax zone, which has a slippery surface that makes it difficult to escape, ultimately leading them into the fluid at the bottom of the pitcher. The plant produces digestive enzymes within the digestive zone, which assist in breaking down the trapped prey (Dkhar et al. 2020). Meanwhile, other studies have also shown a symbiotic interaction between the microbial community in the digestion of trapped prey. The fluid within Nepenthes plants acts as a micro-ecosystem that hosts various bacterial and arthropod communities. Research conducted by Chan et al. (2016) on Nepenthes fluid isolated from Malaysia identified 616 species of bacteria belonging to 18 different phyla through metagenomic analysis. The dominant bacterial phyla in this sample were Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Planctomycetes. These five phyla are closely related to the processes involved in the decomposition of organic matter. Similarly, a study by Kanokratana et al. (2016), which examined Nepenthes fluid from Thailand, identified 20 phyla, with Bacteroidetes and Actinobacteria being the most prevalent. Additionally, Takeuchi et al. (2015) studied four Nepenthes species and found 17 phyla with 195 genera, again predominantly featuring Proteobacteria and Bacteroidetes.

Several studies indicate that Nepenthes produce antibacterial compounds, allowing only certain bacteria to grow well within their fluid. Research conducted by Chan et al. (2016) showed the presence of enzymatic activity from successfully cultured microorganisms. Enzymatic activities in bacteria isolated from Nepenthes fluid are proteolytic, amylolytic, cellulolytic, xylanolytic, and chitinolytic enzymes. In addition, some bacteria that live in Nepenthes fluid have chitinase and lipase enzyme activities, supporting the hypothesis of a symbiotic relationship between these bacteria and the carnivorous plants (Morohoshi et al. 2011).

The bacterial community and biocatalytic activity are influenced by geographic location and altitude. Variations in these factors affect climate and temperature conditions, which in turn influence the types of insects or prey captured and alter the composition of pitcher plant fluid (Gilbert et al. 2020a, 2020b). Despite their importance, the role of bacteria in the digestive processes of Nepenthes pitcher fluid has not been extensively studied. This research aims to investigate the metagenomic profile of bacteria found in the fluid of *N. adrianae* and to assess the biocatalytic activity of bacteria that can grow in culture media. The findings of this study enhanced our understanding of how these bacteria contribute to prey degradation.

MATERIALS AND METHODS

Study area and sample collection

Pitcher plant fluid samples (*N. adriani*) were collected from pitcher plants around the Mount Slamet hiking trail, Central Java, Bambang route at two different altitudes. The first altitude is around 900 meters above sea level (m asl) to post one at an altitude of 1947 m asl. While the second altitude, sampling at higher altitudes was taken from the post two hiking trail with an altitude of between 2000 and 2500 m asl. The pitcher fluid was put into a 15 mL sterile centrifuge tube. Pitcher samples were collected in triplicate at each altitude. Then the tube is wrapped to avoid contamination during the trip to the laboratory. Samples that have been collected into centrifuge tubes are stored in an ice box during the trip. In the lab, the pitcher fluid is preserved by storing at low temperatures (4 °C in refrigerator).

DNA extraction and metagenome sequencing

Total genomic DNA was extracted as much as 250 µl obtained from each *N. adriani* pitcher fluid sample that were homogenized, followed by centrifugation, and collecting the remaining microbial DNA or cell into one tube with the DNA extraction protocol using ZymoBIOMICS DNA Microprep (Zymo Research Corp). Genomic DNA samples were stored in sterile microcentrifuge tubes and sealed tightly sent to Novogene for sequencing with the Next Generation Sequencing (NGS) platform Illumina Inc. (USA). Amplification and sequencing were performed on the V3-V4 hypervariable region with specific primers (515F-806R) 515F GTGCCAGCMGCCGCGGTAA; 806R GGACTACHVGGGTWTCTAAT with 250 bp paired-end readings. The library preparation of bacterial community samples was carried out following the NEBNext Ultra™ DNA Library Prep Kit protocol for Illumina. All DNA samples were then quantitatively checked by Qubit instrument and qPCR before being loaded into the Illumina sequencer platform.

