



Faculty of Resource Science and Technology

**Isolation of Amylolytic Fungi and Characterisation of Amylase in
Aspergillus niger KBA2 and *Penicillium* sp. BB3 from Starchy and Agro-
Industrial Waste Locally Collected in Kuching and Samarahan,
Sarawak, Malaysia**

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**Master of Science
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Isolation of Amylolytic Fungi and Characterisation of Amylase in *Aspergillus niger* KBA2 and *Penicillium* sp. BB3 from Starchy and Agro-Industrial Waste Locally Collected in Kuching and Samarahan, Sarawak, Malaysia

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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

Amylases are enzymes that catalyse hydrolysis of starch into simple sugars and the production of these enzymes largely depends on microbial sources, making it important to continuously screen for potential producers of amylases. Previous studies have reported on isolating amylolytic fungi from sources such as soil samples, farmlands and different types of waste. Agro-industrial and food wastes are major contributors to the generation of large amount of organic waste. Food is also largely wasted due to spoilage, causing spoiled food waste to be a significant issue worldwide. Despite the potential of these wastes as sources of amylolytic fungi, limited studies have explored their diversity and enzymatic properties under local context. The study therefore aims to isolate amylolytic fungi from various starchy waste, optimise amylase production in potent isolates and characterising the partial purified enzyme. Six types of wastes were collected for this study, which were sago pith waste (agro-industrial waste), washed rice water (food waste), and various spoiled foods such as potato, bread, banana cake and glutinous rice cake. Fungal isolation was conducted using serial dilution and direct plating on potato dextrose agar, followed by identification through morphological and molecular methods. Screening on starch agar was performed to identify amylolytic fungi and followed by the study of amylase activity through determining the enzymatic activity index (EAI). Potent isolates were then selected for study of initial medium pH and incubation period optimisation for amylase production. Amylase was then partial purified from two further chosen species through ammonium sulphate precipitation and molecular weight of the enzyme was determined through SDS-PAGE, while native-PAGE was done for investigating the hydrolytic activity. The partially purified amylase was also characterised to determine its optimal activity at different temperatures and pH values. A total of 41 isolates were obtained from the wastes, classified into 12 genera and 23 species.

The *Aspergillus* genus, with 15 isolates from 9 different species, was the most prevalent and diverse genus, followed by *Penicillium* genus with 8 isolates of 3 species. Out of 41 isolates, 34 of them were extracellular amylase producers and subsequent study revealed that most members of *Penicillium* as well as several *Aspergillus* species had higher activity compared to other isolates. Therefore, further studies were focused on these two genera. Two isolates with the highest EAI were selected from each genus, namely *Penicillium* sp. BB3 (1.45 ± 0.10), *P. citrinum* KBA4 (1.36 ± 0.02), *A. europaeus* BNN1 (1.19 ± 0.04) and *A. niger* KBA2 (1.16 ± 0.02). *Penicillium* sp. BB3 and *A. niger* KBA2 stood out as the isolates which produced the highest value of amylase under optimised condition of pH 7 under a week of incubation and pH 5 under 5 days of incubation respectively. Amylase from strain BB3 was purified in 0.51-fold with a recovery of 26.47% while the enzyme from strain KBA2 was purified in 2.08-fold with a recovery of 28.85%. SDS-PAGE and native-PAGE revealed strain BB3 might be producing several isoforms or types of amylases, with estimated molecular weights of 80 kDa, 65 kDa, 42 kDa, 40 kDa and 35 kDa respectively. On the other hand, amylase of strain KBA2 formed a single band on both SDS-PAGE and native-PAGE with molecular weight of approximately 130 kDa. The enzyme of strain BB3 showed optimal activity under pH 7 and at a temperature of 40 °C, while amylase of strain KBA2 worked best at pH 5 and 50 °C. The results indicate that strain BB3 and KBA2 are promising amylase producers, with potential applications such as in food processing, biofuel production and waste management. Further studies should focus on aspects such as scaling up and genetic modification to enhance activity and stability of the enzyme for practical applications.

