



Faculty of Resource Science and Technology

**Isolation and Identification of Fungi in Raw Wet Sago Starch from
Sarawak**

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Isolation and Identification of Fungi in Raw Wet Sago Starch from Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Sago starch is a type of carbohydrate source which, extracted from the pith of *Metroxylon sagu* (true sago palm) and is a traditional staple food for some Sarawak communities. Fungi are widely distributed in the agricultural settings, and certain strains are known for causing infections that can affect the producers' crops and the consumers' health. Due to limited research conducted, the current food safety status of raw wet sago starch, remains undetermined and may pose significant risks. The aim of this research was to isolate and identify fungal species present in sago starch. The screening of fungi was conducted by using the serial dilution technique to isolate the fungi present in fifty (n=50) samples of sago starch. Identification of fungi was done by molecular analysis through species-specific PCR which targeted the ITS region. The results of all sago starch samples exceeded limit established by the Malaysian Standard for edible sago starch and complied for the International Commission for Microbiological Specification of Foods, with the highest count was 2.1×10^4 CFU/g, the lowest count was 1.3×10^2 CFU/g, and the mean count was 8.6×10^3 CFU/g. There were 45 isolated fungi derived from 10 genus, namely *Aspergillus* spp., *Candida* sp., *Ceratocystic* sp., *Paecilomyces* spp., *Penicillium* spp., *Peniophora* sp., *Perenniporia* sp., *Phaeacremonium* sp., *Pleurostoma* spp., and *Trichoderma* spp. that were detected from the samples. Based on the molecular identification, a total of eighteen (n=18) fungal species were identified, including *Ceratocystic paradoxa*, *Trichoderma pleuroti*, *Trichoderma harzianum*, *Penicillium rolsii*, *Penicillium brasilianum*, *Penicillium sumatraense*, *Penicillium verruculosum*, *Penicillium citrinum*, *Phaeoacremonium* sp., *Pleurostoma richardsiae*, *Aspergillus sydowii*, *Aspergillus caesiellus*, *Aspergillus terreus*, *Penicillium citrinum*, *Paecilomyces variotii*, *Paecilomyces niveus*, *Peniophora malaiensis*, *Candida blattae*, and *Perenniporia tephropora*. In conclusion, all fifty raw wet sago starch samples

collected and purchased from wet market from different districts and regions in Sarawak such as Mukah, Dalat, Matu, Pusa, Betong, Bintulu, Marudi, Miri, Sibu and Selangau were contaminated with fungi as it did exceed the permissible limits for human consumption. The outcomes from this study provided a baseline on level of fungi contamination in sago starch for future risk and mitigation assessment planning and established detailed knowledge of the food safety status in sago starch production from Sarawak, which will form a foundation for future studies.

Keywords: Sago starch, raw, wet, fungi, food safety, PCR, ITS

Pengasingan dan Pengenalpastian Fungi dalam Kanji Sagu Mentah Basah Berasal dari Sarawak

ABSTRAK

Kanji sagu adalah sejenis sumber karbohidrat yang diekstrak daripada batang Metroxylon sagu (palma sagu asli) dan merupakan makanan ruji tradisional bagi sesetengah masyarakat Sarawak. Jangkitan kulat kerap berlaku dalam bidang pertanian dan beberapa strain kulat yang terkenal dengan jangkitan berbahaya boleh menjejaskan pengeluaran tanaman dan kesihatan pengguna. Disebabkan penyelidikan yang terhad, status keselamatan makanan terkini bagi sago basah mentah, masih belum ditentukan dan mungkin menimbulkan risiko yang ketara. Tujuan penyelidikan ini adalah untuk mengasing dan mengenal pasti spesies kulat yang terdapat dalam kanji sagu. Pengasingan kulat dijalankan dengan menggunakan teknik pencairan bersiri untuk mengasingkan kulat yang terdapat dalam lima puluh ($n=50$) sampel kanji sagu. Pengenalpastian kulat dilakukan melalui analisis molekul melalui PCR khusus spesies yang menyasarkan kawasan ITS. Keputusan untuk semua sampel kanji sagu melebihi had yang ditetapkan oleh Piawaian Malaysia untuk kanji sagu yang boleh dimakan dan bagaimainapun mematuhi had boleh diterima yang dirancang oleh Suruhanjaya Antarabangsa bagi Spesifikasi Mikrobiologi Makanan, dengan kiraan tertinggi ialah 2.1×10^4 CFU/g, kiraan terendah ialah 1.3×10^2 CFU/g, dan kiraan min ialah 8.6×10^3 CFU/g. Terdapat empat puluh lima ($n=45$) strain spesies kulat terpencil daripada sepuluh ($n=10$) genus kulat, Aspergillus spp., Candida sp., Ceratocystic sp., Epulorhiza sp., Paecilomyces spp., Penicillium spp., Peniophora sp., Perenniporia sp., Phaeacremonium sp., Pleurostoma spp. dan Trichoderma spp. telah dikesan daripada sampel. Berdasarkan pengenalpastian molekul, lapan belas ($n=18$) spesies kulat telah dikenalpasti iaitu Ceratocystic paradoxa, Trichoderma pleuroti, Trichoderma harzianum,

Penicillium rolfsii, Penicillium brasilianum, Penicillium sumatraense, Penicillium verruculosum, Penicillium citrinum, Phaeoacremonium sp., Pleurostoma richardsiae, Aspergillus sydowii, Aspergillus caesiellus, Aspergillus terreus, Penicillium citrinum, Paecilomyces variotii, Paecilomyces niveus, Peniophora malaiensis, Candida blattae dan Perenniporia tephropora. Kesimpulannya, kesemua lima puluh sampel mentah basah kanji sagu yang diambil dan dibeli daripada pasar pasar daripada daerah dan kawansan berbeza di Sarawak seperti Mukah, Dalat, Matu, Pusa, Betong, Bintulu, Marudi, Miri, Sibu and Selangau adalah tercemar dengan kulat kerana ia melebihi had yang dibenarkan untuk pemakanan manusia. Hasil daripada kajian ini menyediakan data asas tentang tahap pencemaran kulat dalam kanji sagu yang boleh digunakan untuk penilaian risiko serta kaedah mitigasi. Selain itu, terperinci tentang status keselamatan makanan berasaskan kanji sagu dari Sarawak, boleh dijadikan asas untuk kajian selanjutnya.

Kata kunci: Kanji sagu, mentah, basah, fungi, keselamatan makanan, PCR, ITS

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LIST OF ABBREVIATIONS

µl	Microlitre
°C	Degree Celsius
bp	base pair
CFU	Colony Forming Unit
DNA	deoxyribonucleic acid
g	gram
ITS	Internal Transcribed Sequence
kb	kilobase
mins	minutes
mL	millilitre
no.	number
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
s	seconds
mm	millimetre

CHAPTER 1

INTRODUCTION

1.1 Study background

Sago starch is a type of carbohydrate source extracted from the pith of *Metroxylon sagu* (true sago palm). A high starch concentration can be found in the pith core of sago palm due to the conversion of the stored nutrient into sago starch and accumulated in the trunk before flowering (Uthumporn et al., 2014). Sago palm is natively found in tropical Southeastern Asia regions such as Papua New Guinea, Indonesia, Philippines and Malaysia (Lim et al., 2019). The natural habitat of sago palm is mainly in tropical lowland forests and freshwater swamps, usually near sea level. It is renowned for its ability to adapt to damp growth conditions, and its most significant advantage lies in its capacity to thrive in challenging swampy peat environments (Mohamad Naim et al., 2016). There are several ways that sago starch plays a crucial part in influencing the local people's lifestyle. The sago starch has traditionally served as a crucial dietary staple and holds cultural significance among indigenous communities for the coastal Melanau and Iban populations in Sarawak (Mohamad Naim et al., 2016).

Sago starch is utilised in the preparation of many culinary creations, such as sago porridge, sago noodles, and traditional confections like "pancong" or "kerupuk sago." "Linut", known as "Ambuyat" in Sabah, is a viscous porridge-like dish prepared by pouring boiling water over a bowl of sago starch and typically eaten with sambal (Magiman et al., 2020). "Tebaloi", a renowned Melanau delicacy, is mostly composed of sago starch, eggs, desiccated coconut, sugar, turmeric, and various other flavours (Shin & Collins, 2015). Sago starch serves as a substitute for potato and corn starch in alternative starch products. Sago

starch is incorporated into the making of "kerepok lekor" in Terengganu (Hamzah et al., 2017). In addition to being a food source, sago starch plays a significant role in determining the texture of many dishes. It is commonly utilized in the food and industrial sectors as a thickening, colloidal stabilizer, gelling agent, bulking agent, and water retention agent (Hirao et al., 2018).

In some areas, local people consider sago starch as their primary dietary carbohydrate, while in some areas, they practise commercialization of sago starch as means of earning cash (Toyoda, 2018). Sago starch has gained significant importance as an essential agricultural commodity in Southeast Asian nations like Papua New Guinea, Indonesia, Philippines and Malaysia (Mustafa Kamal et al., 2017). Malaysia is the third largest sago producer worldwide in terms of hectares, with approximately 59,000 hectares, followed by Indonesia with 2.94 million hectares and Papua New Guinea with 1.02 million hectares. Together with one another, these three nations account for almost 94.6% of the world's sago production (Mohamad Naim et al., 2016). Mukah division in the State of Sarawak is the largest sago-producing area in Malaysia, with 42,310 hectares of land cultivated with sago, and 70 per cent are toiled by the small-scale sago farmers (Yaakub et al., 2023). Currently, it is known as one of the world's largest exporters of sago starch, exporting more than 40,000 tons annually to different countries, including Peninsular Malaysia (Amin et al., 2019).

Fungal infections are prevalent occurrences in the agriculture sectors, with numerous fungal strains renowned for their adverse effects on crop production and consumer health. The contamination of crops leads to economic losses in the agricultural industry, affecting both crop producers and handlers. This is due to the necessity to dispose of badly contaminated crops (Isaac Oluseun & Olufemi Adebukola, 2020). Contamination can also

propagate across the food chain, starting from the field and continuing during harvesting, processing, storage, transit, and consumption (Anna & Behdad Shokrollahi, 2020). Additional factors, such as substandard harvesting processes, inadequate processing, packaging, drying techniques, and transportation activities, also impact the proliferation of fungi (Neme & Mohammed, 2017).

The fungal isolates were classically identified at the genus level through a morphological examination. Macroscopically analysis of fungi can be performed with the naked eye by observing the presence of fungal on the media plate. Macroscopic characteristics for example, the colour of the fungi colony, and the structure of the fungi colony on media plate, and growing rate of the fungi are essential in mycological research. This involved assessing characteristics such as the colour, margin, and texture of the fungal colonies, as well as examining the shape of the spore-bearing structures under a microscope (Alsohaili & Bani-Hasan, 2018). Examining the physical characteristics of a fungal culture can assist in determining the identity of the isolates, however this method is limited to identifying them only at the family or genus level (Wang et al., 2016). This identification method is inadequate for determining the specific species of the isolated fungi. Therefore, a molecular technique is used to aid in the identification process (Lockhart et al., 2021).

1.2 Problem statement

Up to the present time, there has been a lack of comprehensive research addressing the precise extent of fungal contamination in sago starch sourced from Sarawak due to limited research conducted. Despite some existing studies on the occurrence of mycotoxigenic fungi in sago-associated products from New Papua Guinea reported by

Greenhill (2006). However, there has been no such comparable investigations have been conducted specifically on the prevalence of fungi in sago starch production from Sarawak. This gap in research is noteworthy, especially considering the significant role sago starch plays as a staple food in certain communities and the potential health risks associated with fungal contamination. Even though there are many studies related to the screening of fungi in other crops (e.g., oil palm, rubber, cocoa, pineapple, and pepper) in Malaysia, none of them have delved into the specific context of sago palm crops. Given the absence of data on fungi prevalence and species identification in sago starch from Sarawak, there exists a concerning gap in our understanding of the food safety status of this vital commodity. Without a clear assessment of fungal contamination levels and the potential risks posed to consumers, there is a looming threat to public health. Therefore, this study was meticulously designed to fill this critical knowledge gap by shedding light on the prevalence of fungi and identifying specific fungal species present in sago starch production, ultimately aiming to safeguard consumer well-being and strengthen food safety standards in Sarawak, Malaysia.

This research will provide a fundamental composition for assessing and mitigating risks related to fungal contamination in sago starch. Furthermore, it will enhance our comprehensive knowledge of the effectiveness of current food safety protocols in sago starch manufacturing in Sarawak, laying the foundation for future scientific investigations in this area. Identifying various fungal species and their abundance in sago starch samples will contribute critical information and deepen our understanding of fungal diversity and distribution in food products within the scientific field of food microbiology. This work will add to the security information on sago starch and investigate possible risks related to fungal contamination, contributing to global initiatives to improve food systems' long-term viability and sustainability. This investigation will confirm the safety of consuming sago starch within

permissible amounts and provide reassurance to consumers and regulatory agencies concerning the safety of this traditional staple food. This study would be the first investigation incorporated with the screening fungi contamination in sago starch from Sarawak hence enhancing the comprehension of the current food safety regulations.

1.3 Research questions

This study is motivated by two research questions, which are:

- a) What is the prevalence and occurrence rate of fungal species detected in sago starch across different regions within Sarawak?
- b) What are the morphological characteristics and molecular identities of fungal isolates obtained from sago starch samples in Sarawak?

1.4 Research hypotheses

This study was set up with two research hypotheses, which were:

- a) It is hypothesized that there will be a significant prevalence and occurrence rate of fungal species detected in sago starch across different regions within Sarawak.
- b) It is hypothesized that there will be revealed morphological characteristics and molecular identities of fungal isolates obtained from sago starch samples in Sarawak.

1.5 Research objectives

This study was conducted based on two main objectives, which are:

- a) To isolate and detect fungi in raw wet sago starch throughout the state of Sarawak, Malaysia
- b) To identify the species of fungal isolates based on molecular analysis targeting the internal transcribed spacer (ITS) region.

CHAPTER 2

LITERATURE REVIEW

2.1 Sago palm

The sago palm is a perennial monocotyledonous plant belonging to the genus *Metroxylon*. There are fourteen genera within the subfamilies of the *Arecaceae* family that have the capacity to produce starch in their trunks. The genus *Metroxylon* is highly proficient in starch production, and it consists of only one species classified under the section *Metroxylon*, specifically *Metroxylon sagu* (Ehara, 2018). Its trunk is famous for its capacity to store substantial amounts of starch. *Metroxylon sagu*, also known as true sago palm, is an exceptionally productive crop in terms of starch synthesis. It is reported that this species is distributed in Southeast Asia and Melanesia (Ehara, 2006). The genus *Metroxylon* has a clump-forming structure, with variations in the presence of spines, either without spines or having spines. These variations are distinguished by variances in the length and density of the spines. The fruit is embellished with 18 scales that are arranged in a vertical alignment. Each scale has a diameter of 5 cm (Ehara et al., 2003).

The term "sago" originates from the Javanese phrase denoting a species of palm tree that serves as a reservoir for starch (Bagasbas & Barroca, 2020). The scientific nomenclature of the sago palm is derived from the Greek terms "metra," signifying pith or parenchyma, and "xylon," signifying wood or xylem. The term "pith" is used to describe the soft tissue located in the trunk of a palm tree. The pith of the sago palm stem has a greater quantity of starch than other sources, such as rice, maize, and wheat (Lim et al., 2019). Sago palm is highly thriving in these districts due to its topography, which can grow from low flooded areas to uplands and acidic to neutral soils (Nisar & Hussain, 2022). The primary benefit of

this plant is in its capacity to flourish in challenging marshy peat conditions, including floods, droughts, and strong winds, thanks to its resilience towards extreme weather fluctuations and moist growing conditions, whereas other major crops cannot grow well. However, sago palms will grow better in upland conditions with sufficient water and show preferable production than under submerged or flooded conditions. This attribute renders it a multipurpose plant that demands minimal maintenance for cultivation. The sago palm thrives in tropical rainforest areas and freshwater marshes and is primarily found in coastal and sub-coastal lowland locations (Hirao et al., 2018).



Figure 2.1: Sago palm, *Metroxylon sagu* (Al Manar et al., 2023)

The species is native to Southeast Asia, specifically Malaysia, Indonesia, New Papua Guinea, and the Philippines (Mustafa Kamal et al., 2017). Sarawak, situated in Malaysia, is renowned for being the central hub of sago production and housing the largest sago palm farms in the country, namely in the Mukah and Dalat regions (Yaakub et al., 2023). Sago represents a valuable commodity with significant potential to boost Sarawak's export revenue. According to Food Industry Commodities and Regional Development Sarawak (2023), the total area dedicated to sago cultivation decreased slightly to 32,747 hectares in 2022, down from 33,407 hectares in 2021. Despite this reduction in cultivated area and production volume, the export value of sago products rose from RM80.34 million in 2021 to RM107.72 million in 2022. As stated by Food Industry Commodities and Regional Development Sarawak (2024), Sarawak exported 35,000 metric tons of sago starch worth RM113 million, mainly to Peninsular Malaysia and Japan, accounting for 41 percent and 45 percent of the total exports in 2023. The Sarawak Department of Agriculture has introduced a sago subsidy initiative with the purpose of encouraging small-scale farmers to participate in sago palm cultivation.

2.2 Sago starch

2.2.1 Production of sago starch

The harvesting of sago palms typically occurs approximately 10 to 15 years after they are planted, and the timing is determined by the fertility of the soil. A full-grown plant can grow up to 25.0 metre tall and typically reach a height of around 10.0–12.0 metres and a diameter of about 0.800–1.00 metre when matured (Bujang, 2006). According to Singhal et al. (2008), sago palm trees with a height ranging from 7.5 to 9.0 metres will be cut down

just after they bloom and just before they start producing fruits. This is because the amount of starch in their trunks decreases significantly during this time. The sago trunk is subsequently divided into approximately 10-12 sections, with each segment measuring 75.0 to 90.0 cm in length of logs. Once sago logs are severed, they are gathered into rafts and transported by boats to sago factories for further processing. In addition, the logs can be transported to the factory across the land, where autonomous agents will gather and transport them in exchange for a charge. Due to their consistent occurrence on riverbanks, sago producers often transport sago logs by floating them down canals and rivers (Amin et al., 2019). Nevertheless, if the canals become water-depleted, automobiles will substitute.

The primary component of the sago palm pith is starch, which needs to be extracted from the cellulose components. As a result, it goes through multiple processing procedures to obtain a substantial amount of high-quality starch (Awg-Adeni et al., 2010). The process of obtaining sago starch can generally be divided into two primary methods: traditional and contemporary. The conventional methodology can be categorised into domestic and limited-scale processing facilities (Amin et al., 2019). In contrast to the processing factory level, the domestic level involves individual farmers who harvest and prepare sago palms in their gardens, hence minimising the need to transport the bulky trunks to manufacturers. On the other hand, the current method requires multiple alterations to be made specifically in small-scale processing facilities. The majority of large firms in Sarawak have implemented this strategy, whereby they have fully automated their operations and can minimise the need for labour-intensive tasks (Karim et al., 2008). However, the fundamental concepts and techniques for extracting starch are comparable in both ancient and modern operations, with the main distinction being the scale of the operation (Mustafa Kamal et al., 2017)

During the processing stage, the initial step entails peeling off the outer layer of the palm, which bears a resemblance to bark. Subsequently, the palm is subjected to maceration using a rasper. The rasping procedure is used manually or mechanically to grind the pith and release the starch particles from the fibre. Subsequently, the starch is extracted from the fibre using mechanical kneading, stomping, or water spraying. Afterwards, the starch undergoes a sequence of centrifugal screening processes to eliminate large fibre. The extraction procedure involves employing cyclone separators to separate the particles. Afterwards, the material is dehydrated using a rotary drum dryer and exposed to hot air drying. The procedure has been recorded in inquiries carried out by (Mustafa Kamal et al., 2017).

The pith tissue of the sago palm trunk functions as the repository for sago starch. The first stage in the extraction of processed starch is the removal of the bark from sago logs, followed by the milling of the pith, and finally, the starch extraction. The starch is then extracted using a method of forced filtration, in which a combination of starch milk and fibre is sprayed over a sieve under applied pressure. The starch slurry is concentrated, refined, and dried (Awg-Adeni et al., 2010). Although sago starch within the pith starts out free from bacteria, it quickly becomes contaminated by microorganisms when exposed to unclean milling equipment during processing (Greenhill et al., 2009). According to studies done by Greenhill (2006) and Pue (2013) have undertaken experiments that have identified two potential sources of contamination: infection of the palm before harvesting and infection during processing after harvesting. Contamination can also happen through contact and from tools, such as the palm leaf sheeting used to place the sago pith.