Screening for amylolytic, proteolytic, cellulolytic, chitinolytic bacteria

Screening of amylolytic, proteolytic, cellulolytic, chitinolytic activity was carried out by growing pitcher plant fluid bacteria on basal media by adding 1 % starch (Merck) for amylolytic, skim milk (nzmp) for proteolytic, carboxymethyl cellulose (CMC) (Himedia) for cellulolytic, and colloidal chitin for chitinolytic adjusted to pH 7.0 (Aryati et al. 2016). The composition of basal media is 0.2 g NaNO₃, 0.05 g MgSO₄, 0.005 g K₂HPO₄, 1 mg FeSO₄, 2 mg CaCl₂, 2 mg MnSO₄ and 2 % agar adjusted to pH 7 (Sari et al. 2016). A sample of *N. adriani* pitcher plant fluid of 0.1 mL was grown on screening media using the spread plate method. Furthermore, the culture was incubated at 28 °C for 7 days.

The formation of a clear zone in the basal media supplied with a specific substrate for bacterial growth indicates a positive result when the bacteria exhibit biocatalytic activity. The formed clear zone occurs because the bacteria growing in the media utilize the available substrate by hydrolising the its bonds. The ability of bacteria to degrade polysaccharides with different levels of complexity was tested using starch and CMC substrates. The ability of bacteria to degrade chitin was tested using a colloidal chitin substrate, and the ability to hydrolise peptide bonds in proteins was tested using a skim milk as a substrate.

In amylolytic screening media that has been treated with iodine, the iodine binds to starch and produces a dark blue color in the substrate area. However, if the bacteria hydrolyze the starch into glucose, the glucose will not bind to iodine, resulting in a clear area surrounding the inoculum (Gopinath et al. 2017; Tan et al. 2023; Ni'matuzahroh et al. 2024). Meanwhile, cellulose degradation by cellulolytic bacteria is characterized by congo red

discoloration, which forms a light yellow zone or clear zone around the colony on cellulose agar (Chan et al. 2016; Dewiyanti et al. 2022; Ni'matuzahroh et al. 2024).

Preparation of colloidal chitin, 10 g of shrimp chitin powder (shrimp shell) is mixed with 100 mL of concentrated HCl to form a thick slurry, which is then cooled in ice water to control heat. The slurry is stirred every 5 minutes for 30 minutes, becoming less viscous over time. After hydrolysis, it is diluted with 2 L of chilled distilled water while stirring, forming a white suspension. The mixture is filtered using an LCCM-based apparatus. The CC paste trapped in the filter is washed multiple times (3–5 repetitions) with distilled water until a near-neutral pH is achieved (Koteshwara 2021).

Calculation of biocatalytic enzyme activity index using the formula:

$$\text{Enzyme activity index} = \frac{\text{clear zone diameter (mm)} - \text{colony diameter (mm)}}{\text{colony diameter (mm)}}$$

The clear zone indicates the ability of each bacterium to hydrolyze the substrate, which is measured by the enzymatic activity index. Extracellular enzyme activity demonstrates a strong response when the ratio of extracellular enzyme to colony is greater than or equal to 2. If the extracellular enzyme ratio falls between 1 and 2 (i.e., $1 < x < 2$), the activity is considered moderate. Conversely, if the extracellular enzyme ratio is less than 1 (i.e., $x < 1$), the response is regarded as weak (Choi et al. 2005).

Molecular identification of biocatalytic bacteria

Bacterial isolates were grown on Nutrient Broth (NB) media and incubated on a shaker for 24 hours. The culture was centrifuged at 12,000x g for 10 minutes to harvest the cells and the supernatant was discarded. According to the manufacturer's instructions, DNA extraction was performed using Quick-DNA™ Fungal/Bacterial Miniprep (Zymo Research Corp). After obtaining the DNA genome, PCR was performed with 50 µL of reaction volume consisting of 25 µL of master mix (GoTaq® Green master mix), 2 µL of 10 pmol forward primer, 2 µL of 10 pmol reverse primer, 2 µL of (100 ng) DNA template, and 19 µL of Nuclease-Free Water. The PCR program is based on research by Zhang et al. (2020) with slight modifications, including initial denaturation at 94 °C for 5 minutes, denaturation at 94 °C for 1 minute with 35 cycles, annealing at 57°C for 1 minute, elongation at 72 °C for 2 minutes and extension at 72 °C for 10 minutes, after which the temperature was lowered and ended at 4 °C. Universal primers 16S rRNA 27F (5' AGATTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The primers amplify the DNA template with an amplicon length of approximately 1460 bp. An aliquot of 3 µL of PCR product was verified by 1 % agarose gel electrophoresis (dos Santos et al. 2019).