Keywords: Amylolytic fungi, starchy waste, *A. niger*, *Penicillium* sp., purification

Isolasi Kulat Amilolitik dan Pencirian Amilase dalam Aspergillus niger KBA2 dan Penicillium sp. BB3 dari Sisa Berkanji dan Agro-Industri Tempatan dari Kuching dan Samarahan, Sarawak, Malaysia

ABSTRAK

Amilase merupakan enzim hidrolitik yang terlibat dalam pencernaan kanji kepada gula. Penghasilan enzim ini banyak bergantung kepada sumber-sumber mikrobial dan penyaringan kulat yang berpotensi untuk menghasilkan amilase adalah amat penting. Terdapat pelbagai laporan yang melaporkan pemencilan kulat amilase daripada sumber-sumber seperti sampel tanah, kawasan perladangan dan sisa-sisa lain. Sisa makanan dan agro-industri merupakan penyumbang utama dalam penghasilan sisa-sisa organik. Makanan juga banyak dibazirkan disebabkan pembasian, dan keadaan ini merupakan masalah yang serius di seluruh dunia. Walaupun bahan-bahan sisa tersebut merupakan sumber yang berpotensi, kajian terhadap kepelbagaian dan ciri-ciri amilase kulat dari pelbagai sisa berkanji tempatan adalah terhad. Oleh itu, kajian ini dirancang untuk memencilkan kulat amilolitik dari sisa-sisa berkanji, mengoptimumkan penghasilan enzim amilase dari kultur yang berpotensi dan mencirikan amilase separa tulen. Enam jenis sisa termasuk hampas sagu (sisa agro-industri), air basuh beras (sisa makanan), serta makanan basi seperti kentang, roti, kek pisang dan kuih bakul telah dikumpul. Kulat-kulat dipencilkan melalui pencairan bersiri dan kultur langsung, manakala pengecaman kulat dilakukan melalui kaedah morfologi dan molekular. Penyaringan kulat penghasil amilase dilakukan dengan medium pepejal kanji dan aktiviti amilase ditentukan melalui indeks aktiviti enzim (EAI). Isolat yang berpotensi kemudian dipilih untuk penentuan pH optimum dan tempoh penkulturan optimum. Penulenan separa amilase dilakukan melalui pemendakan ammonium sulfat, dengan penentuan berat molekular amilase melalui SDS-PAGE dan aktiviti amilase melalui gel elektroforesis natif (native-PAGE). Suhu dan pH optimum

amilase separa tulen juga dikaji. Sejumlah 41 isolat yang dibahagikan kepada 12 genera dan 23 spesis telah dipencil dari 6 sumber. Genus Aspergillus yang mengandungi 15 isolat dengan 9 spesis merupakan genus yang mempunyai sebaran dan kepelbagaian spesis yang tertinggi, diikuti dengan genus Penicillium yang mempunyai 8 isolat dengan 3 spesis. Dari 41 isolat, 34 menghasilkan amilase ekstraselular, dan isolat Penicillium serta Aspergillus dipilih untuk kajian seterusnya disebabkan aktiviti amilase yang tinggi. Dua isolat dengan indeks yang tertinggi dipilih dari setiap genus, iaitu Penicillium sp. BB3 (1.45 ± 0.10), P. citrinum KBA4 (1.36 ± 0.02), A. europaeus BNN1 (1.19 ± 0.04) dan A. niger KBA2 (1.16 ± 0.02). Dalam 4 isolat, Penicillium sp. BB3 dan A. niger KBA2 menghasilkan amilase paling tinggi di bawah medium pH 7 dan pengkulturan selama seminggu dan medium pH 5 dengan pengkulturan selama 5 hari masing-masing. Amilase dari strain BB3 ditulenkan secara 0.51 kali ganda dengan penghasilan sebanyak 26.47% manakala amilase dari strain KBA2 mencapai ketulenkan 2.08 kali ganda dengan penghasilan 28.85%. SDS-PAGE dan native-PAGE menunjukkan strain BB3 menghasilkan beberapa isoform amilase ataupun amilase berlainan jenis dengan berat molekul 80 kDa, 65 kDa, 42 kDa, 40 kDa dan 35 kDa. Sebaliknya, strain KBA2 hanya menghasilkan sejenis amilase dengan berat molekul 130 kDa. Amilase separa tulen dari strain BB3 menunjukkan aktiviti yang tinggi pada pH 7 dan suhu 40 °C manakala enzim dari strain KBA2 menunjukkan aktiviti yang tinggi pada pH 5 dan suhu 50 °C. Hasil kajian ini menunjukkan bahawa strain BB3 dan KBA2 merupakan kulat-kulat amilolitik yang berpotensi untuk digunakan dalam pelbagai industri. Oleh itu, kajian lanjutan adalah dicadangkan untuk fokus kepada peningkatan aktiviti dan kestabilan enzim tersebut.

