



**ELUCIDATING THE FLUCTUATIONS IN
MICROORGANISM RELATED TO *RAFFLESIA*
KERRI IN LOJING HIGHLANDS,
KELANTAN**

by.

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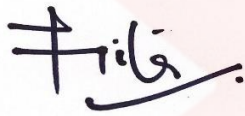
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DECLARATION

I declare that this thesis entitled “Elucidating the Fluctuations in Microorganism Related to *Rafflesia kerri* in Lojing Highlands, Kelantan.” is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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Elucidating the Fluctuations in Microorganism related to *Rafflesia Kerri* in Lojing Highlands, Kelantan

ABSTRACT

This study investigates the microbial dynamics associated with various blooming stages of *Rafflesia kerri*, focusing on the morphological characteristics, density, and biochemical activity of microorganisms in three male flowers. The objective is to assess microorganism density at different blooming stages and determine the biochemical activity of selected microorganism species. Morphological analysis revealed that bacterial cultures exhibited milky, cloudy appearances with filamentous, circular, and irregular shapes across all samples. Microorganism density varied significantly between different blooming stages. The early blooming stage showed the highest microorganism density, decreasing progressively through the mid and late stages. Biochemical tests, including Gram staining, IMViC tests, hydrogen sulfide production, and urease activity, demonstrated that microbial metabolic processes evolved through the blooming stages. In the early stage, the tests showed active metabolic activity with positive results for indole production, Voges-Proskauer reaction, hydrogen sulfide test, and urease activity. For example, in Male Flower 1, urease activity was 50% positive at the 10^{-3} dilution. In the mid stage, test results indicated metabolic changes with positive Gram staining across all dilutions and mixed results for IMViC tests. In the late stage, urease activity remained consistently positive across all dilutions, highlighting its role in nitrogen metabolism. Analysis showed that the highest indole production was found in the early stage (45%), while urea activity decreased from 45% in the early stage to 15% in the late stage. The Voges-Proskauer test showed varying acetoin production with 24% in the early stage, 35% in the mid stage, and 41% in the late stage. The Methyl-Red test showed positive reactions of 40% in the early stage, 20% in the mid stage, and 40% in the late stage. Hydrogen sulfide test results indicated the highest activity in the early and mid-stages (43%), and the lowest in the late stage (14%). These findings provide valuable insights into the changes in microbial density and biochemical activity during the development of *Rafflesia kerri* flowers, as well as differences between the various male flowers. This data helps in understanding the metabolic adaptations of microorganisms and their impact on flower health and development. Overall, this study highlights the complex interactions between microbial communities and the unique blooming processes of *R. kerri*, offering insights into the ecological roles of these microorganisms.

Menjelaskan Fluktuasi Mikroorganisma berkaitan dengan *Rafflesia kerri* di Tanah Tinggi Lojing, Kelantan

ABSTRAK

Kajian ini meneliti dinamik mikrob yang berkaitan dengan pelbagai peringkat pembungaan *Rafflesia kerri*, dengan memberi tumpuan kepada ciri-ciri morfologi, kepadatan, dan aktiviti biokimia mikroorganisma dalam tiga bunga jantan. Objektifnya adalah untuk menilai kepadatan mikroorganisma pada pelbagai peringkat pembungaan dan menentukan aktiviti biokimia spesies mikroorganisma terpilih. Analisis morfologi menunjukkan bahawa kultur bakteria menunjukkan penampilan susu, keruh dengan bentuk filamen, bulat, dan tidak teratur di seluruh sampel. Kepadatan mikroorganisma berbeza dengan ketara antara peringkat pembungaan yang berlainan. Peringkat pembungaan awal menunjukkan kepadatan mikroorganisma tertinggi, berkurangan secara progresif melalui peringkat pertengahan dan akhir. Ujian biokimia, termasuk pewarnaan Gram, ujian IMViC, penghasilan hidrogen sulfida, dan aktiviti urease, menunjukkan bahawa proses metabolik mikrob berkembang melalui peringkat pembungaan. Pada tahap awal, hasil ujian menunjukkan aktiviti metabolik yang aktif dengan hasil positif pada penghasilan indole, reaksi Voges-Proskauer, ujian hidrogen sulfida, dan aktiviti urease. Misalnya, pada Bunga Jantan 1, aktiviti urease adalah 50% positif pada dilusi 10^{-3} . Pada tahap menengah, hasil ujian menunjukkan perubahan metabolik dengan pewarnaan Gram positif pada semua dilusi dan hasil campuran untuk ujian IMViC. Pada tahap akhir, hasil aktiviti urease tetap positif secara konsisten di semua dilusi, menunjukkan peranan penting dalam metabolisme nitrogen. Analisis menunjukkan bahawa penghasilan indole tertinggi ditemui pada tahap awal (45%), sementara aktiviti urease menurun dari 45% pada tahap awal kepada 15% pada tahap akhir. Ujian Voges-Proskauer menunjukkan penghasilan asetoin yang bervariasi dengan 24% pada tahap awal, 35% pada tahap menengah, dan 41% pada tahap akhir. Ujian Methyl-Red menunjukkan reaksi positif 40% pada tahap awal, 20% pada tahap menengah, dan 40% pada tahap akhir. Hasil ujian hidrogen sulfida menunjukkan aktiviti tertinggi pada tahap awal dan menengah (43%), dan terendah pada tahap akhir (14%). Temuan ini memberikan wawasan penting tentang perubahan dalam kepadatan mikrob dan aktiviti biokimia sepanjang perkembangan bunga *Rafflesia kerri*, serta perbezaan antara bunga jantan yang berbeza. Data ini dapat membantu dalam memahami adaptasi metabolik mikroorganisma dan kesannya terhadap kesihatan dan perkembangan bunga. Secara keseluruhannya, kajian ini menekankan interaksi kompleks antara komuniti mikrob dan proses pembungaan unik *R. kerri*, sambil menawarkan pandangan tentang peranan ekologi mikroorganisma ini.

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Rafflesiaceae is a family of holoparasite flowering plants. Most famous for its flowers, known as biggest flower in the world. *Rafflesia* is a holoparasitic plant, a known host plant for *Rafflesia* is *Tetrastigma*: Vitaceae (Nickrent, 2002). The *Rafflesia* flowers are huge and have become a world-class tourist attraction. The flower is a unique, not only in terms of its physical appearance, but the nature of the mystery that has been said to have fascinated scholars, conservationists, and nature lovers to see for themselves the beauty. Furthermore, *Rafflesia* is grown without other vegetative organs such as roots, leaves and stems. Clustered in a group of parasites (holoparasites), *Rafflesia* lives on a host plant (*Tetrastigma* sp.).

Rafflesia is referred to as *Bunga Pakma* in Bahasa Malaysia. Studies revealed additional habitat for *Rafflesia kerri*, including in Lojing Highlands, Mt. Tepuh, Mt. Chamah, Mt. Stong, and Mt. Basor, as well as in Betis River Forest Reserve in Kelantan and Pengkalan Hulu in Perak (Asfarina et al., 2022). *R. kerri* is recognised as the biggest species in Peninsular Malaysia. Having to depend on *Tetrastigma* vines for its existence, it is amazing that its flowers can grow to 110 cm in diameter. But *R. kerri* unable to process food on its own because it depends on the host plant.

In the context of *Rafflesia*, a parasitic flowering plant known for producing the largest individual flower in the world, the relationship with microorganism is significant. *Rafflesia* lacks roots, stems, and leaves, relying entirely on its host plant,

typically a Tetrastigma vine, for nutrients and support. This parasitic lifestyle makes *Rafflesia* dependent on other organisms for its survival, including. The pollination process of *Rafflesia* is fascinating and relies on specific mechanisms for successful reproduction. *Rafflesia* flowers emit a strong odor resembling rotting flesh to attract flies, which act as pollinators. The flies are attracted to the scent and enter the flower in search of food or a place to lay eggs. Within the *Rafflesia* flower, the flies come into contact with the reproductive structures, including the stamens and stigma. Pollen is transferred from the stamen to the stigma of the same or another flower, leading to fertilization and seed production. The role of microorganism in this process is crucial. Microorganism residing within the *Rafflesia* flower contribute to the breakdown of organic matter, including the decaying flesh-like odor produced by the flower. This decomposition process not only aids in the attraction of pollinators but also provides essential nutrients for the plant (Syarifahhaniera et al. 2021)

1.2 Problem Statement

The intricate relationship between *Rafflesia kerri* and the surrounding microorganism in the Lojing Highlands of Kelantan remains inadequately understood. Despite the ecological significance of *Rafflesia* as a parasitic flowering plant, there is a lack of comprehensive knowledge regarding the specific fluctuations in microorganism associated with its pollen. This knowledge gap hinders a thorough comprehension of the ecological dynamics within the *Rafflesia* habitat.

The investigation is prompted by the need to examine how *R. kerri* pollen interacts with and influences the microorganism composition in the Lojing Highlands. Understanding these fluctuations is crucial for gaining insights into the broader ecological implications, including the potential impact on the reproductive success of *Rafflesia* and the overall biodiversity of the region. Additionally, a detailed assessment of the microorganism related to *Rafflesia* can contribute to a more comprehensive understanding of the intricate ecological balance within this unique ecosystem.

Therefore, our knowledge of the variations in the microorganism linked to *R. kerri* pollen in the Lojing Highlands is still lacking, underscoring the necessity of thorough research to close this knowledge gap and improve our comprehension of the ecological dynamics in this unique habitat.