Data analysis

For metagenomics: Adapter and PCR primer sequences from the paired-end reads were removed using Cutadapt (Martin 2011). DADA2 was used to correct sequencing error, remove low quality sequences and chimera errors (Callahan et al. 2016). The Amplicon Sequence Variants (ASVs) data was used for taxonomic classification against SILVA (silva_nr99_v138.1) database. Downstream analysis and visualisation were performed using packages in RStudio (R version 4.3.2) (<https://www.R-project.org/>). Data visualisation, including histogram charts, venn diagram, alpha diversity (Simpson diversity, Shannon diversity, and Inv-Simpson) was performed to illustrate the taxonomic composition and distribution across samples. Diversity indices, including Shannon, Simpson, and inv-Simpson, were calculated using statistical packages in R Studio (version 4.2.3) (<https://www.R-project.org/>) to assess the alpha diversity of microbial communities within the pitcher fluid samples.

Biocatalytic activity was analyzed through an experimental approach using bacterial isolates cultured from pitcher fluid samples. enzymatic activity tests were conducted qualitatively on specific media: skim milk agar for proteolytic activity, starch agar for amylolytic activity, chitin agar for chitinolytic activity, and cmc agar for cellulolytic activity. each test was performed in triplicate to ensure the validity and consistency of the results. the formation of clear zones around bacterial colonies was used as a positive indicator of enzymatic activity, and the test results were analysed descriptively. For biocatalytic bacterial identification: Forward and reverse sequences from Sanger sequencing were assembled using BioEdit. Subsequently, the 16S rRNA gene sequences of bacterial isolates were analyzed with the BLAST program, which searched for similarities in the GenBank database on the NCBI website (<https://blast.ncbi.nlm.nih.gov/>).

RESULTS AND DISCUSSION

Metagenomics profiles of bacterial communities

The *N. adrianae* pitcher fluid represents an extreme microhabitat that offers a unique environment for specific microbial communities, including bacteria. Altitude is a key environmental factor that can influence the distribution and characteristics of microorganisms due to variations in temperature, air pressure, humidity, and resource availability. Therefore, comparing the bacterial communities at low and high altitudes provides valuable insights into how environmental conditions affect the structure of these microbial communities.

Next-generation sequencing (NGS) analysis of *N. adrianae* pitcher fluid at two different altitudes showed variations in the relative abundance of the bacterial community. The relative abundance of the bacterial community of the *N. adrianae* pitcher fluid is displayed through a histogram (Figure 1). In both datasets, bacteria from the Proteobacteria phylum dominate with the most significant proportion (more than 50 %), followed by phyla such as Firmicutes, Bacteroidota, Actinobacteriota, and Verrucomicrobiota in smaller amounts. The dominant Proteobacteria phylum in both locations reflects its role as the main bacterial phylum in the pitcher fluid ecosystem, which decomposes organic compounds. These results are consistent with previous studies conducted by Chan et al. (2016), Kanokratana et al. (2016), Sickel et al. (2016), and Gilbert et al. (2020b), which showed that the phyla Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota were the dominant phyla found in pitcher plant fluid. At the genus level, bacteria from the genus *Pseudomonas* were the most dominant at lower altitudes, with a proportion of about 33 %, followed by genera such as *Rhodopedomonas* and *Acidosoma* and other genera in lower numbers. At higher altitudes, bacteria from the genus *Rhodoferax* were the most dominant, with a proportion of about 11 %, followed by genera such as *Clostridium sensu stricto* 3 with a proportion of about 8 %.

The dominance of Proteobacteria can be explained by the ability of members of this phylum to adapt to environments with varying nutrient availability (Zhou et al. 2020). Proteobacteria, especially *Pseudomonas*, which dominated at lower altitudes, are known as highly metabolically flexible bacteria, both as decomposers of organic matter and producers of secondary metabolites, also able to survive in extreme environmental conditions such as high organic compound content or low pH (Chauhan et al. 2023). Meanwhile, the dominant genus *Rhodoferax* at high altitudes reflects its ability to adapt to environmental stress conditions such as low temperatures and limited light. The presence of the genus *Clostridium sensu stricto* from the phylum Firmicutes at both altitudes indicates its essential role in the anaerobic degradation process, which is relevant for the function of the *Nepenthes* pitcher in digesting prey.