Kata kunci: Kulat amilolitik, sisa berkanji, A. niger, Penicillium sp., penulenan

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic Acid
DNS	3,5-Dinitrosalicylic Acid
EAI	Enzymatic Activity Index
GH	Glycoside Hydrolase
HFCS	High-Fructose Corn Syrup
HMCS	High-Maltose Corn Syrup
ITS	Internal Transcribed Spacer
MWCO	Molecular Weight Cut-Off
Native-PAGE	Native Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SmF	Submerged Fermentation
SSF	Solid State Fermentation

CHAPTER 1

INTRODUCTION

1.1 Study Background

Enzymes, derived from the Greek word meaning ‘in yeast’ (May, 2019), are biological catalysts that accelerate biochemical reactions in living organisms (Porter et al., 2016). The field of enzymology was established in the early of 19th century, with early contributors from scientists like Réaumur and the enzyme ‘diastase’ being among the first to be studied (Heckmann & Paradisi, 2020). The descriptive phase of enzymology followed, focusing on the discovery of new enzymes and quantifying their catalytic activities (Vitolo, 2020). Currently, over 6,000 enzymes have been identified and studied (Kuddus, 2019), with applications spanning across numerous industrial sectors (Arnau et al., 2020). To date, enzymology has reached the planned application phase, where development in enzyme knowledge led to the broad application of these biocatalysts (Vitolo, 2020).

Enzymes are classified based on the types of reactions they catalyse (Garske et al., 2017). Hydrolases (EC3) are the most widely studied group (El-Gendi et al., 2021), with amylase being a key enzyme within the glycosylase subgroup (Lee & deMan, 2018). Amylase works to hydrolyse starch into simple sugars (Rebello et al., 2019), and is one of the most widespread enzymes (Balakrishnan et al., 2019). Amylases differ in their action on polysaccharide bindings of the substrates, which has led to their classification into endoamylases, exoamylases and debranching amylases (Ahmad et al., 2020).

Living organisms are the major source of enzymes, with fungi and yeasts contributing over half of global enzyme production (Sharma et al., 2021). It has always been of interest to screen for natural occurring potential fungi (Ja’afaru, 2013), where various

wastes such as agricultural waste and kitchen waste were previously used as sources. Vast consumption of starchy food (Sanabria et al., 2021) and agroindustry (Brojanigo et al., 2020) constantly contributed to high amounts of organic wastes. Waste is also produced through microbial colonisation of food, causing spoilage (Sharma & Sharma, 2024). Filamentous fungi, in particular, thrive on solid materials, producing enzymes that break down these substrates (Ogbonna et al., 2014). Given that carbohydrates are major components of food waste (Taha et al., 2019), isolating amylolytic fungi from such waste sources is a justified and valuable approach.

1.2 Problem Statement

Starchy and agro-industrial waste materials represent a potential and underutilised but promising resource for isolating amylolytic fungi as previous studies have mainly focused on studying hydrolysis of organic wastes by amylolytic isolates often not indigenous to the wastes. Sarawak, as the major exporter of sago starch in Malaysia and a major global producer, generates large quantities of sago pith waste rich in starch (Naim et al., 2016). This waste offers a favourable environment for the growth of amylolytic fungi. However, previous studies focused on utilising this waste as a substrate for microbial hydrolysis by non-indigenous isolates instead of exploring the diversity of amylolytic fungi on the waste. Similar to sago pith waste, studies on food wastes were extensively focusing on introducing non-indigenous fungi in hydrolysing the wastes rather than investing the potential of the wastes as source for indigenous amylolytic fungi.

Investigating this diversity is crucial, as the colonisation patterns and metabolic potential of fungi are strongly influenced by both the type of organic matter and the surrounding climatic conditions (Almeida et al., 2017; Větrovský et al., 2019). Similar fungus, when isolated from different sources, could have variation in their metabolism as

fungi possess the ability to adjust their metabolism according to the availability of nutrients in their surrounding environment (Xiong et al., 2017). Hence, this characteristic causes variation in amylolytic potential of fungi from different sources. However, studies of fungi on local wastes only limited to the utilisation of the wastes as substrates by introduced species instead of investigating indigenous fungi. Therefore, studying local fungal isolates from diverse waste materials may reveal novel amylolytic fungi with unique properties.

Furthermore, these indigenous fungi could play a vital role in sustainable waste management and valorisation. To realise their potential, it is essential to characterise the optimal conditions for amylase production as well as the biochemical and physicochemical properties of the enzymes produced. However, requirements for optimal amylase production of these indigenous potential isolates, as well as biochemical and physicochemical properties of the amylase produced by these isolates remain poorly characterised, hindering the scalability and industrial applications of these fungal enzymes. The knowledge gap restricts the ability to effectively use these fungi for both local sustainable waste management and biotechnological applications. Therefore, this study aims to explore the diversity of amylolytic fungi from underutilised local starchy and agro-industrial waste, identify novel fungal candidates with high amylolytic potential, and evaluate their enzyme production characteristics for applications in local waste management and industrial biotechnology.