1.3 Objectives

The specific objectives of the study are as listed below:

- i) To assess the microorganism density in response varied to blooming stages conditions of *Rafflesia kerri*.
- ii) To determine the biochemical activity of selected microorganism species associated with *Rafflesia kerri*.

1.4 Scope of study

The study was conducted in the *Rafflesia* Conservation Park, Lojing Highlands, Kelantan. The field survey was done during the period of November 2023 to March 2024. This study only focused on one species: *Rafflesia kerri* male flower, primarily chosen for its accessible pollen. Involves how microorganism worked in *R. kerri*. The samples collected were transferred to the lab in Universiti Malaysia Kelantan Jeli Campus for further analysis. The process involves the used of the cast plate method to effectively isolate the microbial colony, followed by quantification involving counting the units that form the colony (CFUS). After that, the dominant colony was selected for a comprehensive biochemical test, which allows accurate identification of the various microbial species associated with the *R. kerri* male.

1.5 Significant of The Study

The study holds significant importance for the state, local community, and the scientific community for the following reasons:

Biodiversity Conservation: *Rafflesia kerri* is a unique species in the Lojing Highlands, contributing to the country's biodiversity. Lojing Highlands is a place to understand the population and the study of *R. kerri* breeding is important for the preservation of the region's natural heritage.

Tourism and Economy: *Rafflesia* is an attractive attraction and a source of international tourism. Comprehensive studies of the population or microorganism of *R. kerri* can contribute to sustainable tourism practices and stabilize the economy of the surrounding area.

Conservation Strategies: The findings can inform evidence-based conservation strategies not only for *R. kerri* but also for other plant species in similar ecosystems. This can have implications for conservation science and management practices in tropical highland environment.



CHAPTER 2

LITERATURE REVIEWS

2.1 *Rafflesia* in General

Rafflesia is becoming a popular ecotourism attraction in Peninsular Malaysia. Their distribution are extending from northern Perak to Kelantan, Pahang and Terengganu. *R. kerri* is a member of the family Rafflesiaceae a family of holoparasitice flowering plants as it is one of the largest flowers in the world after *R. arnoldii*. With fourteen species recorded in Malaysia, nine are found in Peninsular Malaysia and five in Sabah and Sarawak (Norhazlini et al., 2021). Most forests in Peninsular Malaysia are managed by the State Forestry Departments, with most falling under federal law. *Rafflesia* habitats in Peninsular Malaysia are mostly protected and conserved by being designated as High Conservation Value Forests (HCVFs) or High Conservation Value Areas (HCVAs). The international independent non-profit Forest Stewardship Council (FSC) created HCVFs in 1993 to encourage responsible forest management by forest-based industries (Areendran et al., 2020).

Rafflesia is an endoholoparasite flowering plant that grows without real leaves, stems, or roots, with flowers being the only visible structure. *Rafflesia* is known as the flower of the corpse because of its unpleasant smell, *Rafflesia* is a parasite to the wild grapes of the genus *Tetrastigma*, which provides everything necessary for the *Rafflesia* survive (Sargent, 2022).

2.2 Morphology of *Rafflesia*

Typically, the taxonomy of *Rafflesia* is entirely based on floral morphology (form and structure), with an emphasis on outer appearance. The current species delimitation of *Rafflesia* is based on eight major characters, which are listed by Nais (2001). Based on a comparison of the specimen with the protologue specimen and type images, significant differences are apparent in most morphological features, with few remaining similarities.

Rafflesiaceae are in the family endophytes holoparasitic. *R. kerri* has their vegetative and reproductive bodies showing extreme changes. They occupy among the most diminished vegetative bodies of all angiosperms although they have no known roots and only modified fine white filament bodies containing parts of the cell (Nikolov, 2014). Holoparasitic plants depend on the conditions of their hosts to sustain their lives. When the host plants are harmed or are unable to compete with other plants, holoparasitic plants become completely dependent on them and eventually perish. The accessibility of water resources in the appropriate habitat are also crucial for the survival of holoparasitic plants. *Rafflesia* survival and status are closely correlated with the conditions of their host plants (Akhriadi et al., 2010).

2.3 Reproductive System of *Rafflesia*

Rafflesia's reproductive column now consists of alternating longitudinal ridges and grooves. These features start at the ring and extend to the disc base. The purpose of the grooves is to male flowers have separate anthers, while female flowers also have them. However, this characteristic has a significantly smaller stamen whorl in female flowers. Furthermore, *Rafflesia's* reproductive success is known to be extremely low because of a number of factors, including their rarity, low percentage of buds reaching

the mature stage, a brief anthesis phase, a significant gender difference and the infrequent occurrence of both male and female flowers blooming at the same time (Nais., 2001). *R. kerri* are sapromyophilous flowers that release an unpleasant odour similar to that of rotting meat (Mailina Jamil et al., 2020). For greater *Rafflesia* flower reproduction, this pollination is essential. Pollination agents, like flies, are typically in charge of controlling pollination. This brings the microorganism that will be bred in the male *Rafflesia* flower. *R. kerri* has slimy pulp (anther exudates) under the rim of the disk on male flowers that will be used by flies (Norhazlini et al., 2020).

2.4 Life cycle of *Rafflesia kerri*

The life cycle of *Rafflesia*, a parasitic flowering plant, involves several stages as depicted in the diagram as shown in Figure 2.4.1. The stages include seed germination, emergence from the host, inoculation of the host, swollen host organ, bract abscission, mature fruit, flower beginning, fully open flower, and seed dispersal. The entire life cycle spans from 48 months (Stage II) to 160 days (Stage VIII). The life cycle of *Rafflesia* spans from 48 months to 160 days.

The life cycle of *Rafflesia* is complicated since it includes the unseen aspect of growth (Syarifah et al., 2021). Interestingly, *Rafflesia* doesn't have roots, stems or leaves like most plants (Sargent, 2022). These flowers can be more than three feet in diameter, releasing a strong smell resembling a damaging corpse to attract flies or flowering agents for spread.

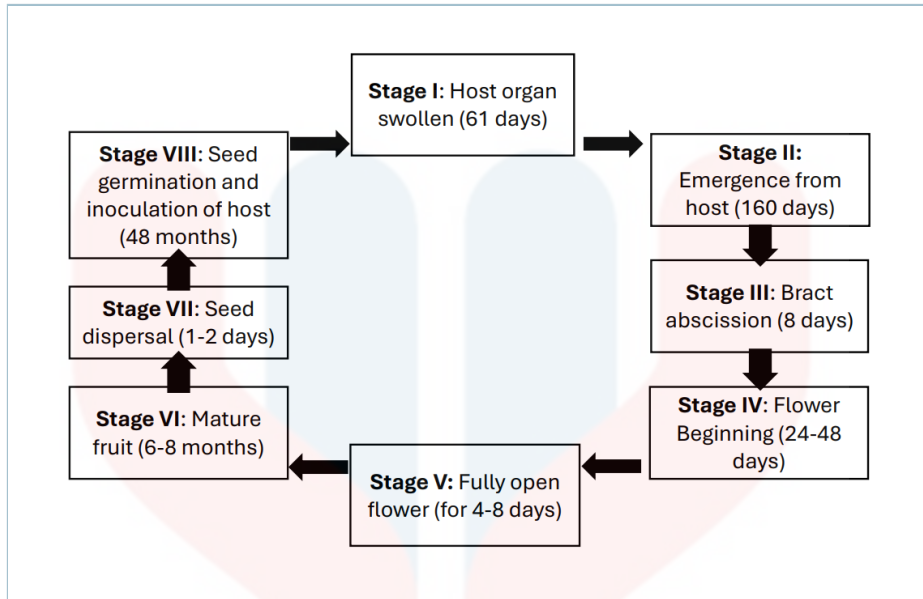


Figure 2.4.1. Life cycle of *Rafflesia* (redrawn from Nais (2004) by N. Sofiyanti).

2.5 Microorganism

Microorganism refers to the community of small living organism microorganisms that inhabit a diverse type of environment, such as soil, water, or the human body. These microorganisms include bacteria, fungi, viruses, and other microscopic organisms. Microorganisms play crucial roles in various ecosystems and biological processes, contributing to nutrient cycling, decomposition, and overall ecosystem balance. In the context of plants, microorganism are often associated with the root system and can influence plant health and growth. Microbes are a valuable extreme in biology, playing a crucial role in ecosystem processes and maintaining life. They represent the richest treasury in nature, establishing biogeochemical cycles and food chains. Over billions of years, they have evolved into various niches, forming essential relationships among themselves and higher organisms (Onen., 2020).

The microbial symbiont, together with the plant host, form the holobiont, a partnership that involves the host and the related microorganism community that are

united with each other (Rosenberg, 2018). Microbial symbionts include endophytes that live in plant tissues that can enhance growth, development and health of plants by increasing nutrient intake, producing phytohormones, performing nitrogen fixation, and/or priming master defences against pathogens (Felestrino et al., 2017). *Rafflesia* is a threatened endophytic holoparasitic plant that spends most of its life in the tissue of its sole host plant, *Tetrastigma* (Molina et al., 2024). Following imbibition, *Rafflesia* seeds exhibit transcriptional activity, which may signal that they are ready to germinate if they receive host stimulation.