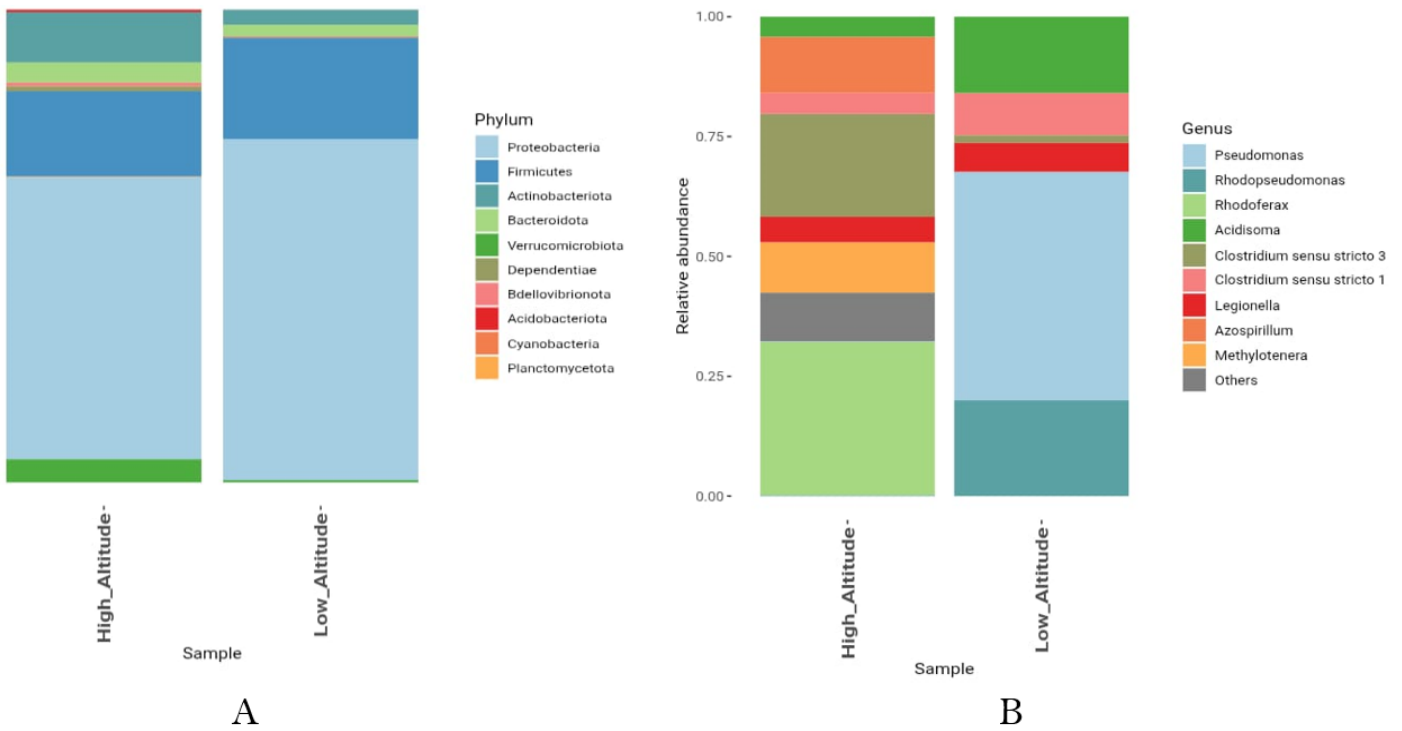


Figure 1. Visual histogram percentage relative abundance bacteria in the pitcher plant fluid of *N. adrianae* (A) phylum taxa, (B) genus taxa.

Differences in community composition at two altitudes indicated the influence of environmental factors on the structure of microbial communities (Zakavi et al. 2022). Higher altitudes have lower temperatures, lower oxygen pressure, and limited carbon sources. The results of this study revealed that *Nepenthes* pitcher fluid microbes play an essential role in supporting plant ecological functions, especially in organic degradation and nitrogen provision. These differences were shown at each altitude, reflecting the adaptation of microbes to specific environmental conditions.

The number of bacterial species observed in the pitcher fluid at both altitudes which is stated as had a number of unique Amplicon Sequence Variants (ASVs) and shared ASVs detected shown with a Venn diagram (Figure 2).



Figure 2. Venn diagram illustrating the unique number of bacterial taxa from each altitude and both in the pitcher fluid.

NGS 16S rRNA amplicon metagenomic sequencing analysis revealed the presence of 25 shared (ASVs) in both pitcher fluid samples, indicating a core microbial community that is adaptable to varying altitude conditions. However, each elevation exhibited unique microbial diversity, with samples from higher elevations showing 386 unique species, while samples from lower elevations had 229 unique species. This variation in diversity is likely influenced by the differing environmental conditions at each altitude. These findings aligned with the research conducted by Zakavi et al. (2022), which examined bacterial communities across different altitudes. Their study suggested that altitude is a significant factor influencing microbial community composition. Although microbiome diversity tends to increase with altitude, highland bacteria are also more resistant to heat and changes in pH. The presence of unique ASVs in each sample indicate microbial niche specialization, where each species has specific adaptations to capitalise on the resources available in its environment.