1.3 Objectives

This project aimed to establish knowledge of amylolytic fungi isolated from various local starchy waste through achieving the following objectives:

- i. To isolate and identify indigenous amylolytic fungi from various local starchy waste materials.

- ii. To evaluate and compare the amylase activity of the isolated fungal strains for selection of potential isolates
- iii. To optimise conditions for amylase production and characterise partially purified amylase from potential fungal isolates for establishment of fundamental knowledge for future application

This thesis consists of six chapters, with Chapter 1 provides a brief introduction regarding the background and objectives of the research. Chapter 2 is the literature review section that discusses on enzymes and amylase, which aims to present the current knowledge and previous research. Chapter 3 hosted the materials and methods applied in this study with clear description for replicability purpose, while Chapter 4 presents the results obtained after the experiments. The comparison of the current findings with previous works and justification of obtained results are presented in Chapter 5, which is the discussion section. Chapter 6 presents the conclusion and recommendation of this study, which focuses on summarising the entire research and also provides suggestions about future works. References used in this thesis will be listed alphabetically after Chapter 6 and the final part of the thesis consists of the appendices section which presents the supplementary information about this work.

CHAPTER 2

LITERATURE REVIEW

2.1 Enzymes

2.1.1 Background

Mesophilic organisms can perform reactions under favourable conditions due to the presence of catalysts (Heckmann & Paradisi, 2020), where these catalysts are enzymes that are defined as biological catalysts which accelerate biological reactions (Porter et al., 2016). The application of these biocatalysts could be dated at least back to 10,000 BC (Chowdhury & Maranas, 2020). As human civilisation progressed, enzyme application had developed in societies around the world, where the Greeks, Chinese and Egyptians were applying enzymes for fermentation and bread making. This era, during which products were produced on a large scale without acknowledging the involvement of enzymes, is referred to as the empirical phase of enzyme technology (Vitolo, 2020).

The early 19th century marks the initiation of enzymology. The term ‘catalyst’ was coined by Jacob Berzelius, where it was formed from the Greek word ‘kata’ meaning wholly and ‘lyein’, meaning to loosen (Punekar, 2018). The first studied enzyme was ‘diastase’ (Heckmann & Paradisi, 2020), dated back in 1833. Payen and Persoz proposed that the addition of ‘ase’ to the substance modified by enzymes could be used in their nomenclature (May, 2019). The term ‘diastase’ originated from the Greek word ‘diastasis’, meaning to make a breach (Punekar, 2018). Later, Wilhelm Kühne suggested that the term ‘ferments’ given to substances that drive the catalysation of fermentation by Pasteur and his colleagues, should be replaced by the name ‘enzyme’, with the definition ‘in yeasts’. Research on the enzyme specificity was pioneered by Emil Fischer through the ‘lock and key’ model in 1894 and later Daniel Koshland with the ‘induced fit’ model (May, 2019). This period of studying

and revealing the catalytic activity of enzymes is reported to be the descriptive phase in enzyme technology development (Vitolo, 2020).

Victor Henri, Michaelis and Menten presented reaction equations of enzymes which contributed to enzyme kinetics (Chowdhury & Maranas, 2020), followed by Briggs and Haldane (Punekar, 2018). Structural studies of enzymes began with Sumner (Garske et al., 2017) and Frederick Sanger later published the complete sequence of amino acids of two polypeptide chains of bovine insulin. In 1965, Philips and his colleagues succeeded in studying the exact structure of enzymes (Garske et al., 2017). Various enzymes were being isolated, purified and characterised within a period of two decades after the work of Stein and Moore in amino acid quantification. As new enzymes were being discovered and enzyme activities were being quantified through a maths model, this era is named as the descriptive and quantitative phase (Vitolo, 2020).

Nowadays, researchers have identified more than 6,000 different enzymes (Kuddus, 2019). The 19th century also marked the start of extensive enzyme application in industry (Wiltschi et al., 2020), where usage of enzymes in industries is increasing rapidly over the years. Presently, the evolution of enzyme technology has reached the fourth phase, which is the planned application phase, where this phase is the period where enzymes are being used widely in industries due to the advances in enzyme knowledge (Vitolo, 2020). Currently, enzymes are involved in 40 industrial sectors (Arnau et al., 2020) such as food and beverages, detergent, and biofuels industries (Prasad & Roy, 2017). Hydrolases make up the most of industrial enzymes, where proteases, amylases and cellulases are used most frequently (Arbige et al., 2019). The establishment of industrial enzyme usage throughout the years was summarised and the estimated growth in the global sales of enzymes will reach approximately 5,500 million Euro in 2025 (May, 2019) as observed in Appendix A.