CHAPTER 3

MATERIAL DAN METHODS

3.1 Description of the Study Area

The study area is located at the *Rafflesia* Conservation Park, near to Kg. Jedip in Lojing Highland, Kelantan as shown in Figures 1 and 2. This area is laying between 4°32' and 4°47' N in latitude and 101°20' and 101°34' E in longitude. Lojing Highlands can be reached through Gua Musang, Kelantan and from Cameron Highlands, Pahang. Besides the *Rafflesia* Conservation Park, the State Government of Kelantan also has declared a 50 hectare (ha) area in Lojing Highlands as High Conservation Value Forest (HCVF) for *Rafflesia kerri* (Fauzan et al., 2021). Lojing Higlands is a 45 minutes' drive from Cameron Higlands to the *Rafflesia* area in Kg. Jedip, Post Brooke. Lojing is a naturally covering Hill Dipterocarp and Montane Forests with stunning views between 1,000 to 2,300 meters above sea level. The mountains in this area have an altitude of about 610–1,500 meters above sea level with a temperature range of 180°–250° (Dony et al., 2014).

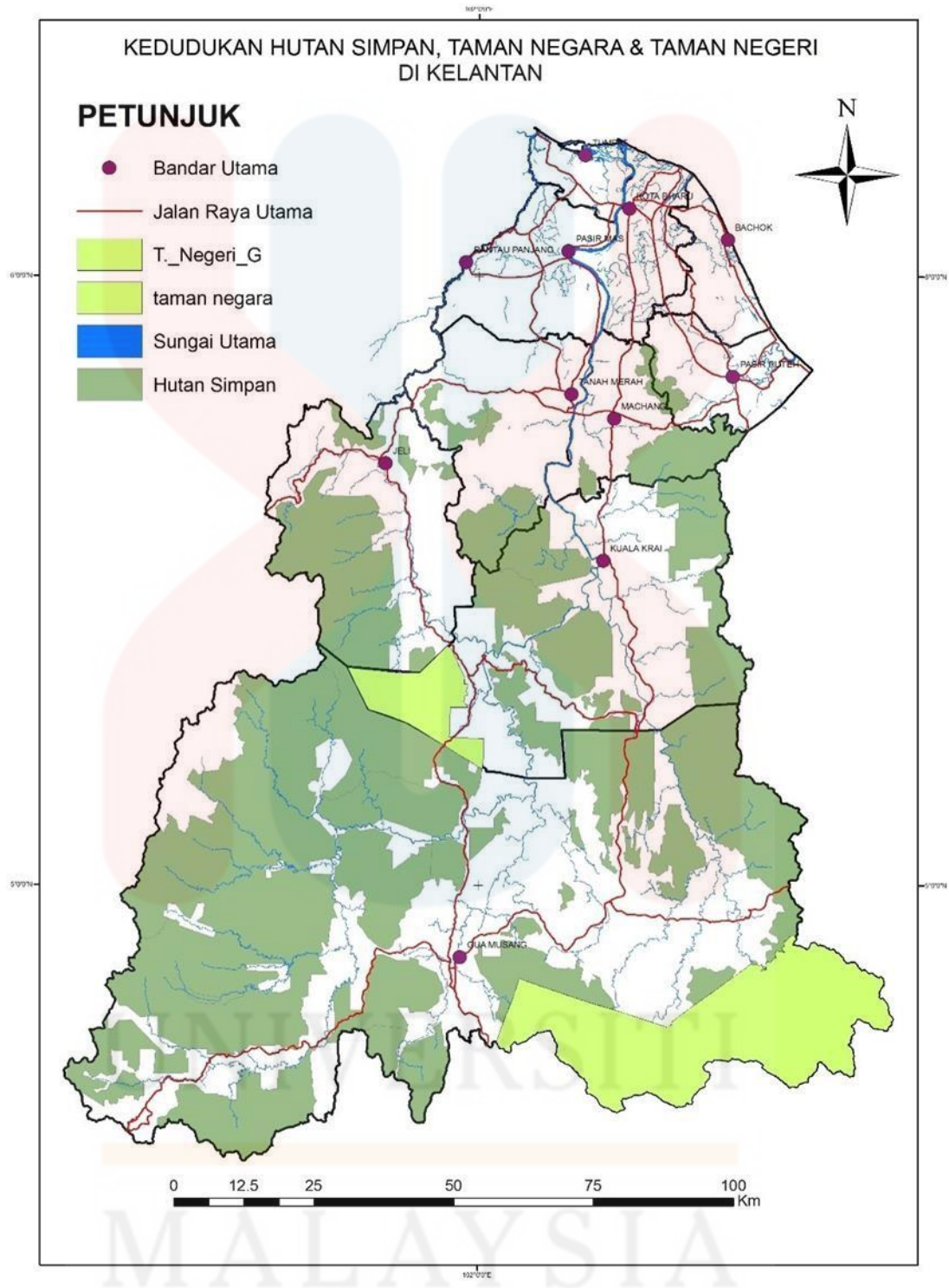


Figure 3.1a. Locations of study area, forest reserves, national parks, and state parks in Kelantan Malaysia (Zulhazman., 2012)

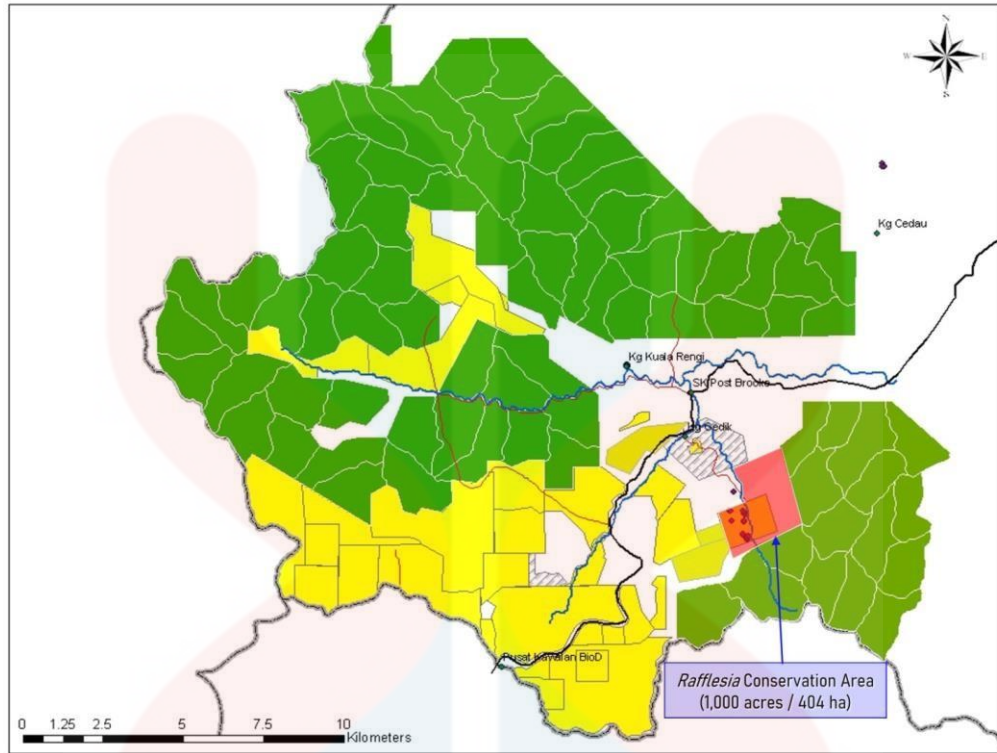


Figure 3.1b. Close up view of study area; *Rafflesia* Conservation Area, Lojing

Highlands Kelantan, Malaysia (Zulhazman., 2012)

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3.2 Material

The equipment used in this study are show in Tables 1 and 2 below:

Table 3.2.1 The equipment used during field survey

No.	Equipment	Functions
1.	The Global Positioning System (GPS)	To get location, velocity and elevation, trilateration collects signals from satellites to output location information.
2.	Sterile swabs	To collect the sample
4.	Alcohol, Formalin (ethanol)	Eliminate external bacteria
5.	Glove	To help keep your hands clean

Table 3.2.2 The equipment used during laboratory analysis

No.	Equipment	Functions
1.	Pipette (10mL)	Place the pipette tip into the liquid you wish to transfer. To transfer precise volumes of liquid. For example, a 10 mL pipette is designed to measure and dispense exactly 10 milliliters of liquid into a container, such as a test tube
2.	Sterile swabs	To detect substance consumption.
3.	Colony Counters	To count the number of colonies, To effectively isolate microbial colonies from the collected samples.