2.2.2 Important of sago starch

2.2.2.1 Food application of sago starch in Malaysia

The consumption of sago starch in Sarawak refers to sago starch derived from the pith of the sago palm. It is utilised in a diverse array of culinary products, beverages, and industrial applications in order to cater to the diverse tastes and dietary needs of consumers, especially the people of Sarawak, Malaysia. Sago starch is a traditional staple food in Sarawak and holds cultural significance among indigenous communities like Melanau (Shin & Collins, 2015). The use of sago starch in Sarawak showcases a blend of traditional customs and modern influences. While sago-based dishes remain popular in rural homes and traditional marketplaces, there is a growing demand for sago goods in urban areas and international markets. The carbohydrate content of sago is higher than that of rice and some other carbohydrate sources and superior in terms of fibre content and glycemic index value, making it an essential provider of energy and dietary fibre (Dewayani et al., 2022), which incorporating sago-based foods into the diet can augment dietary diversity and guarantee nutritional consistency in Sarawak. Sago starch is gluten-free and hypoallergenic, making it suitable for individuals with gluten sensitivity or allergy (Kumari, 2019).

Sago starch is utilised to prepare many culinary creations, such as sago porridge, sago noodles, and traditional confections like "pancong" or "kerupuk sago." "Tebaloi", a renowned Melanau delicacy, mainly comprises sago starch, eggs, desiccated coconut, sugar, turmeric, and various other flavours (Shin & Collins, 2015). "Ambuyat" in Sabah, known as "Linut" in Sarawak, is a viscous porridge-like dish prepared by pouring boiling water over a bowl of sago starch and typically eaten with sambal (Magiman et al., 2020). Sago starch is commonly utilised in West Malaysian cuisine for dishes such as "pengat sago" (a sugary

dessert) and "sago gula Melaka" (a sago pudding served with palm sugar syrup). Sago pearls, made from sago starch, are commonly used in beverages such as "sago gula Melaka" (sago pearls in palm sugar syrup) and "air sago" (sago drink), served cold as a refreshing beverage, especially in hot regions. Sago starch serves as a substitute for potato and corn starch in alternative starch products. Sago starch is incorporated into the making of indulge in popular sago-based snacks and street cuisine, such as "kerepok lekor" (fish crackers) in Terengganu (Hamzah et al., 2017). In addition to being a food source, sago starch plays a significant role in determining the texture of many dishes. It is commonly utilised in the food and industrial sectors as a thickening, colloidal stabiliser, gelling, bulking, and water retention agent (Hirao et al., 2018).

Nevertheless, among the Sarawak coastal Melanau community in Mukah, the traditional significance of sago as a primary food source has gradually been supplanted by rice. The decrease in sago starch consumption in these communities is not attributed to a decline in sago cultivation but rather to the transformation of the role of sago starch from being a food supply to becoming a source of revenue (Mohamad Naim et al., 2016). Instead of consuming sago as their primary food source, the local population can sell their processed sago products to sago mills in exchange for monetary compensation. The sago sector has significant potential, arising from the vast range of applications for sago starch, which commercial food manufacturers can leverage in Sarawak to enhance the texture, extend the shelf life, and boost the nutritional value of processed items through the inclusion of sago starch production.

2.2.2.2 Economical value of sago starch in Malaysia

Sago starch has become an important agricultural crop in Southeast Asian nations such as Indonesia and Malaysia. Malaysia is the leading worldwide exporter of starch derived from the sago palm, producing 47,000 metric tonnes annually (Mustafa Kamal et al., 2019). The sago palm is the traditional agricultural crop and the primary source of sustenance for the Melanau community in Sarawak. As stated by Department of Agriculture Sarawak (2018), it covers eighty-five per cent of the total sago acreage, which is equivalent to 40,641 hectares, and Sarawak exported 42,000 tonnes of dried sago starch in 2017, with a value of over RM 89 million. The Department of Agriculture Sarawak has introduced a sago subsidy initiative to encourage small-scale farmers to participate in sago palm cultivation. The sago industry in Sarawak encounters obstacles, including volatile market pricing, land title disputes, and concerns regarding environmental sustainability, despite its cultural and economic importance (Mohamad Naim et al., 2016).

Sago starch is of utmost importance in the cultural heritage, economy, and food security of Sarawak, Malaysia. The consumption patterns of this product demonstrate a dynamic interaction between traditional practices, innovative approaches, and market influences. The sago industry in Malaysia, specifically in the State of Sarawak, is well-established and has become a prominent sector that substantially contributes to the country's export earnings (Karim et al., 2008). The long-term sustainability of the sago sector relies on implementing sustainable cultivation practices, adopting value-added processing techniques, and expanding market opportunities. According to Uthumporn et al. (2014), it is worth mentioning that 96% of the starch is produced in the state of Sarawak. The sago

business in Malaysia is firmly established and has emerged as a significant sector that contributes to the country's export revenue (Ishak et al., 2021).

Implementing sustainable practices in the management of sago resources, along with the addition of value through processing and the development of markets, has the potential to improve the socio-economic conditions of rural people in Sarawak. Furthermore, this approach ensures the preservation of the cultural heritage associated with sago for future generations, as these meals are integral elements of the local cuisine and have substantial cultural significance.

2.3 Prevalence of fungi in sago starch

As of the present time, there exists a scarcity of comprehensive research addressing the prevalence and occurrence rates of fungi within sago starch. However, notable investigations on fungal contamination in sago starch have been conducted within the region of New Papua Guinea (Greenhill et al., 2007; Greenhill, 2006; Greenhill et al., 2008; Greenhill et al., 2010; Greenhill et al., 2009). According to Greenhill et al. (2007), a significant number of fungi were detected in sago starch from New Papua Guinea. Sixty-two out of sixty-nine samples were contaminated by fungi, with fungal counts ranging from 1.0×10^2 to 3.0×10^6 CFU/g. Some of these fungal counts exceeded the acceptable levels set by the International Commission for Microbiological Specification of Foods (International Commission for Microbiology Specifications International Commission on Microbiological Specifications for Foods, 1974), which recommends 10^2 to 10^5 CFU/g limits. Most isolated fungi belong to the genera *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, and *Paecilomyces*, which are known to produce mycotoxins. Therefore, it has been advocated

that toxigenic fungi or their toxins might be involved in rolling in the aetiology of sago haemolytic disease (SHD).

A further study conducted by Greenhill et al. (2008) sought to investigate the occurrence of toxigenic fungi and evaluated the level of mycotoxins in SHD. No common mycotoxins have been detected among any of the sago starch samples tested, although various genera of filamentous fungus, including toxigenic strains, were identified. This indicates that, although there is a prevalence of toxigenic fungi in sago starch, the toxigenic fungi produced is null or low amounts of mycotoxin as the studies were conducted by two-dimensional thin layer chromatography (TLC). According to Greenhill et al. (2009), a study on the spontaneous fermentation process of traditional sago starch was conducted. The research attempted to identify the significant fermentative organisms in sago and to clarify the involvement of yeasts and filamentous fungus in the fermentation process. The results showed that active fermentation was anticipated to inhibit the growth of filamentous fungus in sago starch. An investigation on hazard analysis of traditional sago starch production and utilize, incorporating observations of the preparation process and insights from sociological and microbiological investigations was carried out by Greenhill et al. (2010). It was concluded that the key critical control points in sago processing were raw wet starch storage methods that led to spontaneous fermentation. The most significant risks were rapid and prolonged fermentation and storage under suitable circumstances. The storage method should encourage quick fermentation by maintaining air-tight and moisture-retaining conditions while preventing exposure to direct sunlight (Uthumporn et al., 2014).

A research by Pue (2013), examined haemolytic chemicals found in fungi of sago and their involvement in sago haemolytic disease in Papua New Guinea. This investigation

follows the study conducted by Greenhill et al. (2008) where fungi isolated from sago have been shown to be haemolytic by in vitro bioassay; however, their haemolytic principle has not yet been identified. The study aimed to identify and isolate the haemolytic toxins by the method of haemolytic activity-guided fractionation utilizing cultures of the highly haemolytic fungi isolate *Penicillium citrinum*. The crude methanol extracts of *P. citrinum* were separated using hexane, purified using C18 solid phase extraction (SPE), and refined by preparative thin layer chromatography and high-performance liquid chromatography. Studies conducted by Pue (2013) and Greenhill (2006) have pinpointed two possible sources of contamination: preharvest infection of the palm and postharvest infection during processing, which can occur through handling and contact with utensils such as the palm leaf sheeting used for sago pith.

2.4 Toxigenic properties in fungi

Mycotoxins are secondary metabolites or poisonous toxic byproducts produced by specific filamentous fungi of the *Ascomycota* phylum (Afsah-Hejri et al., 2013). *Aspergillus*, *Fusarium*, and *Penicillium* are fungal species that are significant producers of mycotoxins, leading to frequent and troublesome contamination of foods and feeds (Alshannaq & Yu, 2017). In adverse environmental conditions, these fungi produce mycotoxins, which are secondary metabolites that may contaminate various plants when in the field or at any stage during the pre-harvesting practices, harvesting technique, post-harvesting process, transportation, or storage of the plants. Mycotoxins can be classified into several groups based on their chemical structures and production sources. Common groups of mycotoxins include aflatoxins, ochratoxins, trichothecenes, fumonisins, and zearalenone, and each group

comprises multiple individual toxins, each with its unique chemical properties and biological effects (El-Sayed et al., 2022).

Aflatoxins are highly carcinogenic mycotoxins produced primarily by fungi of the *Aspergillus* genus, notably the most common and potent aflatoxin-producing moulds, *Aspergillus flavus* and *Aspergillus parasiticus* (Benkerroum, 2020). Aflatoxin B1 is the most potent naturally occurring carcinogen known, posing significant risks to human health through contaminated food and feed consumption. Ochratoxins are a group of toxic secondary metabolites produced by fungi from the genera *Aspergillus* and *Penicillium* (el Khoury & Atoui, 2010). Ochratoxin A is the most well-known and extensively studied subgroup, with documented nephrotoxic, carcinogenic, and immunosuppressive effects in humans and animals. Additional mycotoxins frequently encountered in food include trichothecenes, fumonisins B1 and B2, ergot alkaloids, and zearalenone (Garcia et al., 2018). These toxins can contaminate food and feed, leading to health hazards when consumed by humans and animals.

Mycotoxin contamination of food is a worldwide issue that poses a substantial health risk to humans and animals, regardless of whether they live in developing or developed countries (Awuchi et al., 2021). Mycotoxicosis is a medical condition that arises from the toxicity of mycotoxins, in which refers to ingesting food or feed contaminated with mycotoxins by higher animals (Binder et al., 2007). Ingesting food or feed contaminated with mycotoxins can lead to mycotoxicosis, which can have acute or chronic effects such as estrogenic effects, carcinogenicity, immunosuppression, and teratogenicity (Afsah-Hejri et al., 2013). They exhibit diverse chemical structures and biological activities, ranging from carcinogenic and mutagenic to hepatotoxic and nephrotoxic properties.

Mycotoxins can contaminate crops through two primary pathways. The first is when fungi grow saprophytically on stored crops, and the second is when fungi develop as a pathogen on a plant (Glenn, 2007). Fungal spores can establish and infiltrate crops, producing five mycotoxins during several stages, including preharvest, postharvest, processing, and storage (Bhat et al., 2010). *Fusarium* species, a prominent group of fungi known for their ability to produce mycotoxins, can infest a wide range of crops in Malaysia. The illnesses caused by *Fusarium* species have posed a substantial challenge to farmers, impacting numerous economically important crops (Latiffah et al., 2009). The contamination of crops leads to economic losses in the agricultural industry, affecting both crop producers and handlers. The introduction of mycotoxins into human food sources coincided with early humans' advent of agricultural cultivation and long-term storage practices (Eskola et al., 2020). Contamination can also arise across the entire food chain, starting from the field and continuing during harvesting, processing, storage, transit, and consumption.

Mycotoxin-producing fungal strains and non-toxigenic fungal strains exhibit distinct differences based on their ability to synthesize mycotoxins, which are secondary metabolites with toxicological implications for humans, animals, and plants. Mycotoxins fungal strains are equipped with specific genetic and enzymatic pathways that allow them to produce these toxins in response to favorable environmental factors, such as optimal temperature, humidity, and nutrient availability (Ndiaye et al., 2022). The production of mycotoxins is often linked to the ecological advantage it provides to the fungi, enabling them to inhibit competing microorganisms or deter predation. Among notable mycotoxins are aflatoxins, ochratoxins, and trichothecenes, produced by fungal genera like *Aspergillus*, *Fusarium*, and *Penicillium*, each of which poses significant risks to health due to their diverse biochemical activities. In contrast, non-toxigenic fungal lack the biosynthetic gene clusters necessary for

mycotoxin production, making them less competitive in the same ecological niches where toxin-producing strains might dominate. Without the genetic basis or enzymatic pathways to produce these secondary metabolites, non-toxic strains often coexist with other organisms without exerting the same inhibitory effects observed in mycotoxin-producing counterparts (Yu, 2012).

The mechanism of mycotoxicity in toxin-producing strains involves a complex biosynthetic cascade that generates secondary metabolites with specific cellular targets. Aflatoxins, for example, form adducts with DNA and proteins, disrupting transcription and translation processes, which results in hepatotoxicity, carcinogenesis, and immunosuppression (Zhang et al., 2018). Trichothecenes interfere with protein synthesis by binding to eukaryotic ribosomes, leading to cytotoxic effects and tissue necrosis, while ochratoxins exert nephrotoxic effects by inhibiting cellular respiration and ion transport in kidney cells (Wang et al., 2021). These toxicological mechanisms underscore the pathogenic potential of mycotoxin-producing fungal strains and illustrate the necessity of distinguishing them from non-toxigenic fungal strains for applications in food safety, agriculture, and public health.

2.5 Enumeration and isolation of fungi

The United States Food and Drug Administration (FDA) has produced a standard procedure widely employed for identifying and quantifying fungi in food samples (Tournas et al., 2001). For this standard, the samples were categorised into two distinct plating methods: dilution plating technique (used for homogenised samples like starch) and direct plating technique (used for entire samples such as dry beans and whole spices). The dilution

plating procedure involved preparing the samples by adding 0.1% peptone water to the weighted sample to obtain a 1:10 dilution. The mixture was then homogenised in a stomacher for 2 minutes. Prepare suitable 1:10 (one part sample and nine parts diluent) dilutions in 0.1% peptone water prior to applying on dichloran rose bengal chloramphenicol (DRBC) agar or Potato Dextrose Agar (PDA) medium. The spread plating technique, which involves the application of a diluted sample over a solid medium, is generally regarded as superior to the pour plate approach. The utilisation of the pour plate approach leads to accelerated growth of fungal colonies on the surface, frequently obstructing those located beneath and consequently leading to a less precise enumeration. Surface plating facilitates a more homogeneous proliferation and simplifies isolating colonies.

The standard plate count method is commonly employed to quantify the presence of fungus in the samples. Although it is a complex and time-consuming process, it remains one of the most often used methods for evaluating the extent of food contamination. The standard plate count method involves spreading appropriately diluted food samples onto selective agar, followed by incubation at a specific temperature and duration (Sanders, 2012). Following the incubation period, it is possible to enumerate fungal colonies and determine the number of colony-forming units (CFU). The enumeration of fungal colonies grown on agar plates from serial dilutions of a sample. This method provides an estimate of viable fungal cells present in the original sample and is commonly used in microbiological analyses of food, soil, air, water, and clinical specimens (Zainab et al., 2022). The systematic measurement and enumeration of fungal organisms to determine their abundance, distribution, and dynamics in different habitats or substrates. Enumeration techniques may include dilution plating, membrane filtration, microscopic cell counting, or molecular methods (Ettenauer et al., 2014). The quantitative assessment of fungal populations presents

in a sample or environment. Enumeration methods typically involve counting fungal colonies or cells, either directly on agar plates or indirectly through microscopic examination of stained slides or culture suspensions (Oliveira et al., 2015).

The procedure of selectively recovering fungal organisms from a mixed sample or substrate to obtain pure cultures for further analysis. Isolation methods typically involve inoculating the sample onto selective media, incubating under suitable conditions to promote fungal growth, and isolating individual fungal colonies for subculture and characterization (Sanders, 2012). The technique for the isolation of fungi is by transferring spores or mycelial fragments onto nutrient-rich agar media that support fungal growth. This allows individual fungal colonies to develop, making them easier to identify, characterise, and study under laboratory conditions (Senanayake et al., 2020). The process of purifying fungal cultures to obtain axenic (pure) cultures without contamination by other microorganisms. This involves subculturing individual fungal colonies multiple times on fresh media to ensure the absence of contaminating bacteria, yeasts, or other fungi (Hussain et al., 2020).

2.6 Identification of fungi based on morphology characterization

Morphological characterisation is the process of classifying fungus of fungi, which can be categorised into two distinct methods: macroscopic examination and microscopic examination. As reported by Hibbett et al. (2011), the identification of a significant number of fungi, specifically 8895 species, primarily based on visual characteristics alone, without the use of DNA sequence data, during the period from 1999 to 2009. The macroscopic analysis of fungi can be conducted visually, without the aid of magnification, by observing the presence of fungal growth on the medium plate. The macroscopic properties, such as the

colour and structure of the fungal colony on a media plate, as well as the growth rate of the fungi, are crucial in mycological research (Buffi et al., 2023). Characterisation of the fungi involved considering factors such as colony colour, pigmentation present, and furrow creation. The characteristics are taken into account by observing their morphological features and analysing them microscopically using taxonomic guides and conventional processes such as the existence of mycelia, the colour of the mycelia, whether the hyphae are septate or aseptate, and the structure of the spore-bearing structures (Rohilla & Salar, 2012).

The Lactophenol Cotton Blue technique is a widely employed method for morphological observation that utilises a specific stain. Lactophenol cotton blue dye is a mounting medium and staining agent used to prepare slides for microscopic examination of fungi. This dye is highly suitable due to its composition, which includes lactic acid for preserving fungal structures, phenol for eliminating any organisms that may hinder observation, and Cotton Blue for staining the chitin in the fungal cell wall (Santana et al., 2018). In addition, the species of the grown fungi were determined by considering factors such as the growth rate and the observable changes in the colour of the colony as they mature. Although Lactophenol cotton blue is commonly used, it may be difficult to see particular fungi because they do not produce spores, making it impossible to identify them based on spore features. Furthermore, acquiring a distinct perspective on the fruiting body of the fungi is challenging due to the potential disruption of its structure caused by the inoculation needle during the slide preparation procedure.

The Slide Culture Technique involves culturing the fungus colony on a small section of fresh PDA media and placing it on a microscope slide. The colony can proliferate for a few days before microscopic examination. By employing this method, the integrity of the

fungal spore will remain intact. Both approaches were utilised in this work as some spore structures of a fungal culture exhibited sufficient resilience to be visualised by the application of the lactophenol cotton blue procedure. The slide culture technique is more dependable but requires a more extended incubation period before viewing the slides. Therefore, both procedures were employed to get improved outcomes. For optimal results, it is advisable to inoculate the juvenile mycelia located near the periphery of the colony. The purpose is to guarantee that the harvested mycelia are actively growing so that the selected colony can thrive on the media.

Although morphological techniques are commonly employed in fungal taxonomic research to classify fungi at the ordinal or family level, they may not be as effective for classifying species at lower taxonomic levels (Raja et al., 2017; Yan et al., 2022). In addition, it might be challenging to exclusively identify fungi solely on morphology, mainly when individuals without expertise handle fungal cultures due to the limited number of morphological characteristics available for identification (Raja et al., 2017). For example, certain types of fungal strains that live inside other organisms, such as endophytes and endogenous fungi, are commonly employed to extract secondary metabolites. However, these strains do not usually produce spores when grown in a laboratory, so they cannot be identified based on their physical characteristics. Therefore, as a result of these complexities, the International Code of Nomenclature for algae, fungi, and plants has declared that this method is no longer permissible in the categorisation of species at lower levels (Hibbett et al., 2011; Raja et al., 2017).

2.7 Identification of fungi through molecular analysis

Previously, the identification of fungal species relied heavily on examining their macroscopic and microscopic features, deemed crucial for understanding their morphological history. However, it is now acknowledged that these techniques might not consistently produce exact classifications within an evolutionary framework, often restricted to the family or genus level (Wang et al., 2016). Furthermore, the physical characteristics may sometimes be deceptive because of the simultaneous evolution of fungal cultures and hybridization (Naranjo-Ortiz & Gabaldón, 2019). Hence, identifying fungi through molecular analysis is a new technique based on sequence analysis, which enables the targeted detection of DNA from various organisms or tissues, facilitating the identification procedure at the species level (Hariharan & Prasannath, 2020). This technique requires various components, including DNA Polymerase, Primers, DNTP (Deoxynucleotide triphosphate) mixture, nuclease-free water, buffer, and DNA templates obtained from sample extractions. The combination of all these components is referred to as a Master Mix (Raja et al., 2017).