2.1.2 Properties of Enzyme

Almost all enzymes are proteins (Kuah et al., 2016) as some of them are nucleic acid molecules that possess catalytic ability. Some enzymes are only made up of protein, while most enzymes consist of non-protein components such as lipids, metals, and phosphates (Lee & deMan, 2018). RNA and antibodies can function as catalysts referred to as ribozymes and abzymes (Kuddus, 2019). Enzymes are formed through a combination of 20 amino acids, with the presence of additional structure such as carbohydrates that will affect their catalytic and physical properties (Kuah et al., 2016). Molecular weights of enzymes ranged widely, between 12,000 Da and to more than 1 million Da (Bhatia, 2018). The structure of enzymes is achieved through several folding during post-translational modification (El-Gendi et al., 2021). Differences are observed in the sequences and characteristics of amino acids in an enzyme, and these are related to the activity and stability of the enzyme (El-Gendi et al., 2021).

Enzymes are responsible for catalysing more than 5,000 biological processes (Kuah et al., 2016) by providing alternative paths that require lower activation energy (Garske et al., 2017). At the end of the reaction, enzymes would not be degraded (Choudhury, 2020), and their action would not result in any permanent modifications (El-Gendi et al., 2021). The amount of enzyme required for reaction is small and is lesser than the number of substrates (Bhatia, 2018). Enzymes are reported to be highly specific in carrying out reactions and there is a site for catalysis, referred to as the active site (Garske et al., 2017). Every enzyme is responsible for catalysing a particular reaction or a class of reactions that possess similar properties. Selectivity of enzymes could minimise the production of side-products, thus ensuring the interested products are free from contaminants (Prasad & Roy, 2017).

Enzymes perform their actions under a mild environment (Porter et al., 2016) which are easier for regulations (Choudhury, 2020). However, catalytic properties of enzymes will be lost if they are exposed under certain agents, for example strong acids, organic solvents or other states that could cause denaturation (Garske et al., 2017). Enzymes are environmentally friendly due to their biodegradability which could lower the pollution load (Choudhury, 2020) and also due to their non-corrosive characteristics (Choudhury, 2020). Enzymes could be produced through fermentation with renewable substrates in a large scale, and they could be modified and improved to comply with the requirements of their applications (Garske et al., 2017).

2.2 Amylase

Amylase is one of the significant enzymes with vast applications. Long before Payen and Persoz studied the enzyme ‘diastase’, which was amylase, this enzyme had already been discovered earlier by Kirchhoff in 1811 (Savaner & Sohani, 2020). However, while Erhard Leuchs identified α -amylase in saliva in 1831, Kirchhoff did not observe the presence of enzymes. Kirchhoff reported that the production of sugar from starch during grain germination was similar to the hydrolysis of starch to sugar facilitated by acid (Punekar, 2018). The enzyme ‘diastase’ reported by Payen and Persoz in 1833 was a mixture of amylases, and now referred to either α -, β -, or γ -amylase (Heckmann & Paradisi, 2020; May, 2019). In 1930, Ohlsson proposed the use of anomeric type of sugars in classifying starch hydrolysing enzymes in malt, which resulted in the classification of α - and β -amylases (Kumar & Chakravarty, 2018). Amylase gained recognition as an enzyme with well-established knowledge through years of research (Ahmad et al., 2020), and is one of the most effective enzymes (Kumar & Chakravarty, 2018).

These enzymes are categorised under the group hydrolase (EC3), where hydrolases cleave chemical bonds of substrates to generate two products in the presence of water (Garske et al., 2017). Apart from breaking the bonds and removing groups, hydrolases are also capable of transferring the removed groups to other molecules (Silva, 2018). The members of EC3 are commonly named with the addition of the suffix ‘-ase’ to the name of substrates. Itabaiana and Souza (2018) mentioned that the enzymes under the hydrolases group can be divided into several subgroups according to their specificity. Within hydrolase group, amylases are categorised under the glycosylase subdivision, EC3.2 (Itabaiana & Souza, 2018), and are the most crucial enzymes in this subgroup (Lee & deMan, 2018). They work on the breaking of the starch into simple sugars, specifically hydrolysing starch into glucose (Drożdowska, 2018; Rebello et al., 2019).