4.	Petri Glass	To grow microorganisms by providing a large storage space and preventing it from being contaminated with other species
5.	Pipette tips (10mL)	To measure and transfer liquid solution
	Sampling bottle (1L)	To store each sample solution
6.	Cold storage box	To store for the sample during transporting to laboratory
7.	Glove	Protect hands
8.	Test tube	Test tubes can be used to store liquid samples temporarily, keeping them safe from contamination and evaporation.
9.	Pipettes	To dispense measured volumes of liquids.
10.	Glass Spreader	Used in Sterile Technique
11.	Bunsen Burner	To sterilize objects on high heat
12.	Alcohol	For various purposes such as solvents, reagents, and starting materials in organic synthesis.
13.	Balance Machine	Balancing rotating machine
14.	Incubator	To grow and maintain cell culture
15.	Biological Safety Cabinet	To protect personnel against biohazardous
16.	Autoclave	To kill harmful bacteria
17.	Inoculating Loop	To transfer and spreading sample
18.	Culture Media: Urea Agar Base, MIU Medium Base, MRVP Medium, Simmons Citrate Base, LIM Medium, TSI Agar	Reagent for IMViC Test procedure

19.	Hirayama HV-10 Autoclave Machine	The primary function of the HV-10 is to sterilize laboratory tools, glassware, media, and other equipment by using high-pressure steam. This process effectively kills bacteria, viruses, fungi, and spores.
20.	Microscope slide	Observing microorganisms with Microscope.
21.	Chemical & Reagent	<p>Kovac's reagent To determine the ability bacteria to split indole</p> <p>MR-VP broth To qualitative procedure for MR-VP test</p> <p>Methyl red to determine pH transition</p> <p>Barritt's reagent A and B To detection of acetoin production by bacterial</p> <p>Crystal violet To stain the nuclei of adherent cells</p> <p>Methylene blue To help make cell show up against their background</p>
22.	Urea Broth	To test for rapid urease-positive bacteria
23.	Blue cap bottle (1L)	To collect the filtered solution
24.	Glycerol stock	To preventing damage to the sample

3.3 Method

3.3.1 Field Sampling

A field survey was conducted to ascertain the blooming habitat of *Rafflesia kerri*. The objective of this survey is to pre-emptively address challenges associated with sample collection during the research process. Another crucial matter is to accurately identify the gender of *Rafflesia* flowers, specifically focusing on the male counterpart in the case of *R. kerri*. Proper differentiation between male and female flowers is essential, and careful reading is required to gather this information comprehensively (Figure 3.3.2).

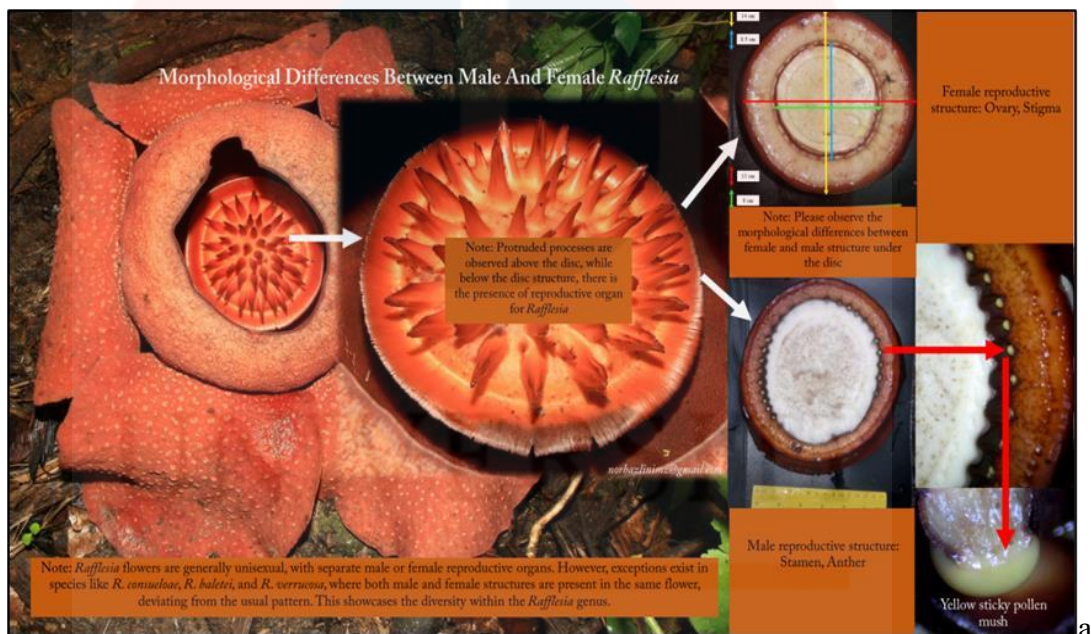


Figure 3.3.2. Morphological Differences Between Male and Female Flower of *Rafflesia*. (Source: Illustrated by Norhazlini M.Z.)

Once the gender has been successfully distinguished, the focus of this study shifts to male flowers. Samples are obtained from inside the male flowers, with a specific area targeted for swabbing. This swabbing process is repeated several times

using a sterile wooden swab stick beneath the disc of *R. kerri*. The sampling is conducted at three distinct stages of blooming *Rafflesia*: (i) Fresh blooming, (ii) Middle blooming, and (iii) Late blooming. Following the collection of all samples, they transported to the laboratory for in-depth analysis.

3.3.2 Media Preparation

3.3.2.1 Luria Broth

To make 1L round LB, 25grams of pre-mixed round powder, which is this powder contains 10 grams of trypton, 5grams yeast extract ang 10grams NaCl. Mix all the powder with 1L H₂O. Rotate the bottle in round motion to mix. Then, Transfer the LB solution to an appropriate autoclave-safe container. Autoclave the solution at 121°C (250°F) for 15-20 minutes to sterilize. Allow the LB broth to cool to room temperature before use.

3.3.2.2 Nutrient Agar

To prepare 1L of nutrient agar, 28 grams of nutrient agar powder was weighted, diluted with 500 mL of distilled water. Another 500 mL of distilled water was added to reach a volume of 1L. The mixture was mixed homogenously. Next, the mixture was autoclaved for 15 minutes at 121°C. After the autoclaved, the media was transferred to laminar cabinet, and the media was poured into sterile petri dish. The plate-media was stored in the chilled freezer at 4 °C and ready to be used.

3.3.2 Laboratory Analysis

3.3.3 Determination of Microorganism Density Association with *Rafflesia kerri*.

The spread plate technique serves as one of the isolation methods employed in microbiology. This particular method is utilized to isolate and quantify microbial colonies from a liquid sample. To execute this technique, various apparatus are required, including test tubes, pipettes, an L-shaped glass spreader, nutrient agar plates, a Bunsen burner and 70% ethanol (EtOH).

The procedure commences by extracting 1 milliliter of the sample using a sterile pipette, depositing it at the center of the agar plate. Before introducing the sample, it is essential to prepare serial dilutions to ensure that the microbial cell count is within a quantifiable range. Once armed with the sample dilutions, it is crucial to label each plate with its respective dilution factor.

Following the addition of the sample to the plate, the glass spreader is immersed in alcohol or acetone and subjected to a flame to sterilize it. After a brief waiting period, the sample is evenly spread across the agar surface. This is achieved by gently rotating the plate and maintaining the spreader at a 45-degree angle. By decreasing hand pressure on the agar surface and utilizing a rotator, the spreading process is facilitated.

The plates were incubated at 37 °C for a period of 24 hours. After this incubation period, the forming colonies were observed and counted.

3.3.4 Examination of Biochemical Activity of Selected Microorganism Species Associated with *Rafflesia kerri*.

The identification of bacteria through biochemical tests involves a series of essential steps. These tests encompass Gram Stain, Starch Hydrolysis, Carbohydrate Fermentation, Indole Production, Methyl-Red (MR), Voges-Proskauer (VP) reaction, Citrate test, Hydrogen Sulfide Test, Urease Test, and Phenylalanine Deaminase test.

3.3.4.1 Gram Stain:

Gram Stain is employed to differentiate between gram-positive and gram-negative organisms. The use of purple crystal dye and iodine forms violet-iodine complex crystals, aiding in the retention of the dye by gram-positive microorganisms due to their higher peptidoglycan content.

3.3.4.2 Starch Hydrolysis:

For Starch Hydrolysis, fresh colonies are sampled and inoculated onto a plate, which is then incubated. Post-incubation, a gram solution of iodine is added, and observations are recorded to detect starch hydrolysis.

3.3.4.3 Carbohydrate Fermentation:

Carbohydrate Fermentation: Add samples to media containing glucose, lactose, and sucrose. Incubate the media at 37°C for 24 hours. After incubation, observe for any changes in the media, including color changes or gas production, indicating acid production due to carbohydrate fermentation. To determine the ability of the microorganisms to ferment specific carbohydrates, which results in the production of acids or gases.

Indole Production: Indole Production is tested by inoculating the SIM tube and incubating it at 37 °C for 24 hours. The Kovac reagent is then used to run the indole test.

3.3.4.4 Methyl-Red (MR):

Methyl-Red involves samples into a labeled medium specifically designed for the methyl-red test, incubating at 37 °C for 24 hours, and conducting the MR test using methyl red drops to assess acid fermentation.

3.3.4.5 Voges-Proskauer (VP) Reaction:

Add samples to a labeled medium designed for the Voges-Proskauer test. Incubate the medium at 37°C for 24 hours. After incubation, perform the VP test by adding Barritt's A-reactant to the medium.

3.3.4.6 Citrate Test:

Citrate Test utilizes Simmon's citrate slant, and observations were made after incubation at 37 °C for 24 hours to detect citrate utilization.

Hydrogen Sulfide Test: Hydrogen Sulfide Test employs SIM medium, and after incubation at 37 °C for 24 hours, the formation of black precipitation on the medium.

3.3.4.7 Urease Testing:

Urease Testing involves inoculating bacteria into urea broth and incubating at 37 °C for 24 hours.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Morphology Characteristic of Microorganism

The results of morphological characteristics of microorganism from three male flowers of *Rafflesia kerri*, namely Male Flowers 1, 2 and 3 were discussed in sub-topics below.

4.1.1 Morphological Characteristics of Microorganism Male Flower 1

The findings of morphological characteristics of microorganism from the Male Flower 1 were displayed in Figure 4.1a and tabulated in figure 4.1.1.



Figure 4.1a. The male of *Rafflesia kerri* (Flower 1), A. The disc of flower. A. The fresh-blooming stages. B. The mid-blooming stage. C. The late-blooming stage.