DNA polymerase is an enzyme that functions as the catalyst for synthesising a new DNA strand complementary to the targeted sequence. The predominant DNA polymerase employed is *Taq* polymerase, also known as Polymerase Chain Reaction (PCR) enzyme, derived from *Thermis aquaticus* (Yamagami et al., 2014). The primers function as the initiation and termination points for the complementary DNA strands synthesised by the polymerase. A typical PCR buffer often consists of Tris-HCl (around pH 8.3), potassium chloride (KCl), or magnesium chloride (MgCl₂). However, the presence and concentration of each component may vary depending on the needs of the experiment (Owczarzy et al., 2008). Water is exclusively employed to attenuate the mixture to an appropriate ultimate concentration of the master mix, and it is imperative that the water utilised is devoid of

nucleases to prevent the deterioration or pollution of the DNA samples. Among the several types of primers available, Internal Transcribed Spacers (ITS) are the most frequently utilised in the field of mycology. This is because ITS has the ability to amplify DNA easily, has a broader range of applications in identifying species, evolves more quickly, and is more typically found in fungal systematic and taxonomy studies (Raja et al., 2017). Hence, selecting suitable primers is essential in PCR techniques to achieve precise species-level identifications of any organism. Finally, the DNA templates are often introduced last because they require the least volume measurement for a single reaction (Garibyan & Avashia, 2013)

The templates are the resulting products obtained from pre-existing DNA extractions. During a PCR procedure, the samples undergo a sequence of phases referred to as denaturation, annealing, and elongation. In the denaturation stage, the reaction temperature is increased to separate the complementary strands. Subsequently, annealing will take place, during which the temperature will be lowered to facilitate the binding of primers to the desired segments. Subsequently, in the elongation phase, the temperature is increased once more to facilitate the extension of primers by DNA polymerase, resulting in the formation of additional strands of DNA. The PCR products generated from PCR are subsequently submitted to Agarose Gel Electrophoresis (AGE) to facilitate the separation of the fragments of the PCR product. AGE is a very effective and efficient method for separating DNA fragments ranging in size from 100 base pairs (bp) to 25 kilobases (kb) (Lee et al., 2012). Once the DNA is placed into the pre-cast well in the gel and an electric current is supplied, the DNA molecules are sorted according to their size. The migration rate of a DNA molecule through a gel is determined by several factors, including the size of the DNA molecule, the concentration of agarose, the conformation of the DNA, the applied voltage, the presence of ethidium bromide, the kind of agarose used, and the electrophoresis buffer. Following

separation, the DNA molecules can be observed using ultraviolet (UV) light after being treated with a suitable dye for staining.

Internal Transcribed Spacers (ITS) denote a particular segment of the ribosomal transcript that undergoes removal and degradation as part of the maturation process (Fischer & Olivas, 2018). ITS is frequently employed for molecular identification of fungi due to its largely conserved length and sequence within the same fungal species, but variability between different fungal species (Vu et al., 2016). Their sequences typically exhibit greater diversity compared to the ribosomal sequence, making them commonly employed primers for phylogenetic analysis and the identification of fungal species and strains due to the observed variance among various strains within some fungal species (Iquebal et al., 2021; Zhang et al., 2018). It offers excellent species resolution in some taxonomic groups. Nevertheless, the nuclear ribosomal small subunit exhibits limited accuracy in distinguishing between different species of fungi, making it marginally less superior than the ITS (Schoch et al., 2012). The typical region employed comprises a fusion of ITS and ribosomal sequences, typically encompassing a fragmentary 18S rRNA sequence, an internally transcribed region (ITS1), the complete sequence of 5.8S rRNA, another internally transcribed region (ITS2), and a partial sequence of 28S rRNA (Al-Hindi et al., 2018). According to Badotti et al. (2017), ITS is the most effective method for identifying a wide range of fungus. It has the best chance of success and can distinguish between different species and variations within a species based on the ribosomal cistron. The barcode gap between interspecific and intraspecific variation is well-defined using this method.

2.8 Implications for food safety and quality assessment

The implication of fungal contamination presents significant challenges to the safety and quality of sago starch-based foods. Sago starch-based foods are often contaminated by fungi like *Aspergillus*, *Penicillium*, and *Fusarium* during preharvest, harvest and postharvest, leading to the production of mycotoxins such as aflatoxins, ochratoxins, and fumonisins (Afsah-Hejri et al., 2013). Consuming sago starch-based foods contaminated with mycotoxins can cause symptoms ranging from mild gastrointestinal issues to more serious health problems such as carcinogenicity, nephrotoxicity, hepatotoxicity, and acute toxicity (Awuchi et al., 2021). The severity of the symptoms depends on the specific species of fungi and the quantity ingested. Chronic exposure to mycotoxins can lead to immunosuppression, developmental abnormalities, and other adverse health effects, making the consumption of contaminated foods a significant public health concern (Janik et al., 2020). Food that has been infected with toxigenic fungi or their toxins may result in foodborne illnesses characterised by symptoms like nausea, vomiting, diarrhoea, fever, and discomfort in the stomach (Benedict et al., 2016). Fungi may trigger allergic reactions in people who are prone to them and can cause symptoms like itching, swelling, respiratory problems, or anaphylaxis in severe instances (Zukiewicz-Sobczak, 2013). Fungal contamination in food can be especially dangerous for populations at risk like newborns, old people, pregnant women, and individuals with weakened immune systems. These people may be more vulnerable to foodborne diseases and other health impacts from fungal toxins.

In addition to mycotoxin production, fungal contamination can also lead to spoilage of sago starch-based foods, causing undesirable changes in the discolouration appearance, slimy texture, and off-odour of food. Visual evidence of fungal contamination is often seen as fungi growth, as it can appear as fuzzy patches, spots, or discolouration on the surface of

various food items. According to a research by Mailafia et al. (2017), the presence of black mould (*Aspergillus niger*) on spoilt fruits vended in Gwagwalada market can cause blackening of the surface, indicating spoilage and fungal contamination. Sago starch contaminated by fungi would develop mould on their surface, resulting in a musty odour and an unappetizing appearance (Greenhill et al., 2007). Fungal growth can degrade the nutritional quality of food by breaking down essential nutrients by reducing levels of vitamins, minerals, and other beneficial compounds in contaminated food items and compromising their bioavailability (Alegbeleye et al., 2022). Improper disposal of fungal-contaminated food can contribute to environmental pollution as fungi in spoiled food may proliferate in landfills, releasing spores and mycotoxins into the air and soil, potentially affecting ecosystems and human health (Mosina et al., 2020). Fungal contamination can lead to food spoilage, rendering it unpalatable, unsafe, or unfit for consumption, decreasing consumer appeal and marketability. Food spoilage also poses economic implications for producers and distributors. This can result in economic losses for food producers and distributors.

Food safety authorities in Malaysia have established standard limits for fungal contamination in various food commodities, including sago starch-based foods, to ensure consumer safety and compliance with food safety regulations. The presence of fungi in sago starch-based foods must comply with regulatory limits set by food safety authorities. Non-compliance with these standards can lead to legal consequences and damage to the reputation of the food manufacturer. Contaminated batches may need to be recalled, leading to financial losses, damage to brand reputation, and legal consequences for non-compliance with food safety regulations (Gunawan et al., 2022). According to Gomez and Marks (2020), foodborne pathogens contamination can have significant economic consequences for the

food industry, including product recalls, loss of consumer trust, legal liabilities, and financial repercussions for affected businesses. Contaminated food products may need to be discarded, leading to wasted resources and lost revenue for the businesses involved. Fungal contamination can result in financial losses at various stages of the food supply chain, including production, processing, distribution, and retail. Incidents of fungal contamination in food can tarnish the reputation of food manufacturers, suppliers, and retailers (Davies et al., 2021). Negative publicity surrounding food safety issues can erode consumer trust and loyalty, impacting sales and brand equity in the long term. It can disrupt international trade relations and impose barriers to the export of food products (Nji et al., 2022).

2.9 Strategy for prevention and control of fungal contamination

Sago starch consumption in Sarawak has seen the potential to help reduce food insecurity. Promoting of awareness of sustainable sago palm production, starch extraction, and utilisation is essential for increasing sago starch productivity. It is necessary to broaden awareness and advocate for the sustainable production and use of sago starch, enhance the implementation of good agricultural practices (GAP) in sago palm cultivation, and encourage good manufacturing practices (GMP) in sago starch production.

The enforcement of GAP policy, such as strict cleanliness practices during sago palm production and harvesting, is required to prevent initial fungal contamination. Based on the Code of Good Agricultural Practice established by Malaysia Palm Oil Malaysian Palm Oil Board (2008), peat land oil palm producers are taught the essential GAP guideline elements, including planting, fertilizing procedures, soil and water management, drainage systems, insect and disease control, harvesting, and post-harvesting procedures. According to Awang

et al. (2021), the empirical results of their research showed that GAP has direct positive effects on farm performance and positive total effects on economic well-being. The implementation of GMP procedures in sago starch processing plants is of the utmost importance for reducing fungal contamination in the production process. This includes upholding cleanliness and sanitation of processing equipment, imposing pest control measures, and using standardised operating procedures to avoid cross-contamination. The studies by Pue et al. (2018) stated that two critical control points in traditional sago production are the preharvest stage for palm selection and the postharvest stage for sago starch extraction. Using clean tools and fresh water to extract the sago is crucial for ensuring the safety of the food.

The development of a precise HACCP plan for sago starch production can assist in recognising and minimising fungal risks during various stages of the production process. Critical control points (CPP) were discovered at multiple stages of sago starch production, such as tree selection, starch extraction, sago starch storage, and cooking (Greenhill et al., 2010). With the integration of natural antifungal substances like organic acids or essential oils into manufacturing practices, fungal development can be prevented, and the shelf life of sago starch products can be prolonged without affecting food safety. According to Tan et al. (2017), synthesised starch derivatives with quaternary phosphonium salts had superior antifungal activity compared with common starch. Another study by Habschied et al. (2021) resulted in improvements in the efficiency of using biological antifungal substances against some specific fungus and can help decrease fungicide pollution in food products.

Meanwhile, appropriate storage conditions are mandatory for inhibiting fungal growth and preserving the quality of sago starch products. Several biotic and abiotic

variables can cause postharvest losses in food commodities. For instance, fungal bio-deterioration is common in storage systems in tropical hot and humid climates (Pandey et al., 2023). By managing appropriate temperature and humidity controls, as well as ensuring adequate packaging to prevent moisture ingress, can mitigate the risk of fungal contamination during storage and transportation. Besides, the creation of awareness through training and education programmes to farmers, processors, and other stakeholders in the sago starch supply chain helps increase understanding of the significance of fungal risk mitigation techniques and compliance with safety protocols. As suggested by Taridala et al. (2013), the future players in the sago agribusiness should actively seek more knowledge regarding both processing and product qualities, which are attributes to achieve consumer satisfaction.

Food safety authorities in Malaysia, such as the Industry Standards Committee on Food, Food Products and Food Safety, which consists of central regulators and enforcers, the Ministry of Health Malaysia, the Ministry of Agriculture Malaysia, local authorities, and customs, have established regulatory limits for fungal contamination in various food commodities including sago starch-based foods to ensure consumer safety and compliance with food safety regulations. According to Malaysian Standard (2019a), the permissible limit for fungal contamination of edible sago starch is a set limit of not more than 10^2 CFU/g. International food safety authorities such as the Drug Administration (FDA) and the European Food Safety Authority (EFSA) also established their standard that can be used as a reference for fungal contamination control. Working collaboratively with regulatory agencies and industry associations to establish and enforce strict safety standards and regulations for sago starch production can guarantee consumer protection and maintain industry integrity.

CHAPTER 3

METHODOLOGY

3.1 Summary of methodology

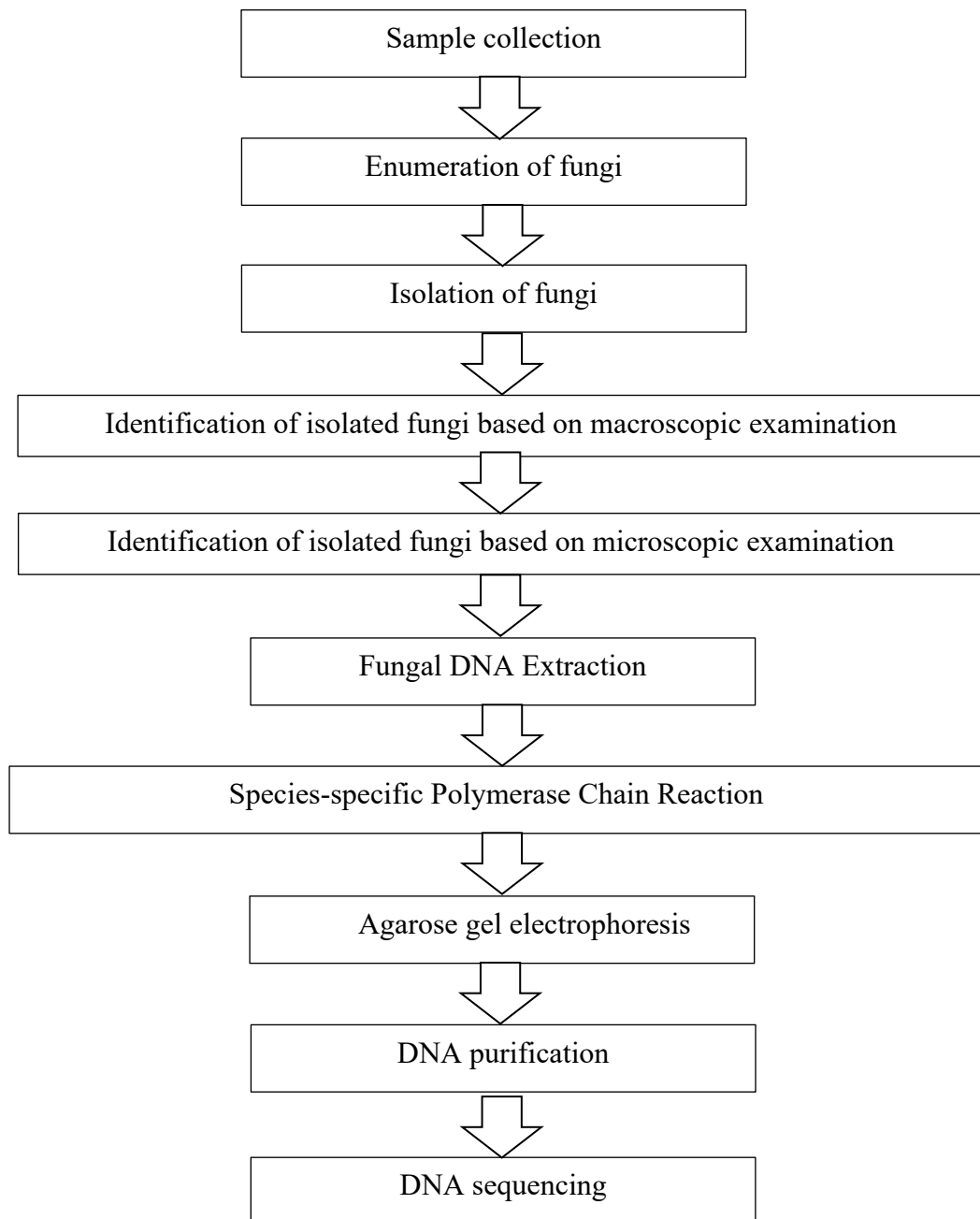


Figure 3.1: The flow chart of methodology involved in this study

Figure 3.1 show the summary of methodology flowchart of this research. Generally, the screening of fungi was conducted by using the serial dilution technique to isolate the fungi present in sago starch samples and to enumerate the prevalence and abundance rate of fungi, which were collected from different districts and regions in Sarawak. Identification of fungal was done based on morphological characterization via macroscopic and microscopic and examination and further confirmation of fungal identification species by molecular analysis through species-specific Polymerase Chain Reaction targeting the internal transcribed spacer (ITS) region.

3.2 Research methodology

3.2.1 Sample collection

Fifty samples of raw wet sago starch were collected and purchased from wet market from different districts and regions in Sarawak such as Mukah, Dalat, Matu, Pusa, Betong, Bintulu, Marudi, Miri, Sibu and Selangau. from September 2020 until January 2021. The details of sago starch samples from respective locations were recorded. These locations were selected as the sampling sites based on the distribution of land were cultivated with sago by small-scale sago farmers or planted by commercial sago plantation located in Sarawak which the data was obtained by the Department of Agriculture Sarawak. Samples were purchased from local wet markets, and some were directly collected from the plantation sites. The collected samples were sealed properly in sterile polythene bags and placed in a cool, insulated container during transportation from the origin location to the laboratory. Sago starch samples were analysed immediately upon arrival at the Microbiology Laboratory, Department of Molecular Biology, Faculty Resource Science and Technology, Universiti Malaysia Sarawak, or refrigerate at 4°C for not more than 48 hours before being analysed.

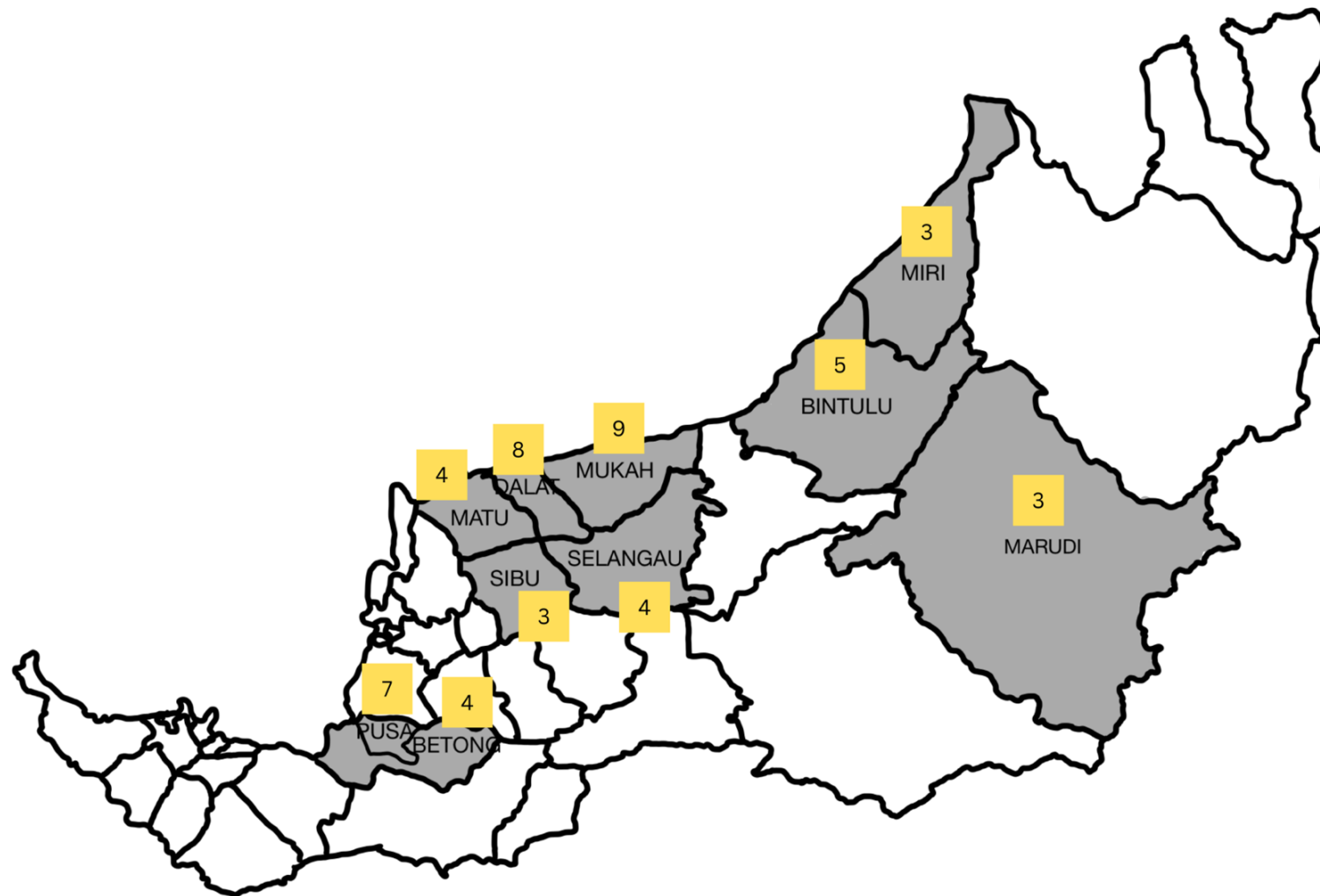


Figure 3.2: Map of Sarawak by districts and the total number of samples obtained from respective locations denoted

3.2.2 Enumeration of fungi

All of the samples were enumerated for fungi colonies using the standard dilution method with the purpose of diluting the fungi in the sample in each dilution (Greenhill et al., 2007). A $10.0 \text{ g} \pm 0.2 \text{ g}$ sample of sago starch was aseptically weighed and added into 90 ml of 0.1% peptone water (HIMEDIA, India) to achieve 1:10 dilution. The sample was then homogenised by using a surface-disinfected food blender at a constant speed for 5 min. Potato dextrose agar (PDA) medium (HIMEDIA, India) with 100 mg of chloramphenicol (ACROS, Belgium) was prepared for this standard dilution method and therefore spread plating was done. Appropriate tenfold serial dilution was prepared by diluting the 1 ml of aliquot sample with 9 ml of 0.1% peptone water, and 1 ml portions of suitable dilutions of the resulting samples suspension were pipetted and inoculated onto PDA medium by spread-plating method using a sterile bent glass rod. Three replicates of PDA plates per sample were made. The plates were incubated at 28°C for 48-72 h, after which time fungi colonies were enumerated for the first time. A further incubation for 48-96 hours was done, and fungi colonies were subsequently enumerated for the second time. The fungal colonies present on the PDA plate were counted, and the mean quantities per gram of the sample were calculated and reported as colony-forming units (CFU) per gram (Al-Hindi et al., 2018).