Bacterial diversity in the liquid of pitcher plant *N. adriani*

The liquid of the pitcher plant is known to have a diversity of bacteria that have their roles in the unique habitat. Species diversity in the bacterial community of pitcher plant fluid is expressed as alpha diversity. Alpha diversity was analysed based on the Shannon and Simpson indices. The Shannon index measures species diversity, with higher values indicating greater diversity. In contrast, the Simpson index assesses species evenness and indicated the dominance of certain species within a community (Table 1). The diversity of bacteria in the *N. adriani* liquid observed at two different altitudes is classified as high, as indicated by the Shannon index value exceeding 3 (Waskita et al. 2024).

Based on the alpha diversity analysis of the data, several metrics are used to measure the diversity of the microbial community, which is stated as the number of observed ASVs, including the Shannon index, the Simpson index, and the inverse Simpson. Each metric provides a different perspective on the structure and complexity of the microbial community. The highland samples had a total of 411 ASVs, while the lowland samples had a total of 254 ASVs. This higher species richness may reflect more heterogeneous environmental conditions, such as microhabitat variations, lower temperatures, or nutrient availability, allowing more species to flourish. Conversely, fewer ASVs observed in the lower plains may be due to environmental homogeneity, such as more stable temperatures or high humidity, which may favor the dominance of certain species.

The Shannon index value is 4.33 at higher altitudes and 3.08 at lower altitudes. The Shannon index measures biodiversity by assessing the number of species and their relative distribution. The higher value at elevated altitudes suggested that the microbial community is more evenly distributed, indicating more balanced representation of species than at low altitudes.

Simpson index value is 0.9705, which is close to 1. This means that even though the community at a high altitude is rich in species and has a considerable Shannon value, showing even more diversity, the Simpson index indicated the presence of fairly dominant species. So, even though this community is rich, the dominance of certain species still occurs. Simpson value at low altitude is 0.8606, lower than the high altitude. This value indicates that species dominance also existed, but the level of dominance was relatively lower compared to the high altitude. The dominance of certain species can occur since of more significant environmental pressures, e.g., lower temperatures or more limited nutrient levels, so only highly adaptive species can become dominant. Lower dominance at lower altitudes may be due to more stable or uniform environmental conditions, allowing more species to share resources relatively more evenly.

Table 1. Microbiome alpha diversity shows the diversity of a single microbial community sample, taking into account the number and abundance of different species.

Liquid of pitcher plant	Number of Species	Shannon Index	Simpson Index	Indesks Inv. Simpson
High Altitude (2.000-2.500 m asl)	411	4.33	0.97	33.95
Low Altitude (900 -1.947 m asl)	254	3.08	0.86	7.18

This metric indicated the number of species that dominate the community. Higher values at high altitudes indicated a community with more diverse structure. High values indicated that the microbial community has many species with equal distribution. This reflects an environment that supports complex ecological interactions. Low inverse Simpson values suggested that a few species dominated the community; one or a few species have a much more extensive coverage than the other species. During the sampling exploration, we noted variations in environmental conditions (Table 2).

There were different temperature and humidity conditions, whereas, at higher altitudes, the temperature and humidity were lower compared to lower altitudes. Meanwhile, the light intensity at lower altitudes had lower intensity. Differences in environmental condition, such as altitude, air temperature, air humidity, and light intensity, can significantly influenced the diversity of bacteria in pitcher plant fluid (Chou et al. 2014; Kanokratana et al. 2016; Gilbert et al. 2020a, 2020b).

Biocatalytic activity of bacteria cultured from pitcher plant fluid

Nepenthes plants can produce their digestive enzymes, as found in the study of An et al. (2002); the study reported that high expression of hydrolytic enzymes was found in the digestive zone of the pitcher. Meanwhile, other studies also showed a symbiotic interaction between the microbial community in the pitcher fluid and plants in the digestion of trapped prey (Bittleston 2018). Biocatalytic test showed the presence of clear zones on the screening media, indicating that the isolated bacteria from the pitcher plant fluid produced enzymes capable of breaking down specific substrates. This proves the presence of biocatalytic activity in the cultured bacteria (Figure 3).