The substrate of amylase is starch, a term originated from the Middle English word ‘sterchen’, meaning ‘to stiffen’ (Balakrishnan et al., 2019). Starch consists of two polymers of glucose, amylopectin, and amylose (Brust et al., 2020). Natural starch contains between 10% to 35% amylose, and it is linear with few branches (Seung, 2020). On the other hand, amylopectin being the major polymer, constitutes between 65% to 90% of starch (Seung, 2020), and is highly branched (Lee & deMan, 2018). Both components have α -1,4-linked glucose chains that are linear, and α -1,6-linked branch points. Glucose residues in amylose could reach an amount of 1,000, while in amylopectin, glucose residues might generally reach 2,000,000 (Hashim, 2019). Amylopectin has a lower degree of polymerisation (less than 100), compared to amylose, that has a degree of polymerisation ranging from 100 to 10,000. Amylose molecules have lower molecular weight of 104 to 106 Da, compared to the molecular weight of amylopectin of 107 to 109 Da (Li et al., 2019).

Amylases are among the most widely dispersed enzymes, present in the bacteria, archaea and eukarya domain (Balakrishnan et al., 2019). The activity of most amylases is the highest at a temperature of 50 °C, most are stable under pH range of 4.0 to 8.0 (Vandenberghe et al., 2020). Further categorisation of amylases is based on their actions upon polysaccharide bindings, which yield three major classes (Ahmad et al., 2020). Amylase are also classified based on the products they produced, apart from the position of enzyme attachment on the starch molecules (El-Gendi et al., 2021).

2.2.1 Endoamylase

Endoamylase is a group of amylases whose members acts randomly and internally within the starch molecule (Gopinath et al., 2017). This randomised cleavage yields both linear and branched oligosaccharides with varying lengths (Kumar & Chakravarty, 2018). Alpha-amylase is a type of endoamylase with the classification number of EC 3.2.1.1, where it is also known as α -1,4-glucan-4-glucanohydrolase (Saini et al., 2017). Detection of α -amylase has a long history, where after the discovery of Leuchs, Magendie also discovered this enzyme in serum in 1846, following by Danilewsky in pancreas in 1862, and Cohnheim in urine in 1863 (Savaner & Sohani, 2020).

The enzyme is categorised under family 13 of glycoside hydrolase (GH) (Balakrishnan et al., 2019). Apart from GH13, α -amylases also belong to the families of GH57, GH119 and GH126 (Zhang et al., 2017). The families are different among one another in their sequences, catalytic machineries, mechanism of action and also catalytic domain fold, where α -amylases that are categorised under the same GH family should exhibit similarities in these characteristics (Janeček et al., 2013). GH13 is the principal family of α -amylases, where it could be found in a wide range of organisms, while GH57 is found in bacteria, archaea, and a little in eukaryotes (Sidar et al., 2020). For GH119 and

GH126, they have only been found in bacteria. These enzymes have molecular weights ranging between 10 kDa and 210 kDa (Rebello et al., 2019). Alpha-amylase with the lowest molecular weight originated from *Bacillus caldoyticus* while the organism with the highest α -amylase molecular weight is *Chloroflexus aurantiacus* (Paul, 2016).

Alpha-amylases are metalloenzymes where incorporation of calcium ions is required for various purposes (Liu & Kokare, 2017), which are maintaining the stability, actions, and the confirmation of the enzymes (Gopinath et al., 2017). One mole of this enzyme contains one gram-atom of calcium (Lee & deMan, 2018). Alpha-amylases are reported to be stable under the presence of several detergents and surfactants, where apart from calcium ions, various enzymes such as potassium and magnesium ions could also activate the enzymes (Sindhu et al., 2017). On the other hand, there are also ions that inhibit the activities of α -amylase, such as copper (II) ions (Sindhu et al., 2017).

Internal hydrolysis of substrates at the α -1,4-O-glycosidic linkages is observed in the action of α -amylase (Liu & Kokare, 2017) and the products is a mixture of oligosaccharides, maltose and glucose (Garske et al., 2017). When acting upon amylopectin, oligosaccharides consisting of glucose with two to six units will be formed (Lee & deMan, 2018). Thus, viscosity of the substrate will be decreased rapidly through α -amylases treatment, but only a small amount of monosaccharide is produced. When acting upon amylose, the enzyme will hydrolyse the substrate completely to maltose, with the formation of some maltotriose. Alpha-amylases will produce oligosaccharides, dextrans, maltose and glucose when acting upon a mixture of amylose and amylopectin. Alpha-amylases will attack the substrate multiple times as the enzymes may stay attached to the substrate and cleavage will occur a few more times before α -amylases dissociate from the substrate (Kumar & Chakravarty, 2018). The enzyme would not change the α -anomeric structure of

the products formed (Zhang et al., 2017). The action of α -amylase is faster compared to other amylases because of its randomised mode of action (Azzopardi et al., 2016).