3.2.3 Isolation of fungi

Following the enumeration of fungi, the representative of grown isolated fungi were taken from the cultured PDA medium and sub-cultured onto a fresh new PDA medium in order to obtain a pure fungal culture. Three replicates of PDA plates per isolated fungi were made. The plates were incubated at 25°C for 5-10 days. Once the isolated fungi had reached

pure culture, sterilised straws were used to pluck disks from the colony edge and stored at 4°C in capped tubes containing autoclaved distilled water to maintain the strains and to be kept as stock cultures.

3.2.4 Identification of isolated fungi genus based on morphological observation

3.2.4.1 Macroscopic examination

A pure culture of isolated fungi was observed by carrying out a macroscopic examination using a stereo microscope (OLYMPUS, Japan) by directly observing the colony morphology of isolated fungi pure culture on PDA in the plates. During macroscopic observation, fungal isolates showing similar characteristics (e.g., size, shape, pigmentation, elevation and marginal) were grouped and labelled. Each of the isolates was subjected to microscopic examination for further observation (Senanayake et al., 2020).

3.2.4.2 Microscopic examination

Conidiophores and conidial morphology were observed through microscopic examination. Slide culture technique and lactophenol cotton blue staining were used for this observation with some modifications (Senanayake et al., 2020). A fresh PDA medium was cut into 5 mm squared blocks using a sterile scalpel. The block of agar was transferred onto a sterile microscope slide, which was placed into an empty sterile Petri dish. The mycelial fragment or spores from the fungi pure culture were scrapped off using a sterile inoculating loop and inoculated on the sides of the block of agar. A sterile cover glass was aseptically placed on the upper surface of the agar block. The Petri dish was sealed and incubated at

room temperature for 48 h. Once the incubation period was completed, the cover glass with fungal growth was removed from the block of agar and placed onto a new microscopic slide with a drop of lactophenol cotton blue stain on the surface of the slide. The sample was then observed under the microscope (OLYMPUS, Japan) and the characteristic of the conidiophores and conidia were recorded. The characteristics that needed to be observed were the structure of the hyphae, hyphal branching, the structure of the fruiting body, and the shape and size of the spores (Barnet & Hunter, 1998).

3.2.5 Identification of isolated fungi species based on molecular analysis

3.2.5.1 Fungal DNA extraction

Heat-shock DNA extraction was done by scrapping off a small portion of isolated fungi pure culture and suspended into a sterile 0.5 ml PCR tube containing 50 µl of TE buffer (1M Tris-CL pH 8.0, 0.5M EDTA pH 8.0) using a sterile inoculating needle (Pandi, 2019). The solution containing the sample was physically crushed using the same sterile inoculating needle to disrupt the cell walls or tough tissue of the sample. The samples were ultra-frozen at -80°C in an ultra-low temperature freezer (HAIER, China) for 30 mins. The samples were partially incubated at 50°C in a water bath (DAIHAN, Korea) for 1 min. Due to rapid changes in extreme temperature, the cell walls of the sample were ruptured, hence releasing the genomic DNA, which is crucial in the identification of species through molecular analysis. The supernatant containing genomic DNA was directly quantified using agarose gel electrophoresis by comparing the sample DNA intensity to that of a DNA quantitation standard. Prior to the high concentration of DNA, the extracted DNA was then diluted into 1× concentration by adding 1 µl of extracted product with 9 µl of TE buffer.

3.2.5.2 Species-specific Polymerase Chain Reaction

The nuclear ribosomal DNA internal transcribed spacer (ITS) gene regions of the isolated fungi were amplified using a set of forward primer and reverse primer, ITS1 and ITS 4 (Haque et al., 2020; Hasan & Zauddin, 2018). This set of primers was chosen due to their high coverage through the diverse taxonomic groups of fungi compared to the other primers (Toju et al., 2012). The PCR Master mix with a total volume of 25 µl was prepared accordingly with accurate volume and concentration, which was composed of all the components listed in Table 3.1

Table 3.1: Name of primers used, sequence and base pair

Primer name	Primer sequence (5' to 3')	Target base pair
ITS1 (Forward)	TCCGTAGGTGAACCTGCGG	~550
ITS4 (Reverse)	TCCTCCGCTTATTGATATGC	~550

Table 3.2: Components of Master mix for PCR amplification

Reagent	Description	Quantity (µl)
ITS1 (Forward)	10 pmol/µl	1.0
ITS4 (Reverse)	10 pmol/µl	1.0
PROMEGA 5X GoTaq Flexi Buffer	-	5.0
PROMEGA MgCl ₂	25mM	2.0
PROMEGA dNTPs Mix	25mM	0.7
PROMEGA GoTaq Flexi DNA Polymerase	5u/µl	0.3
DNA template (from DNA Extraction)	-	3.0
Autoclaved ultra-pure water	HPLC grade	12.0
TOTAL		25.0

Table 3.3: Cycling conditions for ITS region

Steps	Temperature (°C)	Duration	Cycle(s)
Initial denaturation	94	2 min	1
Denaturation	94	1 min	
Annealing	55	1 min	35
Elongation	72	1 min	
Final elongation	72	10 min	1
Hold	4		1

3.2.5.3 Agarose gel electrophoresis

Upon completing the PCR amplification, the PCR products were subjected to agarose gel electrophoresis. A concentration of 1.5% (w/v) agarose gel was prepared by mixing 1.5 g of agarose gel powder (First Base, Singapore) with 100 ml of 1× TBE buffer, which was then heated in a microwave at mid-high temperature until the mixture boiled and the powder dissolved completely. The mixture was cooled down to a suitable temperature and then poured into a casting tray with a gel comb attached. A solidified agarose gel with an unattached gel comb was placed in the electrophoresis chamber and immersed in 1× TBE buffer. The buffer was poured and adjusted until the surface of the gel was fully covered. Five µl of 1 kb DNA ladder (PROMEGA, United States) was loaded into the first well, followed by 5 µl of each PCR product into the subsequent wells. Negative control was included and loaded into the last well. The electrophoresis was set at 80 V with 300 mAh for 1 hour. The gel was stained with 1% (w/v) EtBr solution for 30 min and then visualised using the UV light transilluminator (MAESTROGEN, Taiwan). Any visible DNA band would be cut into fragments for further purification of PCR products.

3.2.5.4 DNA purification

The PCR product was then recovered using Nucleospin Gel and PCR Cleanup Kit (MACHERRY-NAGEL, Germany) and conducted according to the manufacturer manual. The fragment of visible DNA band on 1.5% agarose gel was cut into using a sterile scalpel and placed into a sterile 1.5 ml centrifuge tube containing 300 µl of NTI buffer. The tube was incubated in a water bath at 50°C for 10 min and vortexed in 3-min intervals during the incubation period until the DNA fragmented gel completely was dissolved and mixed with the buffer. Seven hundred µl of mixture samples was aseptically pipetted into Nucleospin Gel and PCR Cleanup column, which was assembled with a sterile 2 ml collection. The sample was centrifuged at $11,000 \times g$ for 30 sec. The flow-through was discarded, and the spin column was reassembled with the collection tube. Seven hundred µl of NT3 buffer was pipetted into the spin column and centrifuged at $11,000 \times g$ for 30 sec. The flow-through was discarded, and the spin column was reassembled with the collection tube. Further centrifugation at $11,000 \times g$ for 1 min was done to ensure the Buffer NT3 residue was completely removed. The spin column was then placed into a new sterile 1.5 ml centrifuge tube, and 25 µl of NE buffer was pipetted into the spin column and incubated at room temperature for 1 min. The tube was centrifuged at $11,000 \times g$ for 1 min, and the spin column was removed. The supernatant containing purified genomic DNA was kept at -20°C before being sent for DNA sequencing.

3.2.5.5 DNA sequencing

The purified DNA products were sent to a sequencing service provider company, Apical Scientific Sdn. Bhd. (604944-X), for both forward and reverse primer Sanger sequencing using an ABI PRISM sequencer. The DNA sequencing results provided by the service company were qualitatively assembled and trimmed based on the quality of electropherograms by using a data analysis programme, Molecular Evolutionary Genetics Analysis Version 10.0 (MEGA X) software (Kumar et al., 2018). The sequences were then subjected to CAP3 Sequence Assembly Program software (Rhone-Alpes Bioinformatics Center) to obtain the consensus sequences from the forward and reverse sequences (Huang & Madan, 1999). The consensus sequences were then deposited to the National Centre for Biotechnology Information (NCBI) GenBank database, and a comparative search was carried out using the Basic Local Alignment Search Tool (BLASTn) algorithm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Enumeration of fungal count

In general, fungal counts in all sago starch samples exceeded the acceptable limit established by the Malaysian Standard for edible sago starch. However, fungal counts of all sago starch samples complied within the acceptable limit drafted by the International Commission for Microbiological Specification of Foods for grains and flours, with the highest count was 2.1×10^4 CFU/g, the lowest count was 1.3×10^2 CFU/g, and the mean count was 8.6×10^3 CFU/g, demonstrating significant heterogeneity in fungal contamination among samples. This distinction underscores the variance in safety thresholds between Malaysian standards and international standards, highlighting the need for contextualizing microbial limits based on intended use and regional considerations. The enumeration of fungi colonies was carried out by using the standard dilution method on PDA plates.

Table 4.1 shows the sample code and the fungal count (CFU/g) of fifty sago starch samples. The fungal counts presented in Figure 4.1 reveal substantial variability, likely attributable to differences in environmental conditions, handling practices, or storage environments at the collection sites. Samples such as MT4 and DL5 showed elevated fungal counts exceeding 2.0×10^4 CFU/g, which may be indicative of higher contamination levels or more favorable conditions for fungal growth. Conversely, samples such as MK5 and MR2, with significantly lower counts, suggest minimal contamination or stricter handling and storage conditions.

Table 4.2 shows the location of sample collection and average fungal count of each location meanwhile Figure 4.2 aggregates fungal count data by location, illustrating average contamination levels across collection sites. Regions such as Pusa, Matu, and Dalat show higher mean fungal counts (exceeding 1.0×10^4 CFU/g), potentially linked to environmental conditions that support fungal growth, including high humidity or suboptimal storage environments. Conversely, locations like Betong and Bintulu display lower average counts, implying reduced fungal load possibly due to more controlled storage practices or less exposure to growth-promoting conditions. This location-based analysis provides a foundation for targeted interventions and underscores the importance of maintaining consistent storage and handling standards to mitigate fungal contamination in sago starch products.

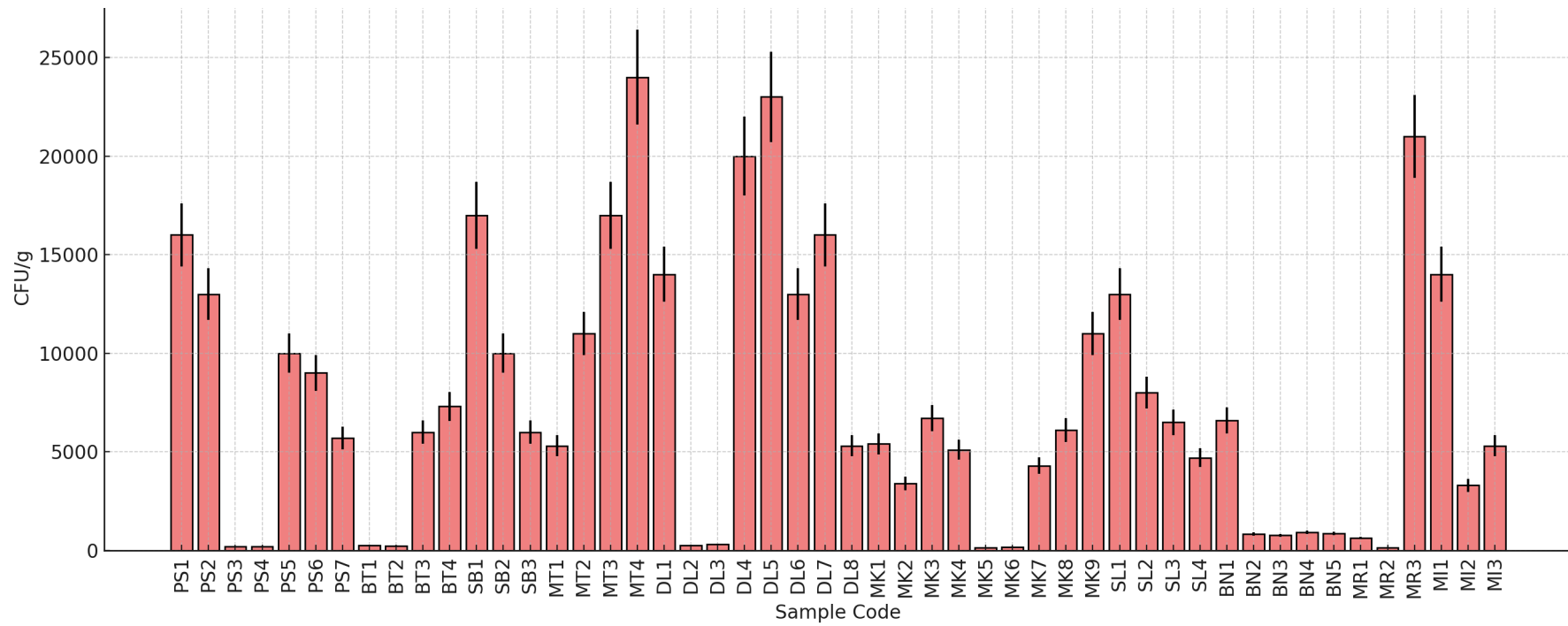


Figure 4.1: CFU/g for each sample code with error bars

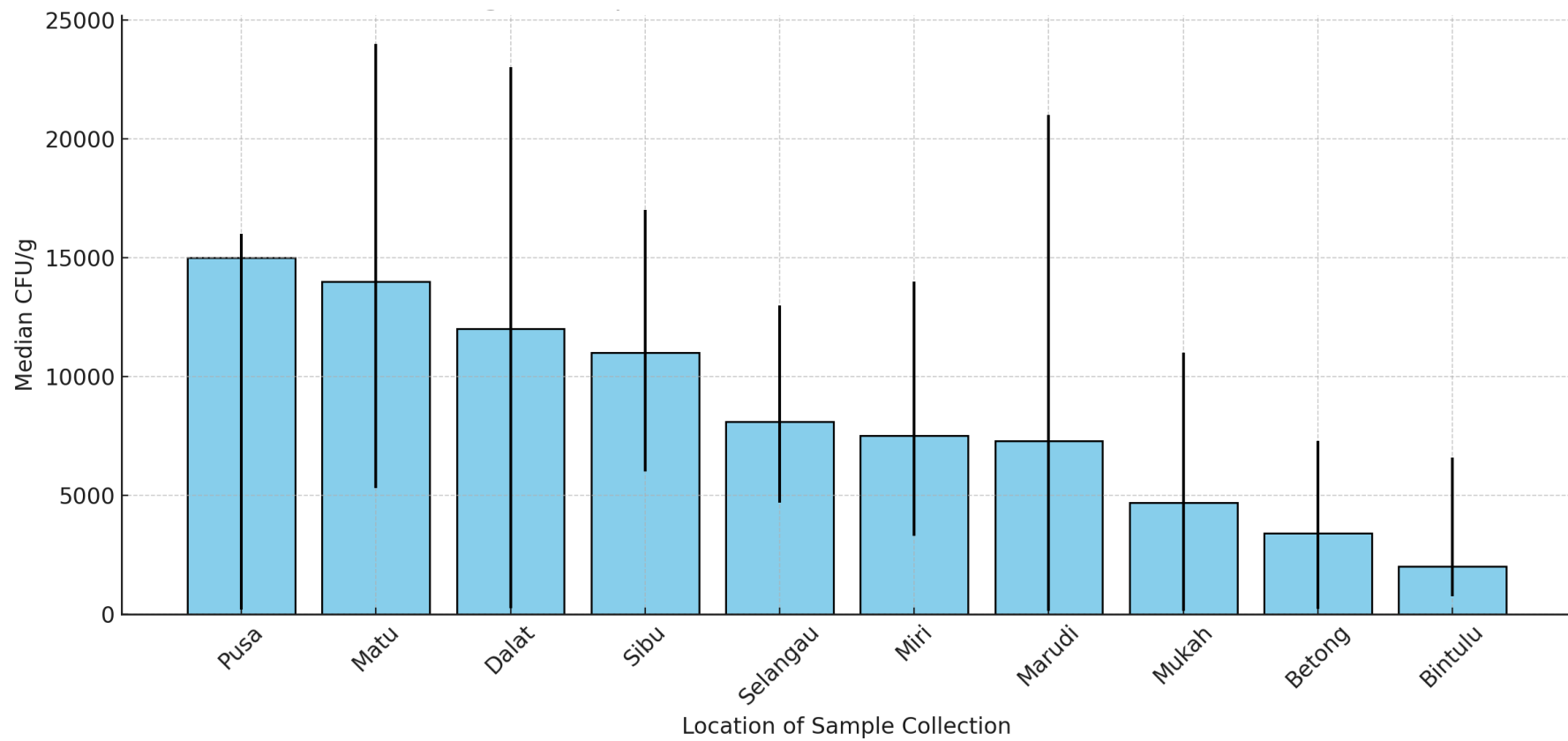


Figure 4.2: Median CFU/g of samples collected from various locations with error bars

Table 4.1: Sample code and the fungal count (CFU/g)

Sample Code	CFU/g	Sample	CFU/g
PS1	1.6×10^4	DL8	5.3×10^3
PS2	1.3×10^4	MK1	5.4×10^3
PS3	1.9×10^2	MK2	3.4×10^3
PS4	2.1×10^2	MK3	6.7×10^3
PS5	1.0×10^4	MK4	5.1×10^3
PS6	9.0×10^3	MK5	1.3×10^2
PS7	5.7×10^3	MK6	1.7×10^2
BT1	2.5×10^2	MK7	4.3×10^3
BT2	2.2×10^2	MK8	6.1×10^3
BT3	6.0×10^3	MK9	1.1×10^4
BT4	7.3×10^3	SL1	1.3×10^4
SB1	1.7×10^4	SL2	8.0×10^3
SB2	1.0×10^4	SL3	6.5×10^3
SB3	6.0×10^3	SL4	4.7×10^3
MT1	5.3×10^3	BN1	6.6×10^3
MT2	1.1×10^4	BN2	8.3×10^2
MT3	1.7×10^4	BN3	7.6×10^2
MT4	2.4×10^4	BN4	9.2×10^2
DL1	1.4×10^4	BN5	8.6×10^2
DL2	2.6×10^2	MR1	6.3×10^2
DL3	3.2×10^2	MR2	1.5×10^2
DL4	2.0×10^4	MR3	2.1×10^4
DL5	2.3×10^4	MI1	1.4×10^4
DL6	1.3×10^4	MI2	3.3×10^3
DL7	1.6×10^4	MI3	5.3×10^3

Notes: PS - Pusa, BT - Bintulu, SB - Sibul, MT- Matu, DL – Dalat, MK – Mukah, SL – Selangau, BN – Bintulu, MR – Miri, MD - Marudi

Table 4.2: The location of sample collection and average fungal count (CFU/g)

Location of sample collection	(CFU/g)
Pusa (PS)	1.5×10^4
Matu (MT)	1.4×10^4
Dalat (DT)	1.2×10^4
Sibu (SB)	1.1×10^4
Selangau (SL)	8.1×10^3
Miri (MR)	7.5×10^3
Marudi (MD)	7.3×10^3
Mukah (MK)	4.7×10^3
Betong (BT)	3.4×10^3
Bintulu (BN)	2.0×10^3

4.1.2 Morphological identification of the isolated fungal

Based on the morphological observation, 45 pure cultures of isolated fungi were grown on PDA, and there were ten different genera of the isolated fungi identified from the sago starch samples. The genera that were identified includes *Aspergillus* spp., *Candida* sp., *Ceratocystis* sp., *Paecilomyces* spp., *Penicillium* spp., *Peniophora* sp., *Perenniporia* sp., *Phaeacremonium* sp., *Pleurostoma* spp. and *Trichoderma* spp. The morphological identification was done via both macroscopic and microscopic examination. The macroscopic examination was carried out by observing the growth rate and morphological characteristics of isolated fungi pure culture on PDA plates and on reverse PDA plates. The growth rate of mycelia was determined by observing the diameter of its growth on PDA over a period of seven days. Fast growing fungi was classified as endophytic fungi that can fill the culture plate within two to three days. Eighty-seven percent of the total isolates showed a fast-growing rate and only six isolates filled the culture plate within four to seven days.