Figure 4.1.1 Petri dish containing bacteria swabbed under discs after being incubated for 3 days at 37° C.

The edge margin, shape and color was determined through light microscope. From the observation, the cultures on the plate had milky and cloudy appearance whereas the shape of the bacteria consists of filamentous, circular and irregular.

4.1.2 Morphological Characteristics of Male Flower 2

The findings of morphological characteristics of the Male Flower 2 were displayed in Figure 4.1b and tabulated in figure 4.1.2

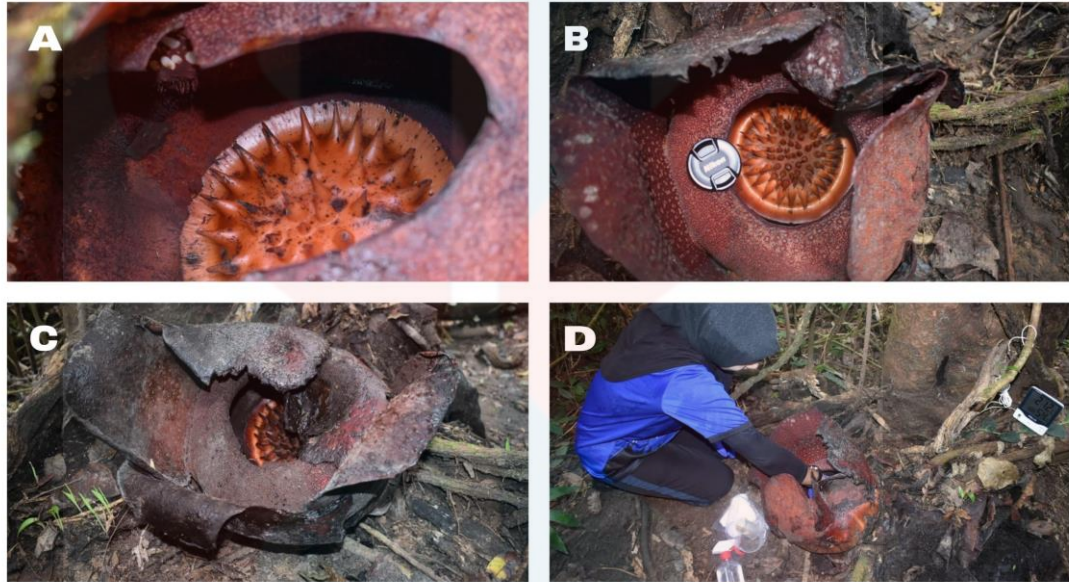


Figure 4.1b. The male of *Rafflesia kerri* (Flower 2), A. The dics of flower. A. The fresh-blooming stages. B. The mid-blooming stage. C. The late-blooming stage.



Figure 4.1.2 showed petri dish containing bacteria swabbed at under dics point after being incubated for 3 days at 37°C.

The edge margin, shape and color was determined through light microscope. From the observation, the cultures on the plate had milky and cloudy appearance, whereas the shape of the bacteria consists of filamentous and irregular.

4.1.3 Morphological Characteristic of Male Flower 3

The findings of morphological characteristics of the Male Flower 2 were displayed in Figure 4.1c and tabulated in figure 4.1.3.



Figure 4.1c. The male of *Rafflesia kerri* (Flower 3), A. The dics of flower. A. The fresh-blooming stages. B. The mid-blooming stage. C. The late-blooming stage

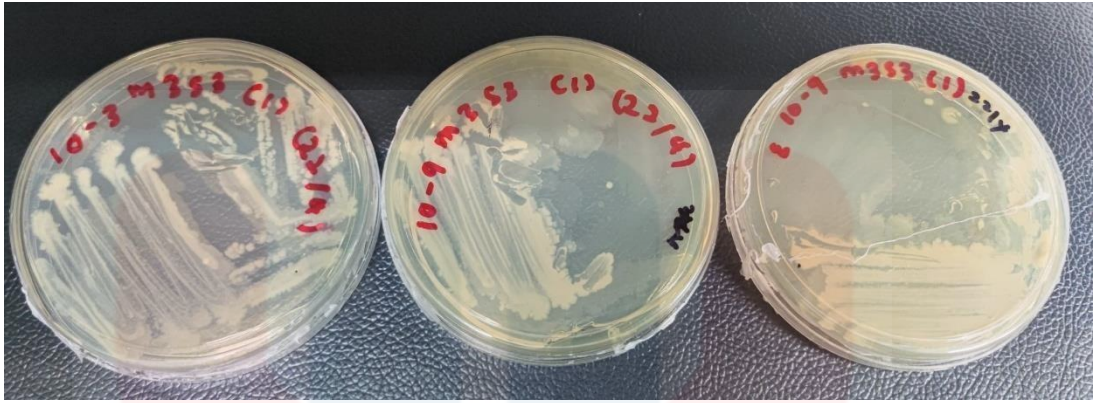


Figure 4.1.3 First, second and third replication of bacteria swabbed under dics after being incubated for 3 days at 37°C of *Rafflesia kerri*.

The edge margin, shape and color was determined through light microscope. From the observation, the cultures on the plate had milky and cloudy appearance, whereas the shape of the bacteria consists of filamentous, circular and irregular.

4.2 Microorganism Density in Three Different Male Flower in Different Stage of *Rafflesia Kerri*.

The results of microorganism density from three male flowers of *Rafflesia kerri*, namely Male Flowers 1, 2 and 3 were discussed in sub-topics below.

4.2.1 Male Flower 1

Table 4.2.1 Microorganism density in response varied to blooming stages conditions of *Rafflesia kerri* for Male Flower 1.

Plate	Tube dilution	ml of dilution plated	Final dilution on plate	Number of Colonies	Bacterial count per ml of sample (CFU/ml)	Average count per ml of sample (CFU/ml)
M1S1	10-3	1	1000	76	76000	76×10^3
M1S1	10-6	1	1000000	34	34000000	34×10^6
M1S1	10-9	1	1000000000	3	3000000000	76×10^9
M1S2	10-3	1	1000	80	80000	38×10^3
M1S2	10-6	1	1000000	34	34000000	56×10^6
M1S2	10-9	1	1000000000	13	13000000000	43×10^9
M1S3	10-3	1	1000	48	48000	48×10^3
M1S3	10-6	1	1000000	15	15000000	15×10^6
M1S3	10-9	1	1000000000	8	8000000000	18×10^9

Based on table 4.2.1, Early stage shows an increasing bacterial count with higher dilutions, indicating a strong microbial presence initially diluted to reveal higher concentrations at subsequent dilutions. For instance, in M1S1, the number of colonies decreases from 76 at 10–3 dilution to 3 at 10–9 dilution. Middle stage or M1S2 also shows a significant increase in bacterial counts with dilution, but slightly less pronounced than M1S1, especially at the highest dilution. For instance, M1S2 show 13 colonies Bacterial count: 13,000,000,000 CFU/ml (13×10^9) and M1S1 show 3 colonies record lowest bacterial count per ml of sample bacterial count: 3,000,000,000 CFU/ml (3×10^9). Late stage (M1S3) has the lowest bacterial counts

across all dilutions, indicating it may have the lowest initial bacterial concentration or less favourable conditions for microbial growth. Early stage (MIS1) consistently shows higher bacterial counts across all dilutions, indicating the most robust initial bacterial population or most favourable conditions for microbial growth.

The bacterial counts across different samples and dilutions provide valuable insights into the microbial density and environmental conditions within each sample. MIS1 shows the highest microbial density, likely due to optimal conditions for bacterial growth. MIS3, on the other hand, displays the lowest microorganism density, possibly due to less favourable conditions or lower initial bacterial counts. The variations between these samples emphasize the importance of environmental factors, nutrient availability, and initial microbial populations in determining bacterial counts in different stages of dilution.

4.2.2 Male Flower 2

Table 4.2.2 Microorganism density in response varied to blooming stages conditions of *Rafflesia kerri*. for Male Flower 2.

Plate	Tube dilution	ml of dilution plated	Final dilution on plate	Number of Colonies	Bacterial count per ml of sample (CFU/ml)	Average count per ml of sample (CFU/ml)
M2S1	10-3	1	1000	100	100000	100×10^3
M2S1	10-6	1	1000000	75	75000000	75×10^6
M2S1	10-9	1	10000000000	72	72000000000	72×10^9
M2S2	10-3	1	1000	88	88000	88×10^3
M2S2	10-6	1	1000000	65	65000000	65×10^6
M2S2	10-9	1	10000000000	40	40000000000	40×10^9
M2S3	10-3	1	1000	65	65000	65×10^3
M2S3	10-6	1	1000000	55	55000000	55×10^6
M2S3	10-9	1	10000000000	34	34000000000	34×10^9

Based on the table 4.2.2, the number of colonies decreases with increasing dilution factor. This is because more diluted samples have fewer bacteria, resulting in fewer colonies. For instance, in M2S1, the number of colonies decreases from 100 at 10⁻³ dilution to 72 at 10⁻⁹ dilution.

The bacterial count per ml of the original sample is calculated by multiplying the number of colonies by the inverse of the dilution factor. As the dilution factor increases, the bacterial count per ml increases significantly due to the large multiplication factor. For example, in M2S1, the bacterial count per ml increases from 100,000 CFU/ml at 10⁻³ dilution to 72,000,000,000 CFU/ml at 10⁻⁹ dilution.