Alpha-amylases from microbial source are stable at pH ranging between 4 to 11 (Mojsov et al., 2018), however microbial α -amylases produced through SSF works best under pH 5.0 with optimum temperature of action at 50 °C (Sindhu et al., 2017). Although these enzymes could be obtained from a wide range of organisms, but those originated from bacteria and fungi played prominent roles in industrial sectors. Alpha-amylases are considered the most crucial enzyme in the initiation of starch hydrolysis (Paul, 2016). By 2024, the global market of these enzymes is expected to achieve USD 320 million from USD 255 million, on account of growing α -amylase usage in India, China, and the Middle East (Ahmad et al., 2020).

2.2.2 Exoamylase

Exoamylases is another group of amylases which hydrolyses amylose and amylopectin externally through acting upon the glucose residues (Hashim, 2019). Both substrates with long and short chains are the targets of exoamylases to produce low molecular weights products, for examples maltose and glucose (Ahmad et al., 2020). These enzymes are able to convert anomeric configuration in their action, thus producing products with β - configuration (Hashim, 2019). Beta- and γ -amylases are categorised under this group (Balakrishnan et al., 2019). Beta-amylases are enzymes with EC number 3.2.1.2 and are also known as 1,4- α -D-glucan maltohydrolase (Mojsov, 2016), glycogenase or saccharogen amylase (Balakrishnan et al., 2019). Beta-amylases are categorised under GH family 14 (Balakrishnan et al., 2019) where this family is mainly found in plants and bacteria (Sidar et al., 2020).

Non-reducing ends of substrates, that are the α -1,4-glucosidic bonds, are the only target of β -amylases (Kocabay et al., 2016). The product of this hydrolysis is mainly maltose, but a minor product consisting of β -limit dextrin is also present (Mojsov, 2016). The enzymes will later convert maltose to beta-maltose through inversion (Duan et al., 2019). Inability of the enzymes to hydrolyse the substrates completely is the reason why limit dextrin is produced, as they could not cleave the branches in polysaccharides (Liu & Kokare, 2017). The point of branching, with α -1,6 glucosidic linkage is where β -amylases cease to cleave (Lee & deMan, 2018). Similar to α -amylases, these enzymes are also showing multiple attacks in their action. Beta-amylases are also reported to not be requiring calcium ions to function and to maintain their stability (Kumar et al., 2016).

Beta-amylases are less sensitive to the acidity of the environment (Zhygunov et al., 2019). Therefore, the optimum pH for β -amylases is lower than α -amylases, which is between pH 4.0 and 5.5 (Savaner & Sohani, 2020). Their optimum temperature is lower than α -amylases as they are more sensitive to temperature (Zhygunov et al., 2019). These enzymes can be found in higher plants such as wheat and soybeans (Lee & deMan, 2018), but they are absent in fungi and bacteria (Thalmann et al., 2019). Beta-amylases take part in important activities in plants' intracellular environment such as in the metabolism of plants and developing the sweet flavour of ripe fruit by hydrolysing starch into maltose (Kocabay et al., 2016). Beta-amylases are stated to be crucial in plants and stated that they are particularly important in the process of seed ripening (El-Gendi et al., 2021). However, microorganisms also served as an important source of β -amylases. Masataka and Shigetaka first isolated microbial β -amylase from *Bacillus megaterium* in 1974 and nowadays many β -amylases producing microbes had been identified and studied (Duan et al., 2019).

Another exoamylase are the γ -amylases, which have EC number 3.2.1.3 and they are also known as glucan 1,4- α -glucosidase (Mojsov, 2016). Gamma-amylases are reported to be having multiple names where they are also being referred to as amyloglucosidase, exo-1,4- α -glucosidase, glucoamylase, lysosomal α -glucosidase, and 1,4- α -D-glucan glucohydrolase (Saini et al., 2017). They are categorised under GH family 15 (Balakrishnan et al., 2019). Gamma-amylases are exoacting amylases targeting both α -1,4 and α -1,6 glycosidic bonds (Mojsov, 2016). The enzymes hydrolyse amylose and amylopectin upon the non-reducing ends (Liu & Kokare, 2017), but with slower cleavage rate at the branching point (α -1,6 linkages) compared to the α -1,4 bonding (Lee & deMan, 2018). Activity of these enzymes on the α -1,6 linkages is only 0.2% of the α -1,4 linkages (Xia et al., 2021). Starch could be degraded completely into glucose through the action of γ -amylases due to their ability to cleave two bonding (Lee & deMan, 2018). The ability of γ -amylase to hydrolyse starch completely after a prolonged incubation period, causing the enzyme to be referred to as a saccharifying enzyme (Karim & Tasnim, 2018). The efficiency of this enzyme is higher when long chain polysaccharides are the substrates (Hashim, 2019).