Meanwhile, the microscopic examination involved comparing the conidiophores, conidial morphology, and fruiting body by using the slide culture technique and lactophenol cotton blue staining with some modifications. To differentiate among all the isolated fungi, a conventional taxonomic keys and authorized monographs were used, and these isolates were organized into appropriate genera. The fungal genera with the highest occurrence were *Ceratocystic* sp. (28.89%), and followed by *Penicilium* spp. (17.78%), *Paecilomyces* spp. (13.33%), *Peniophora* sp. (11.11%), *Pleurostoma* sp. (8.89%), *Aspergillus* spp. (6.67%), *Perenniporia* sp. and *Trichoderma* spp. (4.44% each), *Phaeacremonium* sp. and *Candida* sp. (2.22% each). Table 4.3 shows the summary of the genera found through microscopic view and the percentage (%) of isolated fungi. Table 4.4 shows the summary of the morphological characterisation of the fungal isolates, the growth rate and the frequency of isolation for each of the sampled sago starch.

The data presented in Table 4.3 and Table 4.4 offer significant insights into the fungal diversity within the sago starch samples, elucidating both the frequency of fungal isolation and their morphological characteristics. Table 4.3 provides a detailed breakdown of the prevalence of various fungal genera, with *Ceratocystis* sp. being the most frequently isolated genus (28.89%), followed by *Penicillium* spp. (17.78%) and *Paecilomyces* spp. (13.33%). These findings are critical for identifying the dominant fungal species present in the sago starch samples, which may have implications for both the safety and quality of the product, particularly considering the potential for mycotoxin production by certain genera, which could pose food safety risks. The frequency data presented in Table 4.3 enables the prioritization of fungal genera for further investigation, particularly those with higher isolation rates such as *Ceratocystis* sp. and *Penicillium* spp., which appear in multiple isolates. This is important for assessing the potential risks associated with these genera in

the context of sago starch production and storage, as fungal contamination can lead to product spoilage, reduced quality, and degradation over time.

In contrast, Table 4.4 provides a comprehensive analysis of the morphological characteristics of the fungal isolates, detailing both macroscopic and microscopic features alongside their growth rates. The categorization of fungal isolates into fast-growing and slow-growing species is essential for understanding their ecological dynamics and for formulating effective management strategies. Fast-growing fungi, such as *Ceratocystis sp.*, *Penicillium spp.*, and *Paecilomyces spp.*, are particularly noteworthy due to their rapid colonization of substrates, which increases the likelihood of contamination and spoilage within a short period. Additionally, the detailed morphological descriptions in Table 4.4 provide critical information for the accurate identification of fungal species in future research or industrial monitoring. For instance, the distinctive features of *Ceratocystis sp.*, including powdery black mycelium, dark greenish-brown coloration, and septate branched hyphae with conidiophores bearing conidia, serve as a reliable diagnostic criterion for identifying this species in field samples or during the production of sago starch. The integration of these morphological characteristics with growth rate data offers a more comprehensive understanding of fungal behavior and enhances the precision of fungal species identification.

Table 4.3: Summary of the fungal genera found through morphological identification and the percent (%) of prevalence

Genus	Isolates Code	Frequency of Isolation	
		n	%
<i>Ceratocystic</i> sp.	S1, S2, S3, S4, S7, S18, S23, S37, S40, S41, S42, S43, S45	13	28.89
<i>Penicilium</i> spp.	S6, S13, S15, S16, S19, S24, S28, S33	8	17.78
<i>Paecilomyces</i> spp.	S14, S26, S27, S29, S30, S31	6	13.33
<i>Peniophora</i> sp.	S8, S17, S35, S36, S44	5	11.11
<i>Pleurostoma</i> sp.	S10, S11, S20, S38	4	8.89
<i>Aspergillus</i> spp.	S12, S22, S39	3	6.67
<i>Perenniporia</i> sp.	S32, S34	2	4.44
<i>Trichoderma</i> spp.	S5, S25	2	4.44
<i>Candida</i> sp.	S21	1	2.22
<i>Phaeoacremonium</i> sp.	S9	1	2.22

Table 4.4: Summary of morphological characterisation of the fungal isolates and growth rate and their frequency of isolation in the sago starch sample

No	Morphological characterisation of the fungal isolates		Frequency of isolation		Growth rate	Genus	Sampling location
	Macroscopy	Microscopy	n	%			
1	Powdery black and dark greenish-brown in colour mycelia and black and dark greenish-brown in colour on the reverse side	Septate branched thin-walled hyphae with dense network, simple or branched conidiophores and erected and arise from the mycelium, conidiophores with structures that bear conidia, conidia oval to cylindrical in shape	13	28.89	Fast growth ^a	<i>Ceratocystic</i> sp.	Matu, Dalat, Mukah, Selangau, Sibu
2	Velvety or powdery yellowish-brown and brown and a yellow to yellowish brown in colour reverse for the reverse side	Septate branched thin-walled hyphae with flask-like phialides and closely packed brush-like structures, conidiophores unbranched and arise from the mycelium, spherical or flask-shaped structures and yellow-green spores	8	17.78	Fast growth	<i>Penicilium</i> spp.	Miri, Marudi, Bintulu
3	Velvety or powdery mycelia, and various colours can be observed, mostly brown or wood-like in colour and the mostly brown or wood-like in colour for the reverse side	Septate branched thin-walled hyphae with dense network, conidiophores branched and arise from the mycelium, bear conidia produced in clusters-like structures, conidia oval to cylindrical in shape, dark brown to black in colour spores	6	13.33	Fast growth	<i>Paecilomyces</i> spp.	Pusa, Betong, Sibu

Table 4.4 continued

4	Rough and velvety mycelia and pinkish-brown or wood-like in colour and brown or wood-like in colour for the reverse side	Septate branched thin-walled hyphae with dense and simple network, ellipsoidal or cylindrical basidiospores, basidia bear basidiospores produced in club-shaped, spherical, smooth and colourless spore	5	11.11	Fast growth	<i>Peniophora</i> sp.	Dalat, Mukah, Matu
5	Finely hairy and woolly and greyish brown and white in colour and greyish brown and white in colour on the reverse side	Septate branched thin-walled hyphae with cylindrical-like hyaline conidia and phialides, spherical and ellipsoidal conidiophores, conidia bear conidiophores produced in chains-shaped structures, smooth and ellipsoidal spore	4	8.89	Fast growth	<i>Pleurostoma</i> sp.	Betong, Pusa
6	Cottony and fluffy yellow-green umbonate in colour with whitish areal hyphae and white border and a pale yellow to milk in colour reverse for the reverse side	Septate branched thin-walled hyphae with acute branching filamentous, conidiophores branched and arise from the mycelium, bear conidia produced in clusters-like structures, spherical to ellipsoidal in shade, smooth yellow and green in colour. spores	3	6.67	Fast growth	<i>Aspergillus</i> spp.	Matu, Sibu

Table 4.4 continued

7	Woolly and finely hairy mycelia, and colour can be observed as pale beige to white in colour and pale beige to white in colour on the reverse site	Septate branched thin-walled hyphae and hyaline-bearing clamp connections, ellipsoidal or cylindrical basidiospores, bear basidiospores produced in club-shaped structures, spherical and smooth in shape spores	2	4.44	Slow growth	<i>Perenniporia</i> sp.	Miri
8	Fluffy and soft to granular light green and white coloured aerial mycelia and white-greenish colour for the reverse side	Septate branched thin-walled hyphae with pigmented conidiophores with lateral branches emerging angles, spherical and ellipsoidal conidiophores, bear conidia produced in chain-like structures, green or white colour spores	2	4.44	Fast growth	<i>Trichoderma</i> spp.	Bintulu
9	Creamy to white in colour and may have a smooth, shiny, or wrinkled surface texture and the same structure on reverse side	True hyphae with elongated, cylindrical structures with septate, chlamydospores, which are thick-walled, round, or oval structures.	1	2.22	Slow growth ^b	<i>Candida</i> sp.	Pusa

Table 4.4 continued

							Marudi
10	Velvety beige and white to cream-coloured colour and beige and white to cream-coloured colour for the reverse side	Septate branched thin-walled hyphae with pigmented conidiophores, conidiophores branched and arise from the mycelium, bear conidia produced in chain-like structures, smooth, spherical and ellipsoidal in shape spores	1	2.22	Slow growth	<i>Phaeoacremonium</i> sp.	

^aEndophytic fungi growth fills the culture plate within 3 days,

^bEndophytic fungi growth fills the culture plate within 4-7 days

4.1.2.1 Morphology of *Aspergillus* spp.

Three isolates were identified as *Aspergillus* spp., namely S12, S22, S39. Figure 4.3 shows the representative of macromorphological characteristic of *Aspergillus* spp. on PDA plates and on reverse PDA plates. These fungal colonies appeared with cottony and fluffy yellow-green umbonate in colour with whitish areal hyphae and white border on the PDA, and a pale yellow to milk in colour reverse for the reverse plate of the PDA. These fungal colonies are typically fast-growing. Hyphae are typically thin-walled, branched, septate and hyaline with acute branching filamentous. The mature colony would have been conidiophores branched and arise from the mycelium. Conidiophores are specialized structures that bear conidia produced in clusters-like structures. The spores are usually spherical to ellipsoidal in shade, smooth yellow and green in colour.

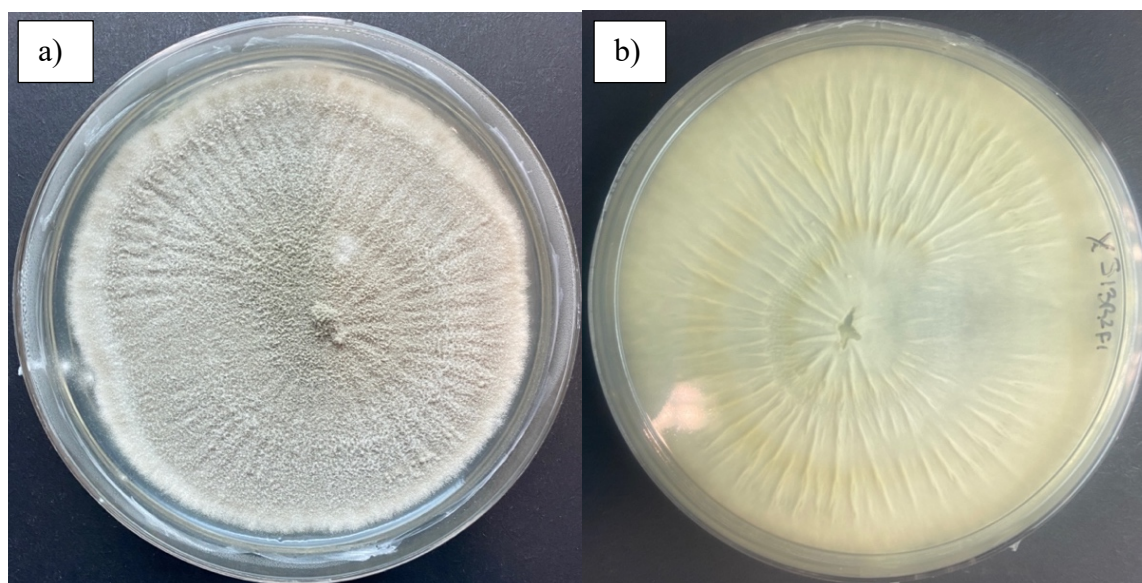


Figure 4.3: The representative of macromorphological characteristic of *Aspergillus* spp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.2 Morphology of *Candida* sp.

One isolate was identified as *Candida* sp., S21. Figure 4.4 shows the representative of macromorphological characteristic of *Candida* sp. on PDA plates and on reverse PDA plates. These fungal colonies appear creamy to white in colour and may have a smooth, shiny, or wrinkled surface texture on PDA and the same structure on reverse plate PDA. These fungal colonies are typically slow-growing. True hyphae with elongated, cylindrical structures with septate that divide the hyphae into individual cells. The mature colony would have chlamydospores, which are thick-walled, round, or oval structures.

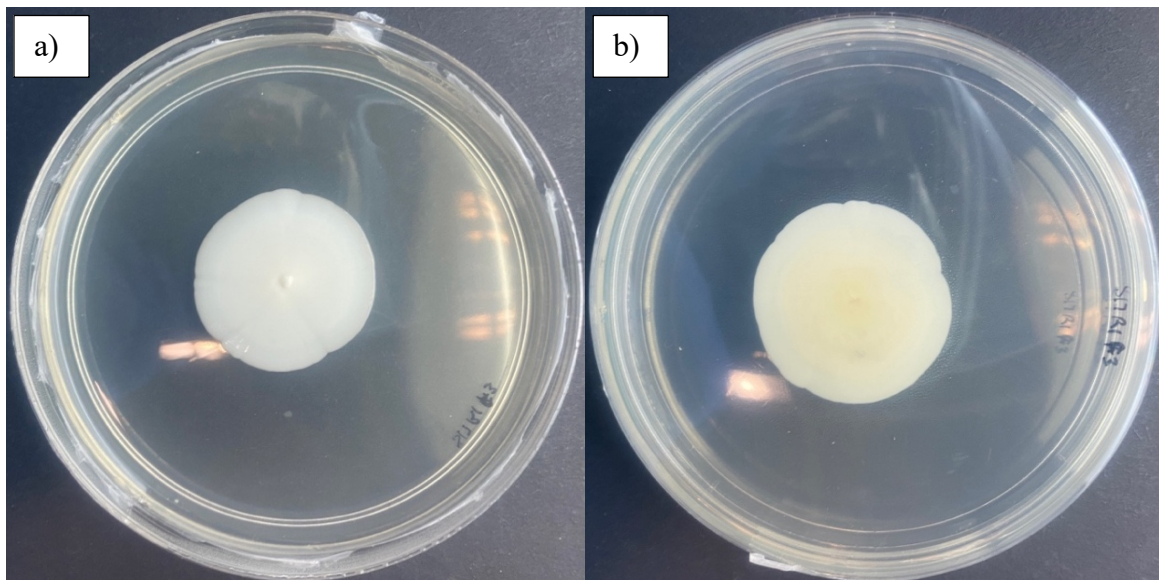


Figure 4.4: The representative of macromorphological characteristic of *Candida* sp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.3 Morphology of *Ceratocystic* sp.

Thirteen isolates were identified as *Ceratocystic* sp., namely S1, S2, S3, S4, S7, S18, S23, S37, S40, S41, S42, S43 and S45. Figure 4.5 shows the representative of macromorphological characteristic of *Ceratocystic* sp. on PDA plates and on reverse PDA plates. These fungal appeared with powdery black and dark greenish-brown in colour mycelia on the PDA and black and dark greenish-brown in colour on the reverse plate of the PDA. The color of the colony can vary from white, grey, to cream-colored initially, later turning dark or black as the fungus matures. These fungal colonies are typically fast-growing. Hyphae are typically thin-walled, branched, septate and hyaline with dense network. Conidiophores are simple or branched and erected and arise from the mycelium. The mature colony would have conidiophores with structures that bear conidia. The conidia shape for most species is oval to cylindrical in shape. The spores are usually dark brown to black in colour and occur densely in a colony.

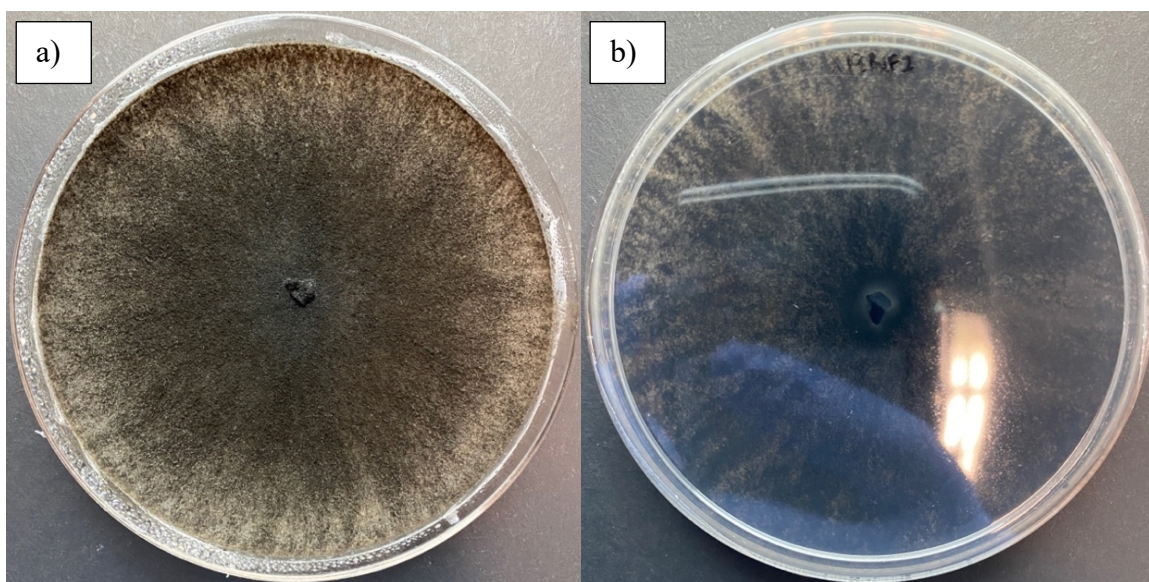


Figure 4.5: The representative of macromorphological characteristic of *Ceratocystic* sp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.4 Morphology of *Paecilomyces* spp.

Three isolates were identified as *Paecilomyces* spp., namely S12, S22, S39. Figure 4.6 shows the representative of macromorphological characteristic of *Paecilomyces* spp. on PDA plates and on reverse PDA plates. These fungal appeared with velvety or powdery mycelia, and various colours can be observed, mostly brown or wood-like in colour on the PDA and the same colour for the reverse plate of the PDA. These fungal colonies are typically fast-growing. Hyphae are typically thin-walled, branched, septate and hyaline-bearing long chains of conidia from the tips of conidiophores. The mature colony would have been conidiophores unbranched and arise from the mycelium. Conidiophores are specialized structures that bear conidia produced in clusters-like structures. The conidia are spherical to ellipsoidal in shape, cream and yellow in colour.