Early stage (M2S1) has the highest bacterial counts at all dilutions, particularly notable at 10⁻⁹ dilution record 72 colonies, indicating a very high initial bacterial density. Male 2 in middle stage (M2S2) record 88 colonies also shows high bacterial counts but is lower than M2S1 record 100 colonies, suggesting a slightly lower bacterial density overall. Male 2 in late stage (M2S3) has the lowest bacterial counts compared to M2S1 and M2S2 at lower dilutions, though it shows significant bacterial density at higher dilutions, similar to the other samples.

Early stage (M2S1) record 100 colonies has the highest bacterial density overall, followed by middle stage (M2S2) record 72 colonies and then late stage (M2S3) record 65 colonies. This suggests that the initial bacterial concentration is highest in early stage and lowest in late stage, with middle stage falling in between. This trend highlights the importance of dilution in accurately estimating bacterial density.

4.2.3 Male Flower 3

Table 4.2.3 Microorganism density in response varied to blooming stages conditions of *Rafflesia kerri* for Male Flower 3.

Plate	Tube dilution	ml of dilution plated	Final dilution on plate	Number of Colonies	Bacterial count per ml of sample (CFU/ml)	Average count per ml of sample (CFU/ml)
M3S1	10-3	1	1000	78	78000	23 x 10 ³
M3S1	10-6	1	1000000	23	23000000	23 x 10 ⁶
M3S1	10-9	1	10000000000	13	13000000000	13x 10 ⁹
M3S2	10-3	1	1000	63	63000	63x10 ³
M3S2	10-6	1	1000000	27	27000000	27x 10 ⁶
M3S2	10-9	1	1000000000	2	2000000000	2x10 ⁹
MIS3	10-3	1	1000	62	62000	62x10 ³
M3S3	10-6	1	100000	16	16000000	16x10 ⁶
M3S3	10-9	1	1000000000	11	110000000	11x10 ⁹

The number of colonies decreases with increasing dilution factor. Higher dilutions lead to fewer bacteria being present on the plate, resulting in fewer colonies. For example, in M3S1, the number of colonies decreases from 78 at 10–3 dilution to 13 at 10–9 dilution.

Male 3 stage 1 (M3S1) is early stage with highest microorganism density. M3S1 exhibits the highest bacterial counts across all dilutions, especially at the 10–9 dilution where it reaches 13,000,000,000 CFU/ml. This result indicates that M3S1 has the highest initial bacterial concentration among the samples. Several factors could contribute to this observation: The sample might have been taken from a blooming stage that provides optimal conditions for bacterial growth, such as high nutrient availability or a suitable microenvironment that supports a dense microbial population.

Middle stage has the lowest bacterial counts at lower dilutions and at 10–9 dilution compared to early and late stage. M3S3 shows high bacterial counts at 10–9 dilution, though lower than early stage (M3S1). The counts at lower dilutions are also

significant, indicating that while late stage (M3S3) has a considerable bacterial density, it is not as high as early stage (M3S1).

4.2.4 Microorganism Density for Three Different Male Flower with Three Dilutions in Early Stage.

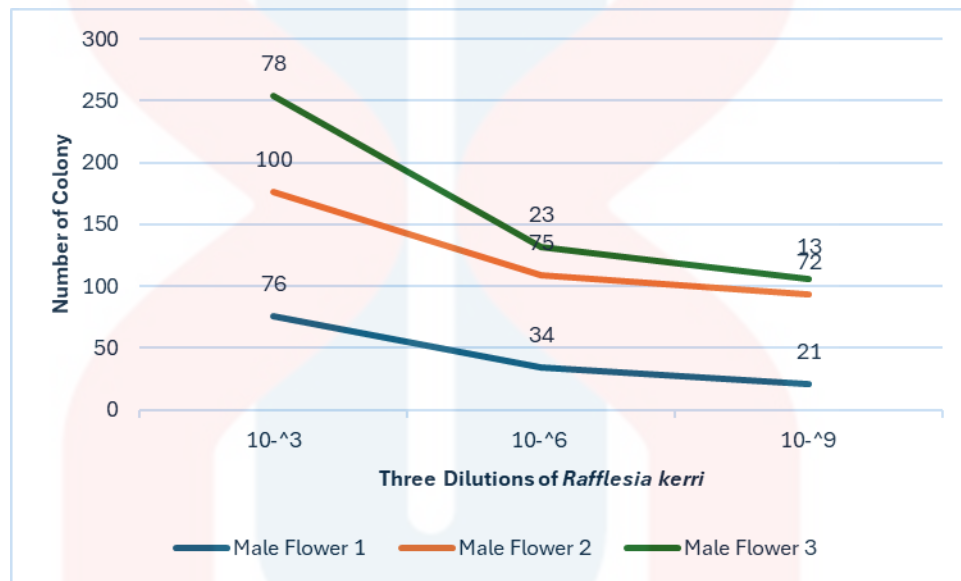


Figure 4.2.4 Microorganism density for three Male Flower in early stage with three dilutions of *Rafflesia kerri*.

The graph effectively shows how bacterial colony counts are influenced by the level of dilution, with distinct patterns emerging for each sample of *R.kerri*. The differences between the samples may reflect variations in the microenvironment or the stage of blooming, which could influence the microorganism density.

Across all three male flowers, the number of colonies decreases as the dilution increases. This is consistent with the expectation that as dilution increases, the concentration of bacteria decreases, leading to fewer colonies forming on the plates. Male Flower 1 shows a steady decrease in the number of colonies with increasing dilution. Male Flower 2 has the highest number of colonies at 10^{-3} dilution and

maintains a relatively high number even at higher dilutions compared to the other two flowers. Male Flower 3 has a significant drop in the number of colonies from 10⁻³ to 10⁻⁶ dilution, and the lowest colony count at the 10⁻⁹ dilution among all three samples.

Higher microflora density could be attributed to factors like genetic predisposition, environmental conditions, or symbiotic relationships with specific microorganisms that promote growth. Lower Microflora density may be due to factors such as competition with other microorganisms, lack of beneficial symbiotic relationships, or environmental stressors affecting growth (Megan et al.,2020).

4.3 Biochemical Activity of Selected Microorganism Species Associated with *Rafflesia kerri*.

4.3.1 Male Flower 1

Table 4.3.1 Biochemical test involving Gram Staining, IMVic TEST, Hydrogen Sulfide Test and Urease Activity for Male Flower 1.

Plate	Gram Stain	IMViCTEST				Hydrogen Sulfide Test	Urease Activity
		Indole Production	Methyl-Red Reaction	Voges-Proskauer (VP) reaction	Citrase Use		
Early Blooming Stage							
10-3	-	+	-	+	-	+	+
10-6	-	+	-	+	-	+	+
10-9	-	+	-	+	-	+	+
Mid Blooming Stage							
10-3	+	+	+	+	-	+	-
10-6	+	+	-	-	-	+	-
10-9	+	+	-	-	-	+	-
Late Blooming Stage							
10-3	-	-	-	+	-	-	+
10-6	-	-	+	+	-	+	+
10-9	-	-	+	+	-	+	+

In the early stage, the results are consistent across all three dilutions, indicating a stable biochemical profile. Most tests (Indole Production, Voges-Proskauer Reaction, Hydrogen Sulfide Test, and Urease Activity) show positive results, except for Gram Stain, Methyl-Red Reaction, and Citrase Use, which are negative. This suggests active metabolic processes early on. There is more variability in the mid stage. While Gram Stain, Indole Production, and Hydrogen Sulfide Test remain positive across all dilutions, other tests show mixed results.

Dilution 10-3 shows positive results for more tests compared to 10-6 and 10-9, indicating increased metabolic activity in a less diluted sample. All dilutions show a negative result for Gram Stain, indicating a shift in cellular characteristics.

Urease Activity is consistently positive across all dilutions in the late stage, suggesting a role in nitrogen metabolism. Indole Production becomes negative, while Methyl-Red Reaction varies with dilution. As the organism progresses through its stages, its metabolic and physiological processes evolve. This results in variations in biochemical activity.

4.3.2 Male Flower 2

Table 4.3.2 Biochemical test involving Gram Staining, IMVic TEST, Hydrogen Sulfide Test and Urease Activity for Male Flower 2.

Plate	Gram Stain	IMViCTEST				Hydrogen Sulfide Test	Urease Activity
		Indole Product ion	Methyl-Red Reaction	Voges-Proskauer (VP) reaction	Citrase Use		
Early Blooming Stage							
10-3	-	+	+	-	-	+	+
10-6	-	+	+	-	-	+	+
10-9	+	+	-	-	-	+	+
Mid Blooming Stage							
10-3	+	-	-	+	+	+	-
10-6	+	-	-	+	+	+	-
10-9	+	-	-	+	+	+	-
Late Blooming Stage							
10-3	-	-	-	+	+	-	+
10-6	-	-	+	-	-	-	+
10-9	-	-	+	-	-	-	+

The early stage shows a relatively uniform biochemical profile with positive results for Indole Production, Methyl-Red Reaction, Hydrogen Sulfide Test, and Urease Activity. This suggests active metabolic processes related to protein and nitrogen metabolism. The consistent negative Gram Stain (except for the 10-9 dilution) indicates the presence of Gram-negative bacteria. The mid stage reveals significant metabolic changes, with positive Gram Stain results across all dilutions, suggesting the presence of Gram-positive bacteria. The consistent negative results for Indole Production and Methyl-Red Reaction, along with positive results for Voges-Proskauer Reaction, Citrate Use, and Hydrogen Sulfide Test, indicate an adaptive metabolic state with enhanced carbohydrate and sulfur metabolism. In the late stage, the biochemical profile becomes more diverse.