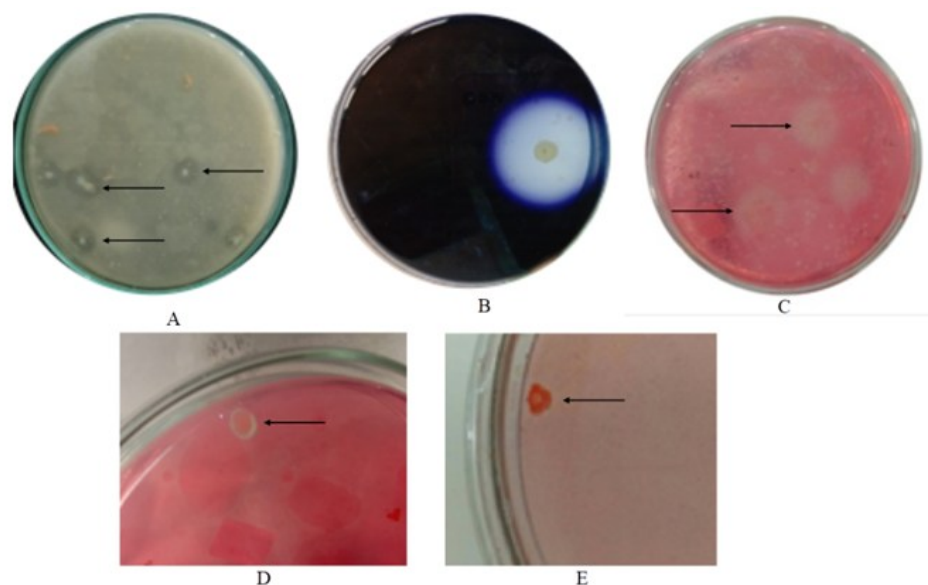


Figure 3. Extracellular enzyme activities showing clear zones shown in the arrows area. (A) proteolytic, (B) amylytic, (C) and (D) cellulolytic, (E) chitinolytic.

The formation of a clear zone on the screening media consisting of amylytic, cellulolytic, proteolytic, and chitinolytic activities indicated that the

Table 2. Environmental condition.

Altitude	Air temperature (°C)	Relative air humidity (%)	Light intensity (lux)
High Altitude (2.000-2.500 m asl)	20.15 ± 0.06	89.75 ± 0.52	2088 ± 218.2
Low Altitude (900 -1.947 m asl)	25.25 ± 1.4	97.25 ± 1.1	1251 ± 34.5

bacteria in the pitcher plant have all four types of biocatalytic activities. Bacteria exhibiting biocatalytic activity found in the pitcher fluid of *Nepenthes* are believed to be closely linked to the ecological function of the pitcher as a trapping and digestive system for prey. The pitcher fluid naturally contains various digestive enzymes (An et al. 2002), such as proteases, lipases, and chitinases, which aid in the breakdown of trapped insect body components. Metagenomic analysis revealed a significant diversity of bacterial communities, including genera known to produce hydrolytic enzymes. The presence of these bacteria supports the hypothesis that they function as symbionts contributing to prey decomposition and nutrient provisioning for the plant. Detected biocatalytic activities include proteolytic activity, which is essential for breaking down protein-rich insect prey. These bacteria are likely involved in the hydrolysis of insect proteins, facilitating nitrogen release for plant uptake. Amylolytic activity, which assists in the degradation of starch and other polysaccharides, along with chitinolytic activity, which is responsible for breaking down the chitinous exoskeletons of insects, further highlights the role of bacteria in degrading structural components of the prey. Additionally, cellulolytic activity suggested a broader enzymatic profile, enabling the breakdown of cellulose originating from plants or insect sources. The presence of these enzyme-producing bacteria indicated a synergistic relationship between *Nepenthes* and its microbiota, where microbial metabolism complements the plant's endogenous enzymes, enhancing the breakdown of complex organic matter and optimizing nutrient acquisition in nutrient-poor environments.

Bacterial Isolates of *N. adriani* fluid with biocatalytic activity have different abilities in hydrolyzing substrates, as indicated by differences in the biocatalytic index of each isolate (Table 3). Analysis of the biocatalytic index of bacterial isolates from *N. adriani* sac fluid showed variations in enzymatic activity in the isolates.