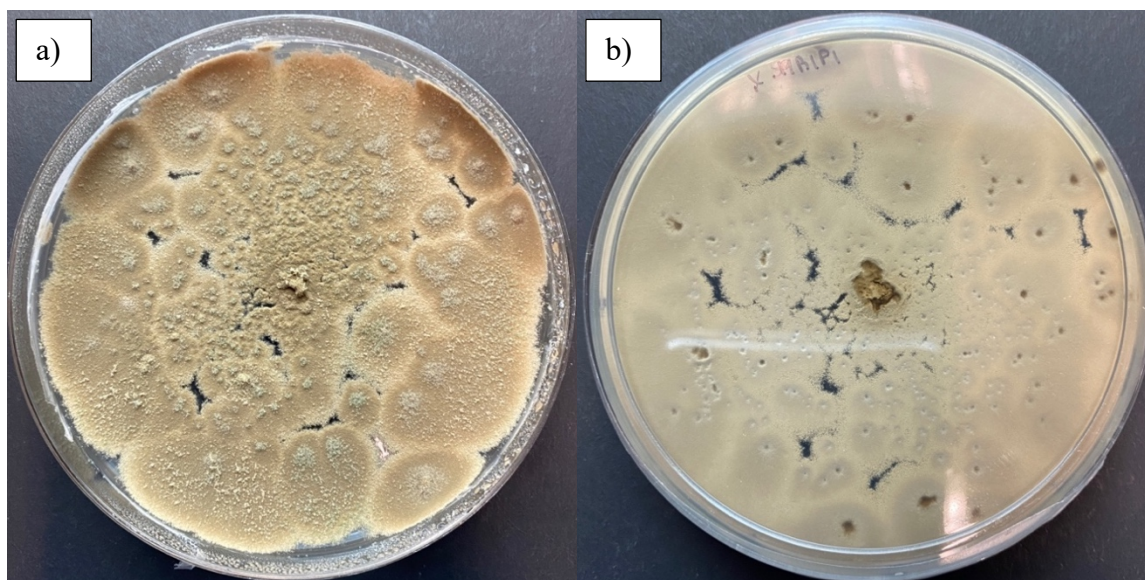


Figure 4.6: The representative of macromorphological characteristic of *Paecilomyces* spp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.5 Morphology of *Penicilium* spp.

Eight isolates were identified as *Penicilium* spp., namely S6, S13, S15, S16, S19, S24, S28, S33. Figure 4.7 shows the representative of macromorphological characteristic of *Penicilium* spp. on PDA plates and on reverse PDA plates. These fungal colonies appeared with velvety and powdery yellowish-brown and brown on the PDA and a yellow to yellowish brown in colour reverse for the reverse plate of the PDA. These fungal colonies are typically fast-growing. Hyphae are typically thin-walled, branched, septate and hyaline with flask-like phialides and closely packed brush-like structures that produce spores. The mature colony would have been conidiophores structured with bear conidia. Conidiophores are unbranched or sparsely branched and arise from the mycelium. The spores are spherical or flask-shaped structures and yellow-green.

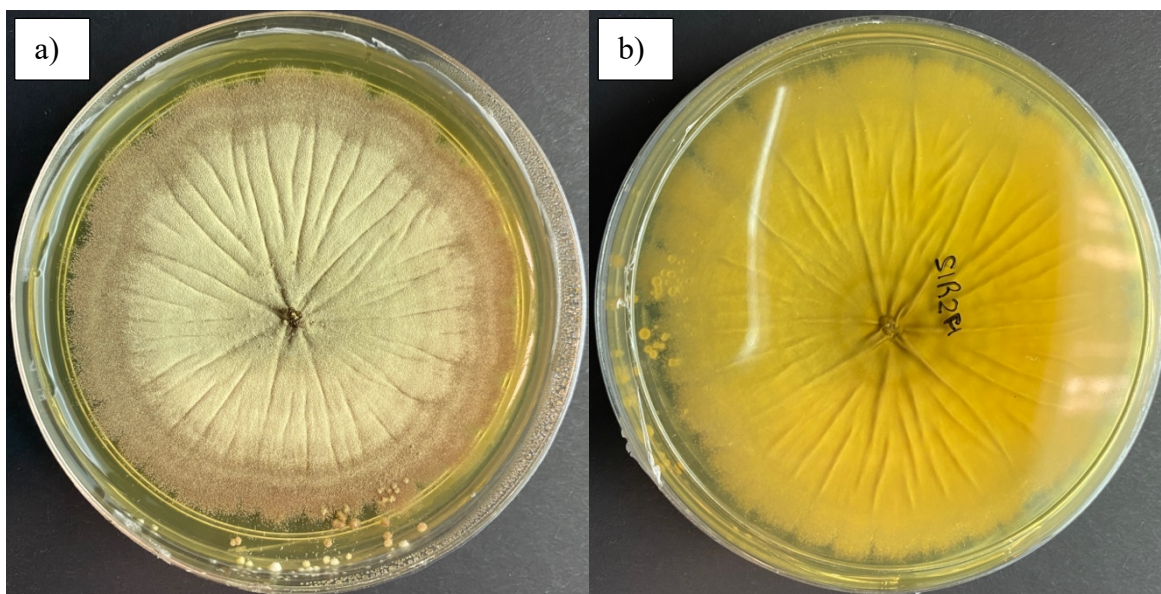


Figure 4.7: The representative of macromorphological characteristic of *Penicilium* spp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.6 Morphology of *Peniophora* sp.

Four isolates were identified as *Peniophora* sp., namely S8, S17, S35, S36, S44. Figure 4.8 shows the representative of macromorphological characteristic of *Peniophora* sp. on PDA plates and on reverse PDA plates. These fungal appeared with rough and velvety mycelia, and various colours can be observed, mostly pinkish-brown or wood-like in colour on the PDA and brown or wood-like in colour for the reverse plate of the PDA. These fungal colonies are typically slow-growing. Hyphae are typically thin-walled, branched, septate, and hyaline, with a dense network and a pinkish-grey hymenial surface. The mature colony would have been basidiospores, usually ellipsoidal or cylindrical. Basidia are specialized structures that bear basidiospores produced in club-shaped structures. The spores are usually spherical, smooth and colourless.

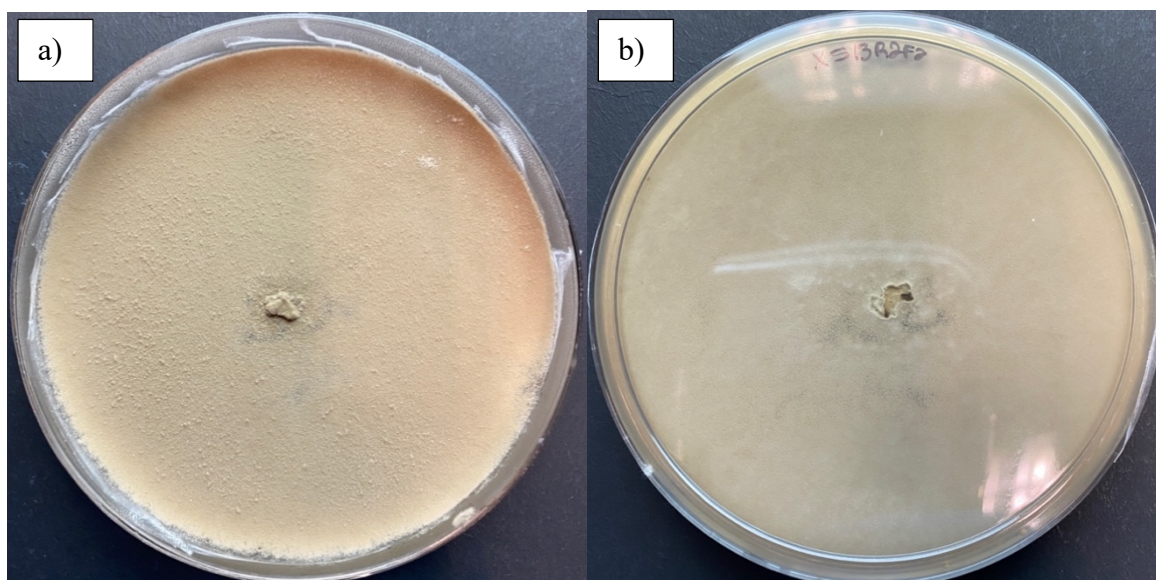


Figure 4.8: The representative of macromorphological characteristic of *Peniophora* sp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.7 Morphology of *Perenniporia* sp.

Two isolates were identified as *Perenniporia* sp., namely S32, S34. Figure 4.9 shows the representative of macromorphological characteristic of *Perenniporia* sp. on PDA plates and on reverse PDA plates. These fungi appeared with woolly and finely hairy mycelia, and colour can be observed as pale beige to white in colour on the PDA and pale beige to white in colour on the reverse plate of the PDA. These fungal colonies are typically fast-growing. Hyphae are typically thin-walled, branched, septate and hyaline-bearing clamp connections. The mature colony would have been basidiospores, usually ellipsoidal or cylindrical. Basidia are specialized structures that bear basidiospores produced in club-shaped structures. The spores are usually spherical and smooth in shape.

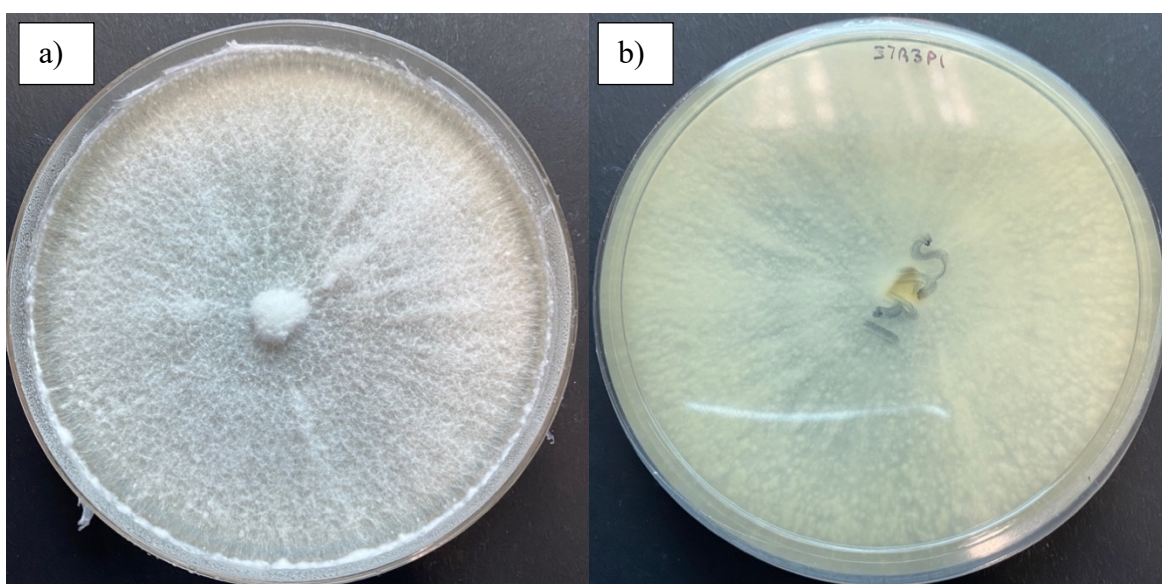


Figure 4.9: The representative of macromorphological characteristic of *Perenniporia* sp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.8 Morphology of *Phaeoacremonium* sp.

One isolate identified as *Phaeoacremonium* sp., namely S21. Figure 4.10 shows the representative of macromorphological characteristic of *Phaeoacremonium* sp. on PDA plates and on reverse PDA plates. These fungi appeared with velvety beige and white to cream-coloured colour on the PDA and beige and white to cream-coloured colour for the reverse plate of the PDA. These fungal colonies are typically slow-growing. Hyphae are typically thin-walled, branched, septate and hyaline with pigmented conidiophores with phialides conidiogenous cells. Hyphae are typically thin-walled, branched, septate and hyaline-bearing clamp connections. The mature colony would have been conidiophores, usually ellipsoidal or cylindrical. Conidia are specialized structures that bear conidiophores produced in chains-shaped structures. The spores are usually smooth, spherical and ellipsoidal in shape.

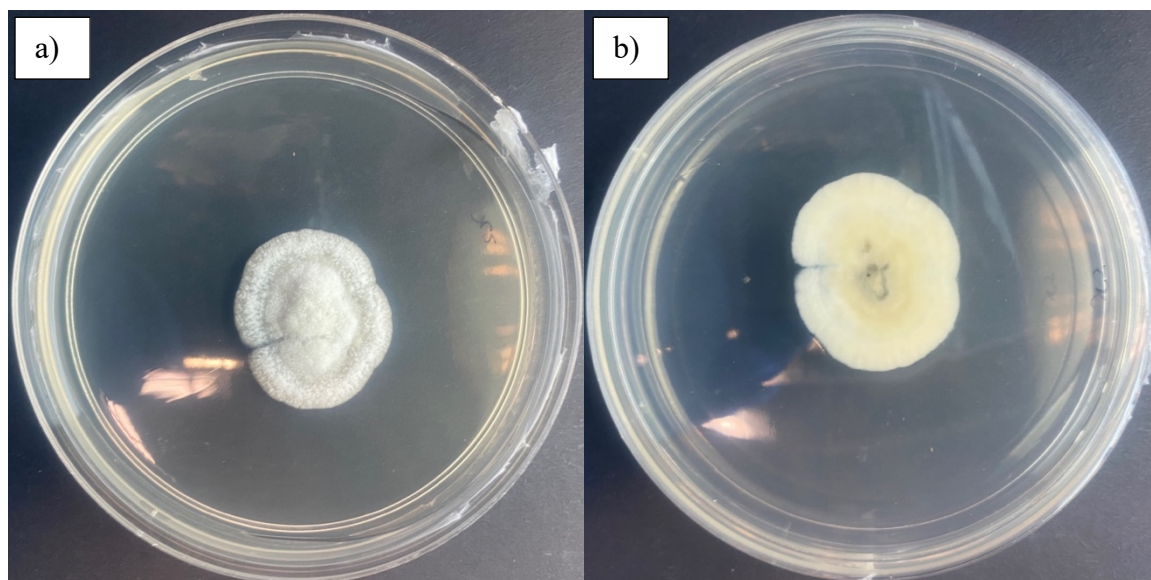


Figure 4.10: The representative of macromorphological characteristic of *Phaeoacremonium* sp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.9 Morphology of *Pleurostoma* sp.

Four isolates were identified as *Pleurostoma* sp., namely S10, S11, S20, S38. Figure 4.11 shows the representative of macromorphological characteristic of *Pleurostoma* sp. on PDA plates and on reverse PDA plates. These fungi appeared finely hairy and woolly and greyish brown and white in colour on the PDA and greyish brown and white in colour on the reverse plate of the PDA. These fungal colonies are typically fast-growing. Hyphae are typically thin-walled, branched, septate and hyaline with cylindrical-like hyaline conidia and phialides. The mature colony would have been conidiophores, usually spherical and ellipsoidal. Conidia are specialized structures that bear conidiophores produced in chains-shaped structures. The spores are usually smooth, spherical and ellipsoidal in shape.

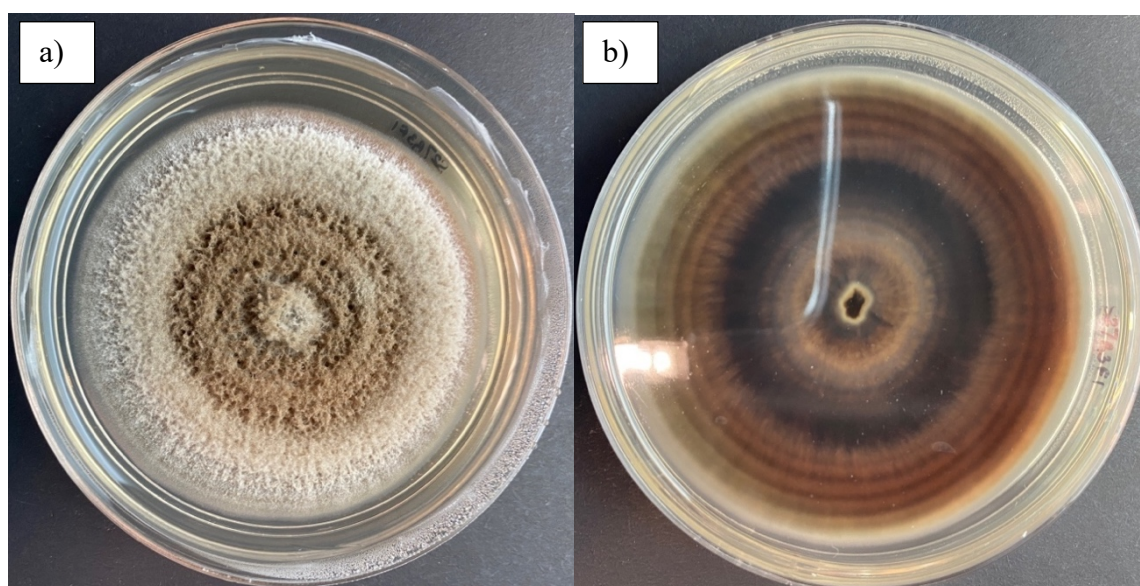


Figure 4.11: The representative of macromorphological characteristic of *Pleurostoma* sp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.2.10 Morphology of *Trichoderma* spp.

Two isolates were identified as *Trichoderma* spp., namely S5 and S25. Figure 4.12 shows the representative of macromorphological characteristic of *Trichoderma* spp. on PDA plates and on reverse PDA plates. These fungi appeared with fluffy and soft to granular light green and white coloured aerial mycelia on the PDA and white-greenish colour for the reverse plate of the PDA. Hyphae are typically smooth-walled, branched, septate and hyalin with lateral branches emerging at different angles. These fungal colonies are typically fast-growing. The mature colony would have been conidiophores, usually spherical and ellipsoidal. Conidia are specialized structures that bear conidiophores produced in chains-shaped structures. Spores are mostly green or white in colour.

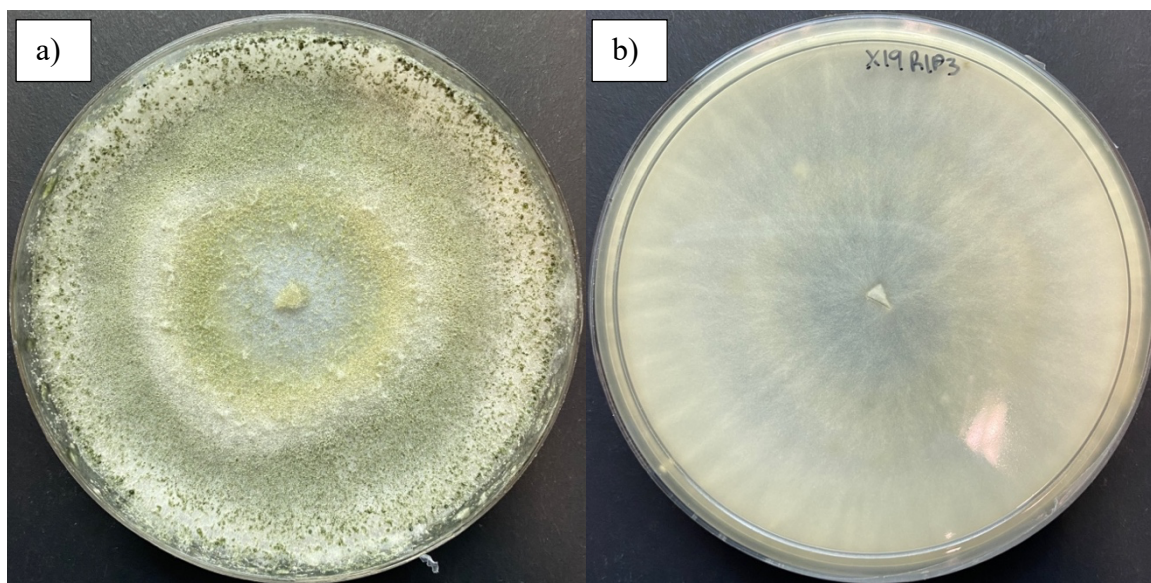


Figure 4.12: The representative of macromorphological characteristic of *Trichoderma* spp. (a) growing on PDA and (b) on reverse plate of PDA

4.1.3 DNA sequencing analysis

The DNA sequencing results provided by the service company were qualitatively assembled and trimmed based on the quality of electropherograms of the forward and reverse sequences by using a data analysis programme, Molecular Evolutionary Genetics Analysis Version 10.0 (MEGA X) software. The sequences were then subjected to CAP3 Sequence Assembly Program software to obtain the consensus sequences from the forward and reverse sequences. The result was carried out by analysing the consensus sequences using the Basic Local Alignment Search Tool (BLAST) to identify the distinctiveness of each isolate.

Figure 4.13 to Figure 4.15 demonstrate the amplification of species-specific PCR products for various fungal isolates, visualized on 1.5% TBE agarose gels. These figures present electrophoresis images of the PCR products from different fungal isolates amplified using ITS1 and ITS4 primers, with lane M serving as the 1 kb DNA ladder for size reference. Each lane represents a specific fungal isolate, and the PCR products exhibit varying sizes that correspond to the presence of specific fungal species, as confirmed by molecular identification. Figure 4.13 reveals distinct bands corresponding to PCR amplification of isolates S1 to S10. Similarly, Figure 4.14, Figure 4.15, Figure 4.16, and Figure 4.17 present the PCR profiles for isolates S11 to S45, confirming the identification of fungal species based on the expected product sizes and their alignment with GenBank database sequences. The successful amplification of these PCR products further validates the efficacy of the ITS1 and ITS4 primers in targeting species-specific regions within the fungal genomes.