The consistent negative Gram Stain suggests a reversion to Gram-negative bacterial characteristics. The variability in test results, with positive Methyl-Red Reaction and Urease Activity in the 10-6 and 10-9 dilutions, and positive Voges-Proskauer Reaction and Citrate Use in the 10-3 dilution, reflects a complex metabolic state, likely due to the organism's maturation and adaptation to its environment.

4.3.3 Male Flower 3

Table 4.3.3 Biochemical test involving Gram Staining, IMViC TEST, Hydrogen Sulfide Test and Urease Activity for Male Flower 3.

Plate	Gram Stain	IMViC TEST				Hydrogen Sulfide Test	Urease Activity
		Indole Production	Methyl-Red Reaction	Voges Proskauer (VP) reaction	Citrase Use		
Early Blooming Stage							
10-3	-	-	-	+	+	-	+
10-6	-	+	+	-	-	-	-
10-9	-	+	+	-	-	-	+
Mid Blooming Stage							
10-3	-	+	+	-	-	-	+
10-6	-	-	-	+	+	-	+
10-9	-	+	-	+	+	-	+
Late Blooming Stage							
10-3	-	-	-	+	+	-	+
10-6	-	-	-	+	+	-	+
10-9	-	-	-	+	+	-	+

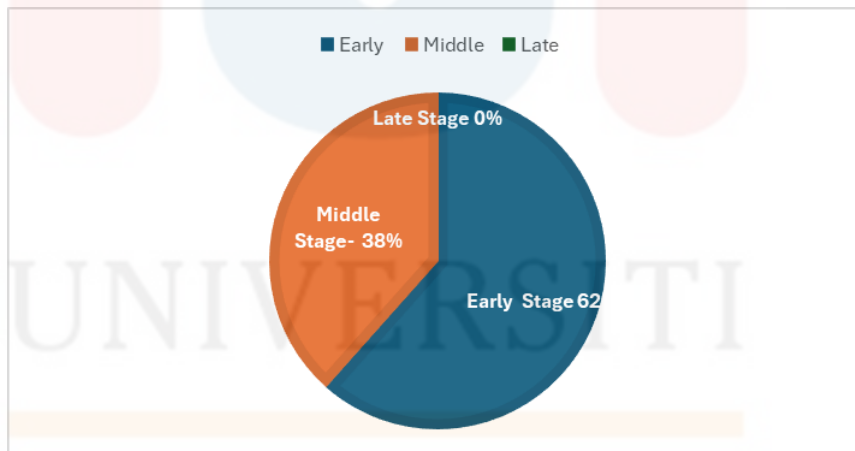
In the Early Stage, the bacteria showed variability in IMViC tests and consistent negative Gram Stain results. The presence of positive Voges-Proskauer reaction and Citrase use in two out of three samples indicates possible metabolic versatility. In the Mid Stage, the bacteria continue to show consistent negative Gram Stain results and mixed results in the IMViC tests.

The presence of positive Urease activity in two out of three samples suggests increased urea metabolism. In the Late Stage, the bacteria showed consistent negative Gram Stain results and consistently positive results for Citrase Use, Voges-Proskauer

Reaction, and Urease Activity across all samples. This suggests a shift towards specific metabolic activities, including the use of citrate and urea, as the stage progresses.

Across all stages, the bacteria from Male 3 show consistent negative Gram Stain results, indicating they are Gram-negative. The IMViC tests and other biochemical activities (Hydrogen Sulfide and Urease Activity) show variability, reflecting different metabolic capabilities at different stages and dilutions. The Late Stage shows more consistency in positive Citrase Use, Voges-Proskauer Reaction, and Urease Activity, indicating stable metabolic traits at this stage. This pattern suggests a progression of metabolic adaptation in the bacterial population over time.

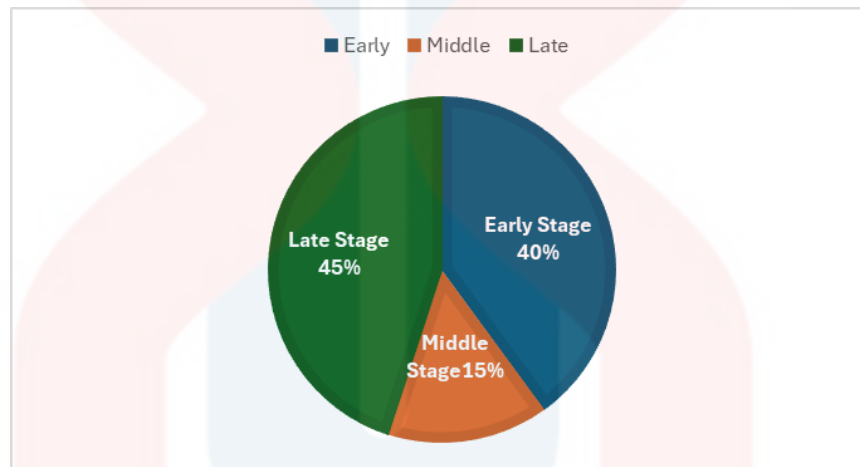
4.4 The Differences of Biochemical Activity in Male flowers 1, 2 and 3.



4.4.1 The percentage of the indole test positive reaction for three different Male Flowers in blooming stage of *Rafflesia kerri*.

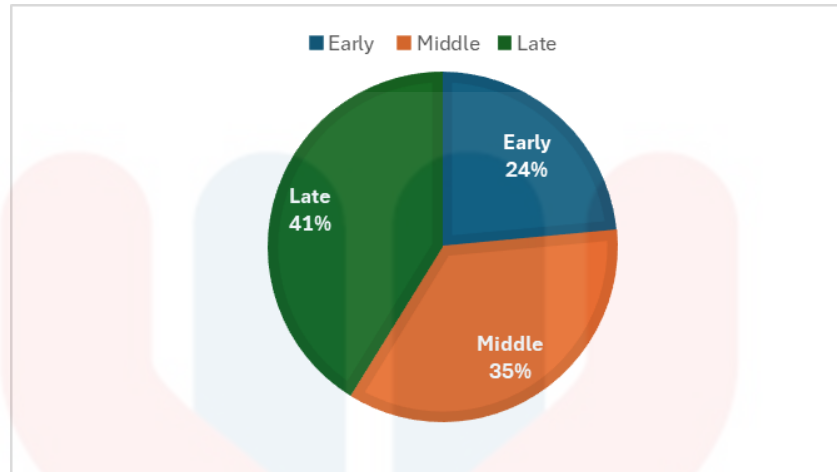
The presence of indole can be detected by adding Kovac's reagent to the culture medium. This indicates that as time progresses, more indole is being produced by the microflora species associated with *R. kerri*. The highest percentage of indole

production is observed in the early stage, followed by the middle stage, and no indole production is seen in the late stage. The indole test examines the organism's ability to destroy the amino acid tryptophan and produce indole. It is used as part of the IMViC procedure, a test battery designed to distinguish between members of the Enterobacteriaceae family (Maria., 2009).



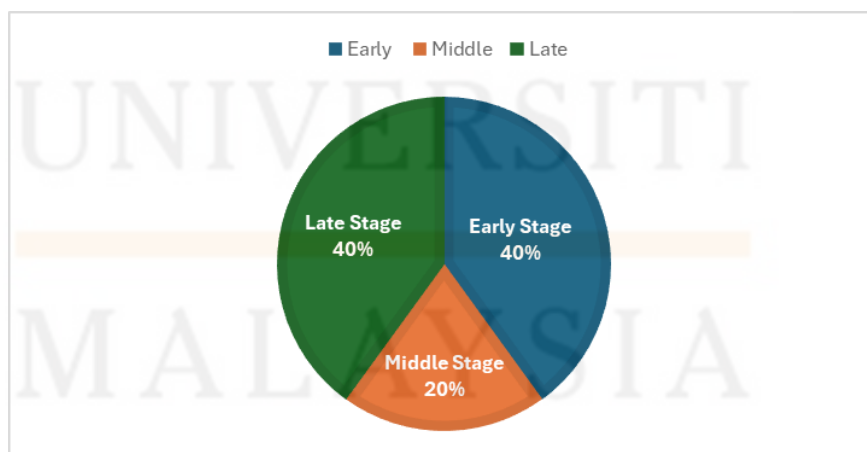
4.4.2 Urea activity positive reaction for 3 different Male Flowers in different blooming stages of *Rafflesia kerri*

Based on the percentages provided, it can be observed that the urea activity decreases as the stages progress from early to late. The highest urea activity is observed in the early stage (45%), followed by the middle stage (40%), and the lowest activity is in the late stage (15%). The trend of decreasing urea activity from early to late stages may indicate a shift in metabolic processes or microbial populations associated with *R.kerri* as it progresses through different developmental stages



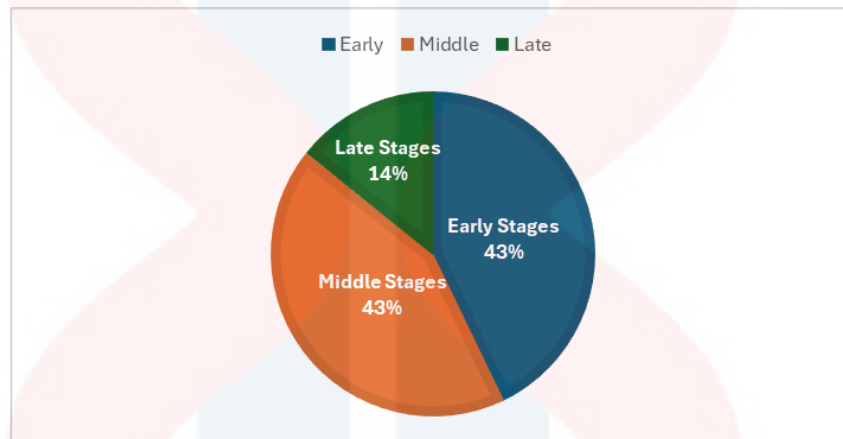
4.4.3 The percentage of Voges-Proskauer positive reaction for 3 different Male Flower in different blooming stages of *Rafflesia kerri*

The results of the Voges-Proskauer test for the selected microflora species associated with *R.kerri* show varying levels of acetoin production at different stages of incubation: 24% early, 41% late, and 35% middle. The Voges-Proskauer test is a biochemical test used to determine the ability of microorganisms to ferment glucose to produce acetoin, which is then oxidized to produce a red color.