Variations in biocatalytic indices indicated different enzymatic abilities of bacteria in the *Nepenthes* pitcher fluid. Proteolytic isolates (P1-P5) tend to have a high biocatalytic index, reflecting the critical role of these bacteria in digesting proteins from insects entering the pitcher. The highest index in isolate P3 (9 ± 0.82) indicated a dominant proteolytic activity. Isolates with cellulolytic activity (C1 and C2) have significant differences in indices, with C2 (3.9 ± 0.62) showing a better ability to degrade cellulose than C1 (0.3 ± 0.03). This suggests that cellulose in the pitcher plant environment can affect the colonization of certain bacteria. Low chitinolytic activity (K1 and K2) may indicate a supporting role in the decomposition of insect chitin, with indices still below 1. Differences in biocatalytic index values, which indicate the ability of bacteria to degrade substrates, can arise from various factors. These factors included pH levels, incubation temperature, the quantity or quality of organic material or substrates (Rutu et al. 2017; Piotrowska-Długosz et al. 2022), the duration of incubation time, and the presence or absence of enzyme cofactors such as Mn^{2+} in the substrate (Rutu et al. 2017). In contrast, amylolytic activity in isolates A2 and A3, although not reaching proteolytic, indicated that these bacteria are also involved in the degradation of complex carbohydrates such as starch. These biocatalytic indices suggested that bacteria in the *Nepenthes* pitcher fluid have enzymatic characteristics that support the function of the pitcher as an insect trap. The dominant proteolytic activity corresponds to the central role of the pitcher in digesting proteins from the

Table 3. Biocatalytic index of cultivable bacterial isolates from the Pitcher fluid of *N. adrianii*.

Isolate code	Activity	Biocatalytic index (mean ± SD)
P1	Proteolytic	3.25 ± 0.5
P2	Proteolytic	3 ± 0.0
P3	Proteolytic	9 ± 0.82
P4	Proteolytic	5.88 ± 0.63
P5	Proteolytic	1.84 ± 0.19
C1	Cellulolytic	0.3 ± 0.03
C2	Cellulolytic	3.9 ± 0.62
K1	Chitinolytic	0.3 ± 0.14
K2	Chitinolytic	0.63 ± 0.25
A1	Amylolytic	0.7 ± 0.18
A2	Amylolytic	1.83 ± 0.35
A3	Amylolytic	3.95 ± 0.33

insect body, while cellulolytic, chitinolytic, and amylolytic activities reflect additional abilities to digest other components such as insect exoskeleton (chitin) and plant compounds that may enter the pitcher fluid. Differences in indices between isolates may reflect microbial adaptation to specific environmental conditions in the pitcher fluid.

Molecular Identification of bacteria with biocatalytic activity

The electrophoregram indicates that the PCR technique using the primers 27F and 1492R successfully amplified the 16S rRNA gene from bacterial isolates found in pitcher plant fluid. A distinct DNA band observed in the range of 1500 bp confirms that the bacterial genomic DNA was effectively extracted and purified for use as a template. The successful amplification of the 16S rRNA gene demonstrates that the primers used are specific to the target gene and that the PCR conditions were appropriately optimized.

BLAST analysis of bacterial isolates from pitcher plant fluid revealed a high level of similarity, with an identity percentage of 99 % to 100 % and a query cover ranging from 99 % to 100 %. The bacterial isolates identified through this analysis were *Leifsonia aquatica*, *Bacillus tequilensis*, and *Bosea lupini*. Additionally, *Pseudomonas sp.* demonstrated proteolytic activity, while *Pseudomonas aeruginosa* exhibited chitinolytic activity. Cellulolytic activity was confirmed in *Pseudomonas* species, specifically *Pseudomonas sp. strain GL24*, while a *Bacillaceae* bacterium was identified as having amylolytic activity (Table 4).

Leifsonia aquatica, *Bosea lupini*, and *Bacillus tequilensis* were bacteria with proteolytic activity found in *N. adrianii* pitcher fluid. These proteolytic bacteria aid in the digestion of proteins from captured insects, breaking them down into simpler elements that the plant can absorb. Additionally, protease play a crucial role in the ecological balance, particularly in maintaining the nitrogen cycle (Razzaq et al. 2019). Isolate P1, in addition to having similarities with *L. aquatica*, also has similarities with *Leifsonia sp. NaF-BtI-2* found in the human oral cavity and *L. xyli* and *Lysinimonas sp. LM-2018* found in soil and plants.

The BLAST search results on NCBI isolate P3 showed that the 16S rRNA sequence analyzed had high similarity to several bacterial species from the genus *Bosea*. The highest identity was found in *Bosea lupini*, with an identity percentage of 99.71 % and a maximum score of 2512. In addition, other species, such as *Bosea vestrisii* and *Bosea sp. Tri-39*, both of which were also found in root nodules, also showed a high level of similarity with identity percentages between 99.71-99.78 %. These results indicate that the isolate analysed is most likely a member of the genus *Bosea*, which belong to the α -proteobacteria group. The genus *Bosea* is known to have diverse habitats and is often found in soil and air and as part of plant microbiota. A high identity

Table 4. Identifies *N. adriani* fluid bacteria isolates based on the 16S gene sequence—the identification based on BLAST similarity searching with the Genbank database.