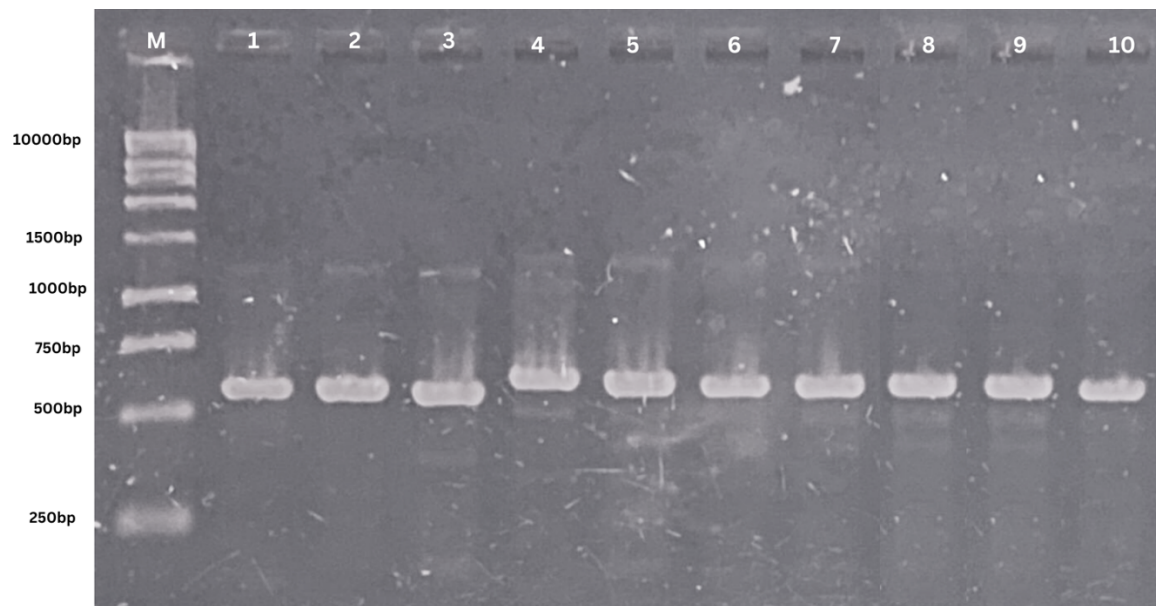


Figure 4.13: Species-specific PCR products visualised on agarose gel amplified using ITS1 and ITS 4 on 1.5% TBE agarose gel at 80V for 60 min. Lane M:1kb DNA Ladder, Lane 1: I1, Lane 2: S2, Lane 3: S3, Lane 4: S4, Lane 5: S5, Lane 6: S6, Lane 7: S7, Lane 8: S8, Lane 9: S9, Lane 10: S10

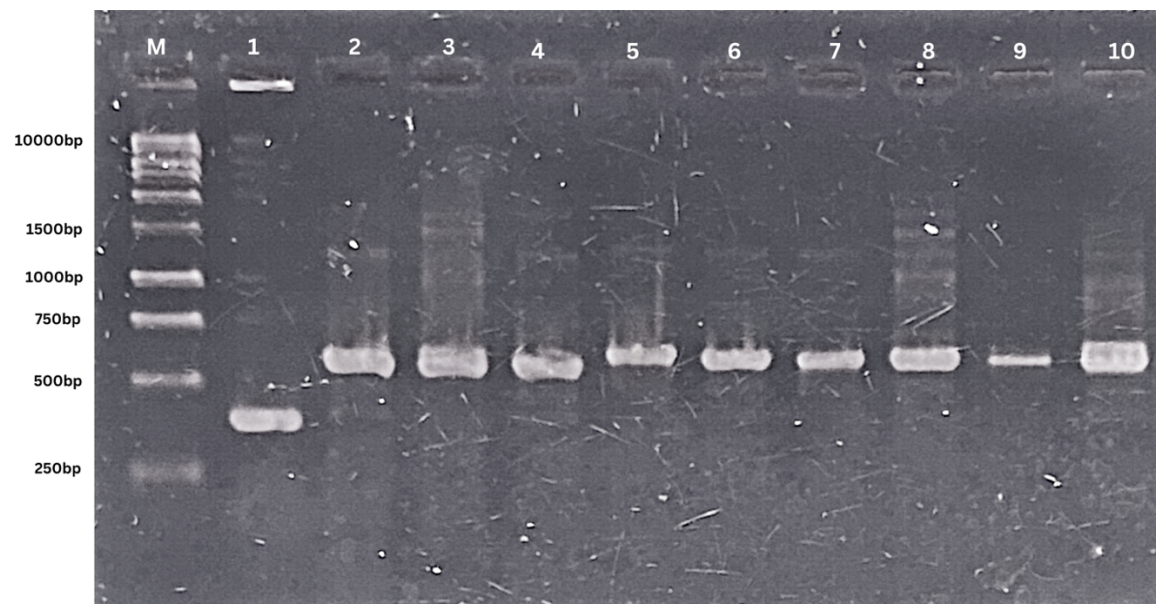


Figure 4.14: Species-specific PCR products visualised on agarose gel amplified using ITS1 and ITS 4 on 1.5% TBE agarose gel at 80V for 60 min. Lane M:1kb DNA Ladder, Lane 1: S11, Lane 2: S12, Lane 3: S13, Lane 4: S14, Lane 5: S15, Lane 6: 16, Lane 7: S17, Lane 8: S18, Lane 9: S19, Lane 10: S20

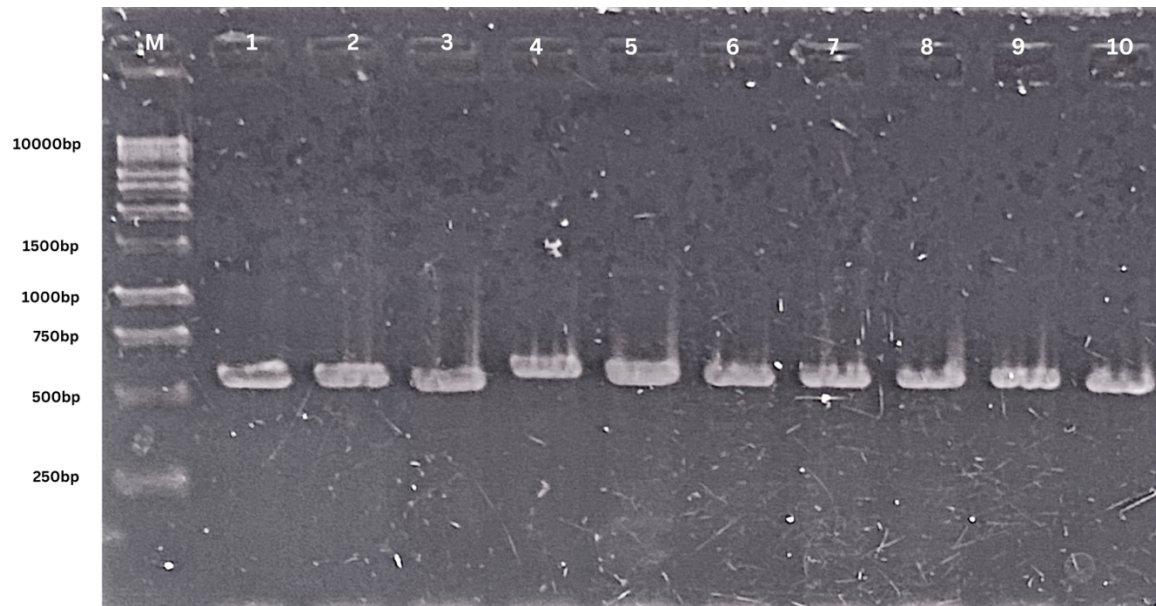


Figure 4.15: Species-specific PCR products visualised on agarose gel amplified using I ITS1 and ITS 4 on 1.5% TBE agarose gel at 80V for 60 min. Lane M:1kb DNA Ladder, Lane 1: S21, Lane 2: S22, Lane 3: S23, Lane 4: S24, Lane 5: S25, Lane 6: S26, Lane 7: S27, Lane 8: S28, Lane 9: S29, Lane 10: S30

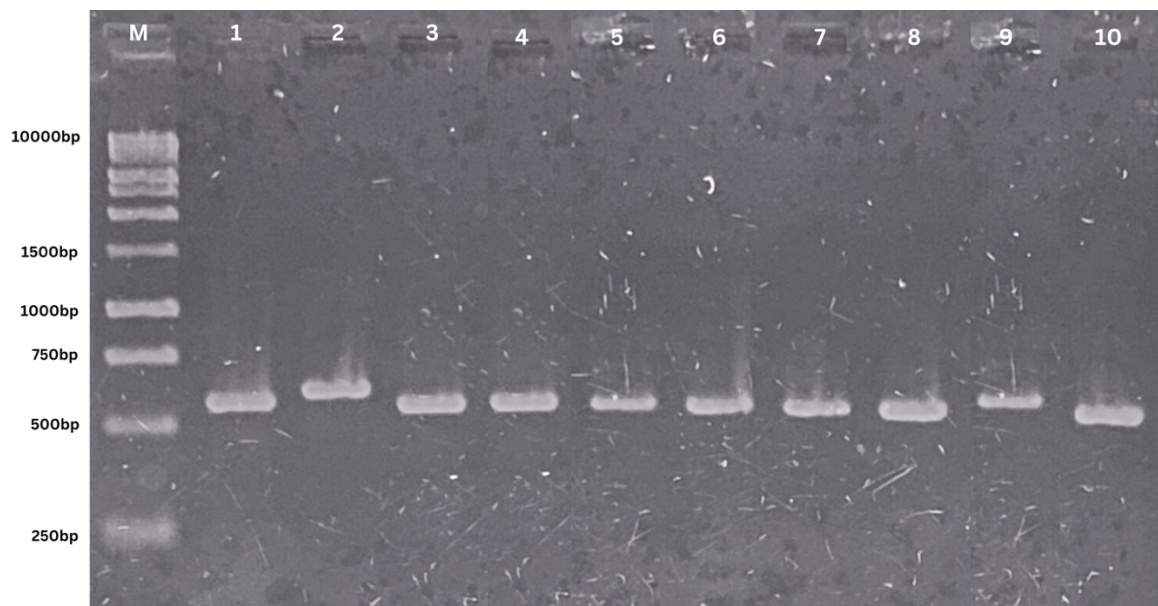


Figure 4.16: Species-specific PCR products visualised on agarose gel amplified using I ITS1 and ITS 4 on 1.5% TBE agarose gel at 80V for 60 min. Lane M:1kb DNA Ladder, Lane 1: S31, Lane 2: S32, Lane 3: S33, Lane 4: S34, Lane 5: S35, Lane 6: S36, Lane 7: S37, Lane 8: S38, Lane 9: S39, Lane 10: S40

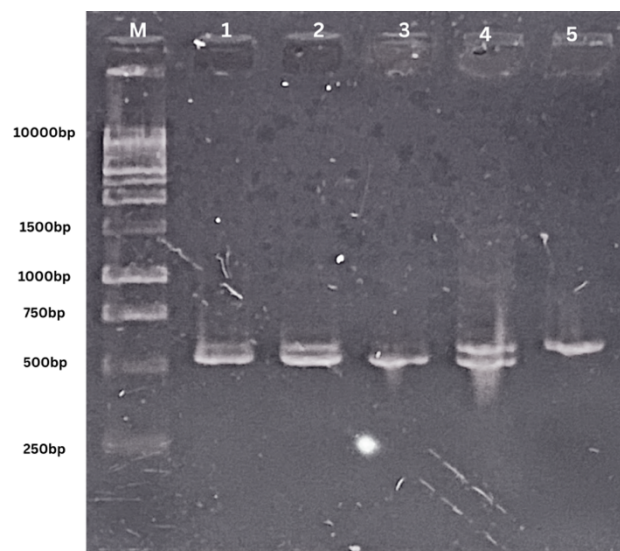


Figure 4.17: Species-specific PCR products visualised on agarose gel amplified using ITS1 and ITS 4 on 1.5% TBE agarose gel at 80V for 60 min. Lane M:1kb DNA Ladder, Lane 1: S41, Lane 2: S42, Lane 3: S43, Lane 4: S44, Lane 5: S45

The consensus sequences were subsequently matched with the sequences obtained from the NCBI GenBank to identify the isolate species. Table 4.5 lists the species and the strain for all forty-five fungal isolates they are most closely related to, their GenBank accession numbers, percentage similarity. The identification of all isolates was conducted using DNA barcoding, resulting in an identity range of 98 - 100%. Based on molecular identification, eighteen fungi species were identified which were *Ceratocystic paradoxa*, *Trichoderma pleuroti*, *Trichoderma harzianum*, *Penicillium rolfsii*, *Penicillium brasilianum*, *Penicillium sumatraense*, *Penicillium verruculosum*, *Penicillium citrinum*, *Phaeoacremonium* sp., *Pleurostoma richardsiae*, *Aspergillus sydowii*, *Aspergillus caesiellus*, *Aspergillus terreus*, *Penicillium citrinum*, *Paecilomyces variotii*, *Paecilomyces niveus*, *Peniophora malaiensis*, *Candida blattae*, and *Perenniporia tephropora*.

The molecular identification results in Table 4.6 provide the species and the frequency of isolation for each fungal isolate, the fungal species with the highest occurrence was *Ceratocystis paradoxa* (28.89%), and followed by *Paecilomyces variotii* and *Peniophora malaiensis* (11.11% each), *Penicillium rolfsii* and *Pleurostoma richardsiae* (8.89% each), *Perenniporia tephropora* (4.44%), and *Penicillium brasilianum*, *Penicillium sumatraense*, *Penicillium verruculosum*, *Penicillium citrinum*, *Paecilomyces niveus*, *Aspergillus sydowii*, *Aspergillus caesiellus*, *Aspergillus terreus*, *Trichoderma pleuroti*, *Trichoderma harzianum*, *Candida blattae*, and *Phaeoacremonium* sp. (2.22% each). Table 4.6 shows the summary of the fungal species found through molecular identification and the percent (%) of prevalence.

When comparing the results from Figure 4.12 to Figure 4.15 with the molecular identification results presented in Tables 4.5 and Table 4.6, a clear correlation can be observed. Table 4.5, which provides a detailed comparison of the sequence similarities between the fungal isolates and their closest related strains from GenBank, which is a further corroborates the gel electrophoresis results. The high similarity percentages (ranging from 98% to 100%) between the obtained sequences and those in the GenBank database provide strong evidence for the species identity of the isolates. Consequently, the correlation between the gel images in Figure 4.12 to Figure 4.15, the sequence similarity data in Table 4.5, frequency of fungal isolates listed in Table 4.6, solidifies the molecular identification process and validates the reliability of the PCR amplification technique employed for fungal species identification in this study.

Table 4.5: Sequence similarities result of isolated fungal compared using NCBI GenBank database

Isolate Code	Species	Strain	Accession Number	Similarity (%)
S1	<i>Ceratocystic Paradoxa</i>	WIN (M)925	DQ318203	98.54
S2	<i>Ceratocystic Paradoxa</i>	WIN (M)925	DQ318203	98.18
S3	<i>Ceratocystic Paradoxa</i>	UAMH 3314	KC305159	99.25
S4	<i>Ceratocystic Paradoxa</i>	UAMH 3314	KC305159	99.25
S5	<i>Trichoderma pleuroti</i>	CBS 124387	MH863369	98.62
S6	<i>Penicillium rolfsii</i>	RHd	KM246748	100.00
S7	<i>Ceratocystic Paradoxa</i>	UAMH 3314	KC305159	100.00
S8	<i>Peniophora malaiensis</i>	CBS 679 91	MH862297	99.84
S9	<i>Phaeoacremonium sp.</i>	EF141	MK842076	99.05
S10	<i>Pleurostoma richardsiae</i>	8937	MK647984	100.00
S11	<i>Pleurostoma richardsiae</i>	8937	MK647984	100.00
S12	<i>Aspergillus sydowii</i>	FJAT30991	KU687805	99.82
S13	<i>Penicillium citrinum</i>	MEBP0017	MT597828	100.00
S14	<i>Paecilomyces variotii</i>	F 0250	MK163442	99.83
S15	<i>Penicillium brasilianum</i>	KAS7777	KY469061	100.00
S16	<i>Penicillium rolfsii</i>	RHd	KM246748	100.00
S17	<i>Peniophora malaiensis</i>	CBS 679 91	MH862297	99.18
S18	<i>Ceratocystic Paradoxa</i>	UAMH 3314	KC305159	99.68
S19	<i>Penicillium rolfsii</i>	DI16 116	LT558938	100.00
S20	<i>Pleurostoma richardsiae</i>	8937	MK647984	100.00
S21	<i>Candida blattae</i>	CBS9871	KY101970	99.21
S22	<i>Aspergillus caesiellus</i>	SRRC 2170	AY373865	100.00
S23	<i>Ceratocystic Paradoxa</i>	UAMH 3314	KC305159	99.68
S24	<i>Penicillium rolfsii</i>	RHd	KM246748	100
S25	<i>Trichoderma harzianum</i>	IPBCC08 606	KC847194	99.67

Table 4.5 continued

S26	<i>Paecilomyces variotii</i>	A1S6 D28	KJ780746	99.51
S27	<i>Paecilomyces variotii</i>	ICMP 146	OL639124	99.83
S28	<i>Penicillium sumatraense</i>	DUCC5751	MT582791	99.61
S29	<i>Paecilomyces variotii</i>	40	MN534797	99.82
S30	<i>Paecilomyces variotii</i>	40	MN534797	99.83
S31	<i>Paecilomyces niveus</i>	U11	MT792005	99.00
S32	<i>Perenniporia tephropora</i>	KoRLI047383	MN341836	98.84
S33	<i>Penicillium verruculosum</i>	KUC1794	HM469420	99.67
S34	<i>Perenniporia tephropora</i>	3168	MT074621	99.84
S35	<i>Peniophora malaiensis</i>	CBS 679 91	MH862297	99.84
S36	<i>Peniophora malaiensis</i>	CBS 679 91	MH862297	99.84
S37	<i>Ceratocystis Paradoxa</i>	UAMH 3314	KC305159	99.63
S38	<i>Pleurostoma richardsiae</i>	8937	MK647984	100.00
S39	<i>Aspergillus terreus</i>	DTO 403 C9	MT316343	100.00
S40	<i>Ceratocystis paradoxa</i>	UAMH 3314	KC305159	99.44
S41	<i>Ceratocystis paradoxa</i>	UAMH 3314	KC305159	99.44
S42	<i>Ceratocystis paradoxa</i>	UAMH 3314	KC305159	99.44
S43	<i>Ceratocystis paradoxa</i>	UAMH 3314	KC305159	99.25
S44	<i>Peniophora malaiensis</i>	CBS 679 91	MH862297	99.96
S45	<i>Ceratocystis paradoxa</i>	UAMH 3314	KC305159	99.62

Table 4.6: Summary of the fungal species found through molecular identification and the percent (%) of prevalence

Species	Isolates Code	Frequency of isolation	
		n	%
<i>Ceratocystic paradoxa</i>	S1, S2, S3, S4, S7, S18, S23, S37, S40, S41, S42, S43, S45	13	28.89
<i>Penicillium rolfsii</i>	S6, S16, S19, S24	4	8.89
<i>Penicillium brasilianum</i>	S15	1	2.22
<i>Penicillium sumatraense</i>	S28	1	2.22
<i>Penicillium verruculosum</i>	S33	1	2.22
<i>Penicillium citrinum</i>	S13	1	2.22
<i>Paecilomyces variotii</i>	S14, S26, S27, S29, S30	5	11.11
<i>Paecilomyces niveus</i>	S31	1	2.22
<i>Peniophora malaiensis</i>	S8, S17, S35, S36, S44	5	11.11
<i>Pleurostoma richardsiae</i>	S10, S11, S20, S38	4	8.89
<i>Aspergillus sydowii</i>	S12	1	2.22
<i>Aspergillus caesiellus</i>	S22	1	2.22
<i>Aspergillus terreus</i>	S39	1	2.22
<i>Perenniporia tephropora</i>	S32, S34	2	4.44
<i>Trichoderma pleuroti</i>	S5	1	2.22
<i>Trichoderma harzianum</i>	S25	1	2.22
<i>Candida blattae</i>	S21	1	2.22
<i>Phaeoacremonium</i> sp	S9	1	2.22

4.2 Discussion

For the enumeration of fungal count, all fifty samples of sago starch were enumerated and calculated, with the lowest count at 1.3×10^2 CFU/g, and highest count at 2.1×10^4 CFU/g, and mean at 8.6×10^3 CFU/g, which were exceeded the permissible limit for fungal contamination edible sago starch set by Malaysian Standard limit of not more than 1.0×10^2 CFU/g (Malaysian Malaysian Standard, 2019b). However, this fungal count were complied within the level of fungi propagules drafted by the International Commission for Microbiological Specification of Foods (ICMSF) of 10^2 to 10^5 CFU/g (International Commission for Microbiological Specification of International Commission on Microbiological Specifications for Foods, 1974).