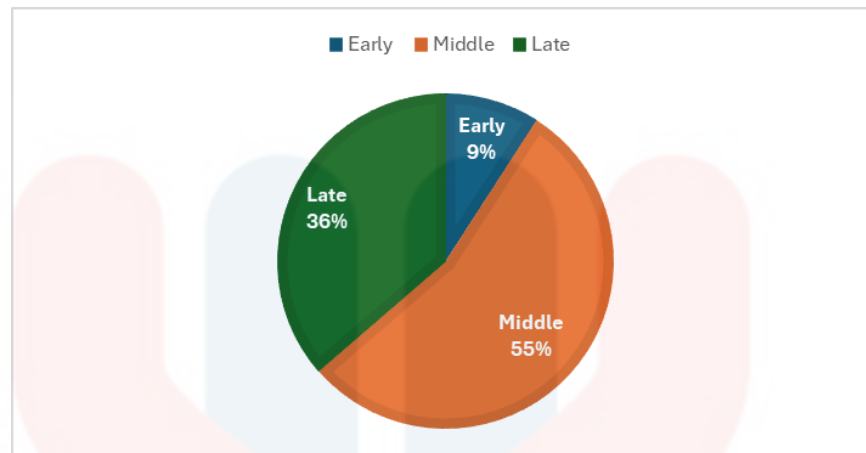
4.4.4 Methyl-Red positive reaction in 3 different Male Flower in different blooming stages of *Rafflesia kerri*

The results of the methyl-red test on the selected microflora species associated with *R.kerri* indicate varying abilities of these microorganisms to ferment glucose into stable acids at different stages of incubation, with 40% showing early positive reactions, 20% in the middle stage, and 40% in the late stage. This causes a negative MR test even though the bacteria can ferment mixed acids (Sagar., 2022).



4.4.5 Hydrogen Sulfide Test positive reaction in 3 different Male Flowers in different blooming stages of *Rafflesia kerri*.

Based on the distribution of microorganism species in the three categories, we can infer that the biochemical activity of the selected microorganism species associated with *R.kerri* is highest during the Early and Middle stages, as they both have the same percentage (43%) of microorganism species. The late stage has the lowest percentage (14%) of microorganism species. The resulting H₂S gas then reacts with ferric ions or lead acetate forming black-colored ferrous sulfides insoluble in water or lead sulfide respectively. This insoluble, black-coloured compound modifies the black-culture media showing positive results for H₂S production tests (Prashant., 2023)



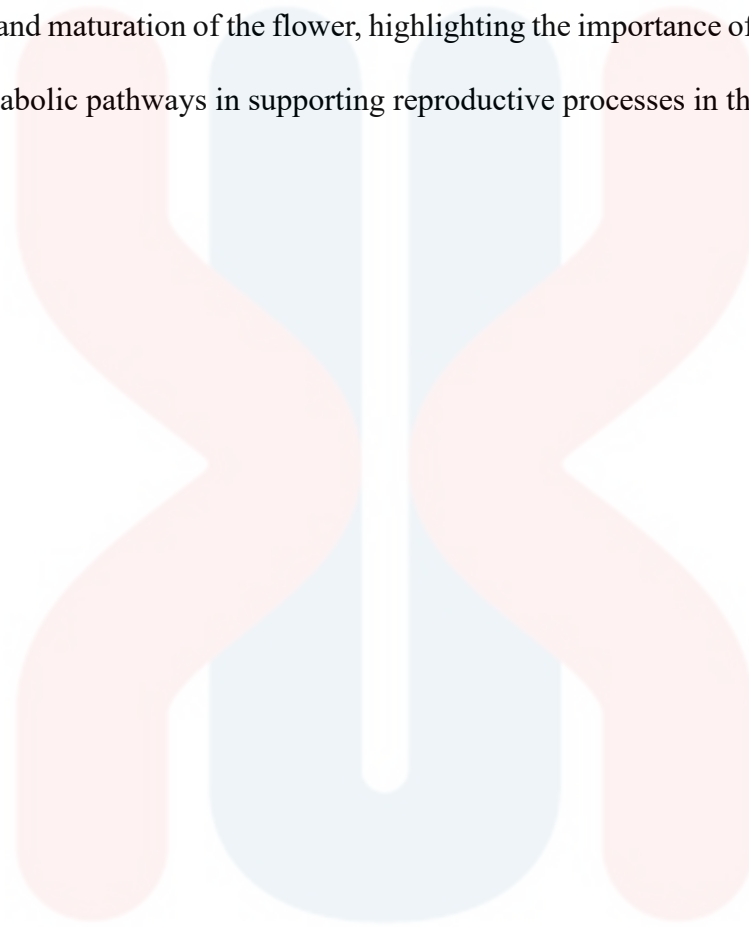
4.4.6 Citrase use positive reaction in 3 different Male Flower in different blooming stages of *Rafflesia kerri*.

The diagram provided shows the distribution of citrase use in male flowers of *R. kerri* across three different stages: Early, Late, and Middle. The percentages of citrase use at each stage are as follows: Early: 9%, Late: 36% and Middle: 55%. In the context of male flowers of *R. kerri*, the distribution of citrase use at different stages may indicate variations in metabolic activity or energy production during the development of the flower.

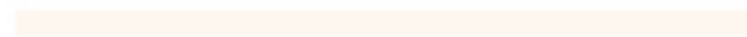
The higher percentage of citrase use in the Middle stage (55%) compared to the Early (9%) and Late (36%) stages suggests that the metabolic activity related to the citric acid cycle is most pronounced during the Middle stage of male flower development in *R. kerri*.

The increased citrase use in the Middle stage may be associated with higher energy demands or specific metabolic processes required for the maturation or reproductive functions of the male flower. This stage could be critical for processes such as pollen development, nutrient uptake, or other physiological activities essential for successful reproduction in *R. kerri*.

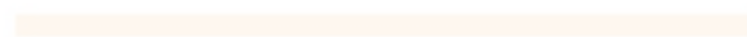
Overall, the distribution of citrase use across the different stages of male flower development in *R.kerri* reflects the dynamic metabolic changes occurring during the growth and maturation of the flower, highlighting the importance of energy production and metabolic pathways in supporting reproductive processes in this plant species.



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CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.0 Conclusion

The study aimed to assess the microflora density in response to different blooming stages of *Rafflesia kerri* and determine the biochemical activity of selected microflora species associated with the pollen. The findings reveal distinct differences in bacterial morphology, density, and biochemical activity across the early, middle, and late stages of blooming in the three male flowers examined.

Morphologically, the bacteria exhibited milky and cloudy appearances, with shapes varying from filamentous to circular and irregular. The microorganism density showed a clear trend of higher bacterial counts during the early blooming stage, gradually decreasing through the middle and late stages. This suggests that the early stage offers optimal conditions for microbial growth, likely due to higher nutrient availability.

Biochemically, the microorganisms displayed diverse metabolic activities, as evidenced by varying results in the IMViC tests, Hydrogen Sulfide Test, and Urease Activity. The early blooming stage exhibited the highest indole production, while urease activity was highest in the early and middle stages but decreased significantly in the late stage. The Voges-Proskauer and Methyl-Red tests indicated shifts in metabolic pathways as the flowers progressed through their blooming stages.

Overall, the study highlights the dynamic interplay between microbial communities and the blooming stages of *R.kerri*, emphasizing the influence of environmental conditions on microbial growth and metabolic activity. These findings

contribute to a deeper understanding of the ecological relationships between *R.kerri* and its associated microorganisms, with potential implications for conservation and management of this rare and unique plant species.

5.1 Recommendation

Rafflesia is known widely as endangered species. *R.kerri* is classified as a vulnerable species due to habitat loss, deforestation, and illegal harvesting. Conservation efforts are crucial to protect this unique plant species. This species should be in attention especially at Lojing Highland, Kelantan due to over exploitation of vegetative production around it. It is recommended to implement strict conservation measures to safeguard the habitats where *R.kerri* grows. Promote research collaborations with academic institutions and conservation organizations to enhance the understanding of *R.kerri* ecological requirements and develop effective conservation strategies. Integrate findings into conservation programs specifically tailored for the Lojing Highland region. To address the unique challenges and requirements of *R.kerri* in this specific location.

By implementing these recommendations, we can enhance our understanding of the ecological dynamics of *R. kerri* in the Lojing Highland and support its conservation and management, ensuring the survival of this remarkable species in Kelantan.

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