Isolate Code	Biocatalytic Activity	Description	Max Score	Total Score	Query Cover	E Value	Identity Percentage	Acc Len	Accession
P1	Proteolytic	<i>Leifsonia aquatica</i> strain A1 16S ribosomal RNA gene, partial sequence	2560	2560	99 %	0.0	99.71 %	1493	JX010948.1
P2		<i>Bacillus tequilensis</i> strain A80 16S ribosomal RNA gene, partial sequence	2636	2636	99 %	0.0	99.93 %	1455	OP43577.5.1
P3		<i>Bosea lupini</i> strain R-45681 16S ribosomal RNA, partial sequence	2512	2512	99 %	0.0	99.71 %	1444	NR_108514.1
K1	Chitinolytic	<i>Pseudomonas aeruginosa</i> strain QK-4 16S ribosomal RNA gene, partial sequence	2615	2615	100 %	0.0	99.93 %	1432	MH746107.1
C1	Cellulolytic	<i>Pseudomonas</i> sp. GC04 16S ribosomal RNA gene, partial sequence	2614	2614	100 %	0.0	99.93 %	1426	KP671493.1
C2		<i>Pseudomonas</i> sp. strain GL24 16S ribosomal RNA gene, partial sequence	2601	2601	99 %	0.0	99.79 %	1432	OR647672.1
A1	Amylolytic	<i>Bacillaceae bacterium</i> strain AFS099849 16S ribosomal RNA gene, partial sequence	2627	2627	99 %	0.0	99.79 %	1508	OP986711.1
A2		<i>Pseudomonas</i> sp. strain GL24 16S ribosomal RNA gene, partial sequence	2615	2615	100 %	0.0	100.00 %	1432	OR647672.1

percentage (>99 %) indicates a close phylogenetic relationship with the species found in the database (Syah et al. 2024). This finding suggested that the microorganisms in the sample may play a role in specific environmental processes, such as the nitrogen cycle or interactions with plants. Isolates C1 and C2 were identified in the genus *Pseudomonas*, a group of gram-negative bacteria with high metabolic diversity. *Pseudomonas* is often found in the environment, including soil, air, and plants. Some BLAST results, such as *Pseudomonas koreensis*, also show closeness, indicating a more specific species. This species is known as an environmental bacteria with the ability to degrade certain organic compounds. *Pseudomonas* sp. have cellulolytic activity. Both belong to the proteobacteria phylum widely found in animal digestion system, where they help degrade organic matter into easily absorbable nutrients. *Pseudomonas* sp. was also confirmed to have cellulolytic activity in *N. mirabilis* (Chan et al. 2016).

Chitinolytic activity in identified bacteria has low enzymatic activity. Chitinolytic bacteria in *N. adriani* fluid identified in this study were *Pseudomonas aeruginosa*. *P. aeruginosa* can be found in soil where it plays a role in biodegradation. Research conducted by Folders et al. (2001) showed that *P. aeruginosa* has chitinase activity with its ability to inhibit the growth of *Fusarium oxysporum* and *Rizoctonia solani* fungi. Chitinolytic bacteria in *N. adriani* plants degrade chitin in prey, such as insects, converting it into simpler molecules to be utilized for their nutrition by *N. adriani* plants. The high abundance of bacteria and biocatalytic activity indicate that bacteria living in *Nepenthes* fluid have a high possibility of degrading prey into simpler molecules that plants can utilize to meet their nutritional needs.

CONCLUSION

This study found that the habitat of *Nepenthes adrianae* at different altitudes affected the diversity of bacteria in its pitcher fluid, with higher altitudes showing greater bacterial diversity. Metagenomic analysis revealed that the fluid contains a rich community dominated by Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota. Several cultured bacterial isolates demonstrated enzymatic activities including proteolytic, cellulolytic, chitinolytic, and amylolytic as evidenced by clear zones on selective media. Notably, *Leifsonia aquatica*, *Bacillus tequilensis*, and *Bosea lupini* showed strong proteolytic activity, while *Pseudomonas sp.* exhibited both cellulolytic and amylolytic activity, and *Pseudomonas aeruginosa* showed chitinolytic activity. These findings highlighted the metabolic potential of pitcher-associated bacteria and enhanced our understanding of how these microbes contributed to prey degradation and nutrient acquisition in *N. adrianae*.

AUTHOR CONTRIBUTION

A.A.N., A.S. and S. designed the research. A.A.N. and A.S. carried out the laboratory work. A.A.N., N.E. and A.S. analysed the data. A.A.N., A.S., and A.P. wrote the manuscript.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest during the research

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