On contrast to Greenhill et al. (2007), fungi were isolated from 65 of the 69 samples, ranging from 1.0×10^2 to 3.0×10^6 CFU/g, which some of the fungi isolated from sago starch were exceeding the drafted standard by the International Commission for Microbiological Specification of Foods. The enumeration of fungi colonies was carried out by using the standard dilution method on PDA plates, which were done exactly as proposed by (Greenhill et al., 2008) with alternative medium used, potato dextrose agar with chloramphenicol instead of using dichloran rose-bengal chloramphenicol medium. The substitution of dichloran rose-bengal chloramphenicol medium with potato dextrose agar (PDA) supplemented with chloramphenicol for fungal colony enumeration presents both advantages and limitations. PDA is a more cost-effective and readily available medium that effectively minimizes bacterial contamination due to the chloramphenicol component, while supporting the growth of a wide variety of fungal species (Black, 2020). This makes it particularly suitable for studies requiring the enumeration of diverse fungal populations. However, PDA is less selective than dichloran rose-bengal chloramphenicol medium, which

may result in faster-growing fungi outcompeting slower-growing species, thereby complicating the accurate counting of individual colonies. Additionally, the alteration in medium composition may influence the morphological characteristics of fungal colonies, further hindering the clarity and precision of colony enumeration.

The fungal isolates in this study were initially identified at the genus level through a morphological examination. Macroscopically analysis of fungi can be performed with the naked eye by observing the presence of fungal on the media plate. Macroscopic characteristics for example, the colour of the fungi colony, and the structure of the fungi colony on media plate and also growing rate of the fungi are essential in mycological research. This involved assessing characteristics such as the colour, margin, and texture of the fungal colonies, as well as examining the shape of the spore-bearing structures under a microscope (Alsohaili & Bani-Hasan, 2018). Examining the physical characteristics of a fungal culture can assist in determining the identity of the isolates, however this method is limited to identifying them only at the family or genus level (Wang et al., 2016). This identification method is inadequate for determining the specific species of the isolated fungi. Therefore, a molecular technique is used to aid in the identification process (Alsohaili & Bani-Hasan, 2018).

In this finding, the morphological identification of fungi revealed ten fungal genera, with the highest occurrence was *Ceratocystic* sp. (28.89%), and followed by *Penicilium* spp. (17.78%), *Paecilomyces* spp. (13.33%), *Peniophora* sp. (11.11%), *Pleurostoma* sp. (8.89%), *Aspergillus* spp. (6.67%), *Perenniporia* sp. and *Trichoderma* spp. (4.44% each), *Phaeacremonium* sp. and *Candida* sp. (2.22% each). *Ceratocystic* recorded the highest percent of isolated fungi with 28.89%, followed by *Penicillium* with 17.78% and

Paecilomyces with 13.33%. Compared to out of twenty-seven fungal genera isolated from traditional sago starch from Papua New Guinea (Greenhill et al., 2007), only three genera were also occurrences in this research which are *Penicillium*, *Aspergillus*, *Trichoderma* and *Paecilomyces*. The genera *Penicillium* spp. and *Aspergillus* spp. were among the most isolated from sago starch, with 61.00% and 25.00% respectively (Greenhill et al., 2008), and they are well known for their mycotoxigenic potential.

Ceratocystis belongs to the class Sordariomycetes, which is one of the major classes within the phylum Ascomycota (Maharachchikumbura et al., 2016). This fungus is commonly referred to as black rot or *Ceratocystis* fruit rot because of the dark coloration it causes in the afflicted area. *Ceratocystis* is a prevalent fungal infection that holds significance in the agricultural and forestry sectors. *Ceratocystis* typically infects fruits and plants that have been injured during harvesting or in the post-harvest stages. During the post-harvest process, both contaminated fruits or plants and healthy ones were immersed and cleansed together in the same location, resulting in the spread of the infection (Sepiah et al., 2003). The pathogen can invade and thrive on many hosts and in diverse geographical environments, predominantly on tropical vegetation and trees, including temperate regions (Gomdola et al., 2022). In 2015, a significant amount of plantation forest in Sabah, East Malaysia, was destroyed as a result of a severe infection caused by *Ceratocystis*. This led to a substantial economic loss for the state (Brawner et al., 2015). The genus mentioned is the primary cause of vascular wilts, stem cankers, and fruit rot.

It is important to undergo further confirmation and identification of isolated fungi, hence the forty-five fungal isolates were subjected to molecular characterization based on ITS sequencing. Heat-shock DNA extraction was done by scrapping off a small portion of

isolated fungi pure culture and suspended into a sterile 0.5 ml PCR tube containing 50 µl of TE buffer (1M Tris-CL pH 8.0, 0.5M EDTA pH 8.0) using a sterile inoculating needle (Pandi, 2019). This approach is widely regarded as fast, inexpensive, and dependable. The conventional CTAB approach involves the use of several chemicals and liquid nitrogen. By employing the current approach, the usage of mortar and pestle, with or without liquid nitrogen, has been avoided. In addition, the disruption of the fungal cell wall and removal of cell contaminants are indispensable for high yield and purity of DNA (Chi et al., 2009). Consequently, this technique is strongly advised for the retrieval of fungal DNA.

The genomic DNA was extracted from the fungal cultures and amplified utilising the internal transcribed spacer region (ITS). The DNA from all fungal isolates was successfully amplified, resulting in PCR products ranging from 500 to 700 base pairs in length. The ITS1 (Forward) and ITS4 (Reverse) primers were chosen for this study because to their wide applicability across many taxonomic groups of fungi (Toju et al., 2012). This enables a precise assessment of the diversity and community compositions of the fungal species. The ITS 1 and ITS 4 primers were used to amplify the target regions of the fungal isolate. The ITS rDNA gene sequence is widely recognised as a pivotal tool for the identification of fungus species present in the environment. This approach has frequently been utilised to discriminate fungal populations and augment conventional identification methods (Alsohaili & Bani-Hasan, 2018). Consequently, it has become a common practice to distinguish the fungal community and enhance traditional identification techniques. Based on molecular identification, eighteen fungi species were identified which were *Ceratocystic paradoxa* (28.89%) with the highest occurrence, and followed by *Paecilomyces variotii* and *Peniophora malaiensis* (11.11% each), *Penicillium rolfsii* and *Pleurostoma richardsiae* (8.89% each), *Perenniporia tephropora* (4.44%), and *Penicillium brasilianum*, *Penicillium*

sumatraense, *Penicillium verruculosum*, *Penicillium citrinum*, *Paecilomyces niveus*, *Aspergillus sydowii*, *Aspergillus caesiellus*, *Aspergillus terreus*, *Trichoderma pleuroti*, *Trichoderma harzianum*, *Candida blattae*, and *Phaeoacremonium* sp. (2.22% each).

Regional patterns of fungal distribution provide valuable insights into the influence of environmental conditions and handling practices on the types and prevalence of fungi found in sago starch samples from various locations. By analyzing the correlation between fungal isolates, sample locations, and environmental factors, this study underscores the importance of establishing precise handling and storage protocols within the sago starch industry. In this study, the fungal genus *Ceratocystis* sp. is identified in multiple regions, including Matu, Dalat, Mukah, Selangau, and Sibul. The widespread occurrence of *Ceratocystis* sp. across these areas suggests that common environmental factors, such as high humidity, elevated temperatures, and typical storage conditions prevalent in tropical climates, may promote the growth and proliferation of this genus. This distribution implies that the handling and storage practices employed in these regions may not be adequate in preventing contamination by this fast-growing fungal species.

Conversely, fungi such as *Candida* sp. and *Phaeoacremonium* sp. are observed in more restricted regions, specifically Pusa and Marudi. This restricted distribution indicates that these fungi may require more specialized environmental conditions or storage practices. For example, *Candida* sp. may thrive under stable, lower humidity environments or in substrates within sago starch that support its slower growth rate. The limited presence of these fungi could also suggest localized sources of contamination or distinct handling processes unique to these regions. This distribution pattern emphasizes how fungi with diverse growth requirements adapt to specific conditions in different locations.

Moreover, regional variations in fungal prevalence highlight the significance of microclimatic differences and storage environments. Areas with higher fungal contamination, such as Matu and Dalat, may face challenges related to persistent high moisture levels, inadequate ventilation, or extended storage durations. These factors create an environment conducive to the growth of a variety of fungal species, including *Ceratocystis* sp., *Penicillium* spp., and *Paecilomyces* spp., known for their rapid growth and adaptability. In contrast, regions like Betong and Bintulu, characterized by lower fungal counts, may benefit from more stringent storage protocols or naturally occurring environmental factors that inhibit fungal development, such as lower humidity or well-ventilated storage facilities. These observed regional patterns of fungal distribution emphasize the need for tailored storage and handling procedures that account for specific environmental conditions in order to minimize fungal contamination. For example, the presence of fast-growing fungi in certain locations indicates that these regions may benefit from moisture control practices or regular fungal monitoring to reduce contamination risks.

Raw sago starch is typically stored in wet forms, and the risk of fungal contamination varies depending on the storage method. Wet sago starch, with its higher moisture content, provides an ideal environment for fungal growth and is particularly susceptible to contamination if stored improperly. The study highlights the importance of monitoring and controlling the moisture content and storage conditions of both forms to mitigate contamination risks. Furthermore, the study suggests that regions outside Pusa, Mukah, and Dalat, where fungal contamination is less prevalent, may face higher risks of contamination if sago starch is stored for prolonged periods. Extended storage, particularly in conditions with high humidity and poor ventilation, creates an opportunity for fungi such as *Ceratocystis* sp. to proliferate, increasing the risk of contamination.

The findings of this study emphasize the need for tailored storage practices that consider the specific environmental conditions of each region. Prolonged storage times, especially in areas with high humidity or inadequate ventilation, can contribute to fungal growth. To minimize the risk of fungal contamination, it is essential to implement moisture control measures, ensure proper ventilation in storage areas, and establish time-sensitive storage protocols. By adhering to optimal storage conditions and monitoring fungal growth regularly, the sago starch industry can reduce the likelihood of contamination and ensure the safety and quality of its products. Ultimately, understanding regional fungal distribution patterns allows for the development of targeted interventions that address the unique challenges posed by different environmental conditions, supporting the production of safer and higher-quality sago starch. Fungal contamination in sago starch can lead to the growth of species that may produce mycotoxins, which can be harmful to consumers if consumed in significant amounts. The study reveals how specific environmental conditions and handling practices influence the types and prevalence of fungi in sago starch samples, highlighting the importance of establishing proper storage protocols to prevent contamination.

Handling procedures play a significant role in reducing fungal contamination risks. If sago starch is handled in a manner that introduces moisture or exposes it to fungal spores, it increases the likelihood of contamination. Improper handling, such as using contaminated equipment or not practicing hygiene in storage areas, can contribute to the spread of fungal species. Moreover, prolonged storage durations, particularly when sago starch is stored beyond the typical recommended period, create opportunities for fungi to thrive. This study suggests that improper handling and extended storage time in suboptimal conditions can lead

to higher contamination levels, particularly in regions with higher humidity or poor storage practices.

In addition to the regional distribution and environmental factors influencing fungal contamination in sago starch, this study also provides valuable insights into the morphological characteristics of the isolated fungi. Comparing these morphological features with those identified in other starch types or similar studies on fungal contamination can help validate the findings and establish broader patterns of fungal behavior and characteristics across different environments and starch products. The morphological characteristics of the fungi isolated in this study, such as *Ceratocystis sp.*, *Candida sp.*, and *Phaeoacremonium sp.*, demonstrate distinct features that align with those identified in previous studies on other starches, highlighting both similarities and unique traits associated with each fungal species. For example, *Ceratocystis sp.* was isolated in multiple sago starch samples across regions like Matu and Dalat, where it thrives in humid and warm conditions. The morphological features of *Ceratocystis sp.*, such as its distinctively dark, thick-walled conidia and characteristic hyphal structure, are consistent with findings from studies on fungi isolated from cassava starch. In research by Okorie et al. (2017), *Ceratocystis sp.* was also found in cassava starch, where it showed similar morphological traits, such as the production of dark, robust conidia under moist, tropical conditions. This similarity suggests that *Ceratocystis sp.* has a common ability to proliferate in starchy environments, particularly under conditions that are conducive to fungal growth, such as high humidity and moisture content.

Similarly, *Candida sp.*, found in more localized regions like Pusa and Marudi, shows characteristic features such as oval or round-shaped yeast cells with budding structures. These morphological traits match those described in studies of *Candida* species isolated from other types of starch, such as maize starch (Sarr et al., 2020). In maize starch, *Candida sp.*

was reported to exhibit similar budding yeast cells and creamy colonies. The similarity in morphology across different starch sources highlights the adaptability of *Candida sp.* to various starchy substrates, although the specific environmental conditions for its growth may vary between regions. *Phaeoacremonium sp.*, which was isolated in Dalat and Pusa, is another example of a fungus with a distinct morphological signature. This fungus produces dark, elongated conidia, often seen in elongated chains. *Phaeoacremonium sp.* was also identified in previous studies on other starches, such as in yam starch (Ogunjobi et al., 2018), where the same dark conidial morphology and branching patterns were observed. The morphological consistency of *Phaeoacremonium sp.* across various starches suggests that it shares similar ecological requirements and can adapt to a variety of starchy environments, likely thriving in substrates with lower moisture levels compared to those preferred by other fungi like *Ceratocystis sp.*

These comparisons of morphological characteristics underscore the consistency of fungal traits across different starch types, supporting the idea that fungi adapt to specific environmental and substrate conditions. The observed similarities between fungal isolates in sago starch and those in other types of starch (e.g., cassava, maize, yam) indicate common ecological niches and environmental factors that promote fungal growth in starch-based products. This understanding can aid in developing more effective prevention and control strategies for fungal contamination in the sago starch industry by recognizing the morphological traits of the fungi and tailoring storage and handling protocols accordingly.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The main objectives of this study were achieved with the (1) isolation and detection of fungi in sago starch throughout the state of Sarawak, Malaysia, (2) identification of species of fungal isolates based on molecular analysis targeting the internal transcribed spacer (ITS).

The result indicates that fungal loads for all fifty sago starch samples examined in this study were recorded within the range of 1.3×10^2 CFU/g to 2.1×10^4 CFU/g, and the mean at 8.6×10^3 CFU/g. These readings were exceeded with the acceptable limit established for fungal contamination in edible sago starch set by the Malaysian Standard limit of not more than 10^2 CFU/g. However, these readings are complied within the range level of fungi propagules drafted by the International Commission for Microbiological Specification of Foods (ICMSF) of 10^2 to 10^5 CFU/g.

The morphological identification of fungi revealed ten fungal genera, namely *Aspergillus* spp., *Candida* sp., *Ceratocystic* sp., *Paecilomyces* spp., *Penicillium* spp., *Peniophora* sp., *Perenniporia* sp., *Phaeacremonium* sp., *Pleurostoma* spp. and *Trichoderma* spp. were detected and isolated from the sago starch in Sarawak. However, further confirmation needs to be done to identify the species of these fungal genera through molecular identification. Based on molecular identification, eighteen fungi species were identified from various sampling location, which were *Ceratocystic paradoxa*, *Trichoderma pleuroti*, *Trichoderma harzianum*, *Penicillium rolfsii*, *Penicillium brasilianum*, *Penicillium*

sumatraense, *Penicillium verruculosum*, *Penicillium citrinum*, *Phaeoacremonium* sp., *Pleurostoma richardsiae*, *Aspergillus sydowii*, *Aspergillus caesiellus*, *Aspergillus terreus*, *Penicillium citrinum*, *Paecilomyces variotii*, *Paecilomyces niveus*, *Peniophora malaiensis*, *Candida blattae*, and *Perenniporia tephropora*.

A stronger emphasis on the connection between fungal distributions and sampling locations enhances the validity and relevance of the findings. By aggregating fungal counts by site, the study illustrates average contamination levels across various collection regions. Notably, areas like Pusa, Matu, and Dalat demonstrate higher mean fungal counts likely due to environmental factors such as high humidity or less-than-ideal storage conditions that encourage fungal growth. In contrast, locations such as Betong and Bintulu exhibit lower fungal counts, suggesting that controlled storage practices or reduced exposure to growth-promoting factors may limit contamination.

The study results have significant implications for food safety, public health, and regulatory compliance, emphasizing the importance of ongoing research and collaboration to address the concerns associated with fungal contamination in sago starch-based foods. The findings from the study provide essential reassurance regarding the safety of consuming raw wet sago starch were exceeded acceptable limits. Identifying several fungus genera and species highlights the requirement for regular inspection and surveillance in sago starch production. The occurrence of cross-contamination may be caused by insufficient post-harvest production, handling, transportation, and storage protocols of sago starch.

The results of this study have established a fundamental framework for assessing and mitigating risks associated with fungal contamination in sago starch. Moreover, it has advanced our detailed comprehension of the effectiveness of current food safety measures

within the sago starch production in Sarawak, providing a cornerstone for future scientific inquiries in this field. Besides, the identification of diverse fungal species and their prevalence in sago starch samples contributes valuable data and expands our knowledge of fungal diversity and distribution in food products in the field of food microbiology. In addition to an established safety profile of sago starch and identifying potential risks associated with fungal contamination, this study contributes to efforts aimed at enhancing the resilience and sustainability of food systems worldwide.

5.2 Recommendations

For future recommendations, there are other suggestions that can be considered. It is advisable to expand the sampling areas and increase the collection of samples from various districts in Sarawak, Malaysia for advanced testing, which resulting more fungi species isolated from different sampling areas could be served as potential fungi inoculants and hence, the genetic variation of the fungi can be seen within different area in Sarawak.

During this study, several fungi were classified as mycotoxin-producing fungi. An additional investigation is being conducted to assess the extent of mycotoxin pollution in sago starch in Sarawak, Malaysia. Although this investigation successfully isolated and identified mycotoxin-producing fungus in the sago starch sample, the extent of mycotoxin contamination remains undetermined. This might cause severe foodborne illness, a prevalent health issue that imposes significant socioeconomic status and emotional distress. Therefore, it is essential to enforce rigorous hygiene standards and strictly follow efficient management and manufacturing methods in sago processing to avoid the incidence of illnesses caused by consuming contaminated sago and sago products.

On the other hand, this study suggests the importance of pursuing ongoing research to address the issues posed by fungal contamination in sago starch-based products. It is crucial to continue studying the factors that affect fungal growth, improve detection techniques, and develop preventive measures to minimize risks to consumer health.

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APPENDICES

APPENDIX 1:

Table shows the reagents for the preparation of Tris-HCl 1 M, pH 8.0 (1 L)

Reagent	Amount
Tris base	121.1 g
HCL	to adjust to pH 8.0
Double distilled water	to adjust the final volume to 1 L

Autoclave the solution and store at room temperature.

APPENDIX 2:

Table shows the reagents for the preparation of EDTA 0.5 M, pH 8.0 (1 L)

Reagent	Amount
Ethylenediaminetetraacetic disodium salt, dehydrate	186.1 g
NaOH	to adjust to pH 8
Double distilled water	to adjust the final volume to 1 L

Autoclave the solution and store at room temperature.

APPENDIX 3:

Table shows the reagents for the preparation of TE Buffer, 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 (1 L)

Reagent	Amount
1.0 M Tris-HCl	10 ml
0.5 M EDTA	200 µl
Double distilled water	to adjust the final volume to 1 L

Autoclave the solution and store at room temperature.

APPENDIX 4:

Table shows the reagents for the preparation of 10X TBE Buffer

Reagent	Amount
Tris base	108.0 g
Boric acid	55.0 g
0.5 M EDTA	40 ml
Double distilled water	to adjust the final volume to 1 L

Autoclave the solution and store at room temperature.

APPENDIX 5: Journal Publication

1. **Bakeri, M. K. S.**, Bilung, L. M., Vincent, M., Tahar, A. S., & Husaini, A. A. S. A. (2025). Occurrence of toxigenic fungi in raw sago starch for commercialisation in Sarawak, Malaysia. *Journal of Stored Products Research*, 110, 102478.