These enzymes are unique compared to α and β -amylases because their optimum pH is low, where they work best under acidic conditions. and mentioned that γ -amylases have an optimum pH of 3 (Saini et al., 2017). Most γ -amylases have optimum temperature between 50 °C and 70 °C, where they are stable under high temperatures (Karim & Tasnim, 2018). There is a wide range of microbes that are able to produce γ -amylases (Karim & Tasnim, 2018), but the enzymes are absent in both plants and animals (Lago et al., 2021). The main source for γ -amylases are the filamentous fungi such as *Rhizopus oryzae* (Karim & Tasnim, 2018).

2.2.3 Debranching Amylase

Pullulanases and isoamylases are debranching amylases, where they target only the α -1,6 bonding (Ahmad et al., 2020) to produce long chain polysaccharides (Hashim, 2019). Pullulanases are enzymes with EC number 3.2.1.41 (Garske et al., 2017), where they are grouped under GH family 13 (Singh & Singh, 2019), and GH 57 (Xu et al., 2021). They are also being known as α -dextrin 6-glucanohydrolase, pullulan 6-glucanohydrolase, limit dextrinase and amylopectin 6-glucanohydrolase (Singhal et al., 2018). The specificity of these enzymes leads to their further classification into two groups, which are pullulanase Type I and II (Møller et al., 2016). The molecular weights between these two types of pullulanases differ quite significantly, where most Type I pullulanases have molecular weights ranging between 65 to 145 kDa, while members of Type II generally weighed more than 100 kDa (Xu et al., 2021). Pullulanases are important during grain germination for the hydrolysis of starch (Li et al., 2019).

One substrate of pullulanases is the pullulan, which is a linear exopolysaccharide that is soluble in water and consist maltotriose units linked with α -1,6 glycosidic bonds (Singh & Singh, 2019). The physicochemical characteristics of this substrate such as high solubility in water and low viscosity are distinguishing due to the uniqueness in its glycosidic linkage pattern. Pullulanases favour closely spaced α -1,6 linkages in their actions (Li et al., 2019). Type I pullulanases cleave exclusively upon α -1,6 bonding of amylopectin and β -limit dextrin (Møller et al., 2016). Hydrolysis of pullulan by these enzymes yield maltotriose while amylopectin is break down to simple sugars when enzymes that cleave α -1,4 linkages are incorporated in the hydrolysis (Xia et al., 2021). The product of Type II pullulanases are maltose and maltotriose, where the enzymes act on α -1,4 and α -1,6 linkages (Hashim, 2019). Efficiency of starch saccharification could be enhanced by Type II pullulanase (Xu et al.,

2021) as α -1,4-glucan chains and α -1,6 branching points are found within amylopectin (Zeng et al., 2019).

Pullulanases could be found in plants, animals and microbes (Singh & Singh, 2019), where these enzymes are mostly obtained from microorganisms (Singhal et al., 2018). Type I and Type II pullulanases are both derived from microorganisms (Xu et al., 2021). Industrial debranching enzymes generally have an optimum temperature of 60 °C (Xia et al., 2021), but Type II pullulanases prefer temperature between 60 °C to 80 °C (Xu et al., 2021).

The other debranching enzyme is the isoamylases, which was first isolated in 1951 from *Saccharomyces cerevisiae* (Xia et al., 2021). With EC number of 3.2.1.68, isoamylases also have the name glycogen-6-glucanohydrolase (Li et al., 2017). These enzymes are being categorised under GH family 13 along with pullulanases and α -amylases (Balakrishnan et al., 2019). Isoamylase, when its amount is reduced, or its function is loss, could cause soluble α -glucans to accumulate (Li et al., 2019).

As observed in pullulanase, α -1,6 linkages are also the target of isoamylases, but they favour widely spaced α -1,6 bonding (Li et al., 2019). While these two amylases act on amylopectin, specificity of substrates are still observed between these two amylases, where pullulanase favour pullulan and isoamylases act on glycogen as well as phytoglycogen (Nabemoto et al., 2016). Pullulanases require the existence of two α -1,4 linked glucose units on sugar chains bonded by the α -1,6 linkage for their action, while isoamylases require a minimum of three α -1,4 linked glucose units (Xia et al., 2021). Isoamylases are mostly found in higher organisms such as plants, while mesophilic and thermophilic microbes serve as the primary microbial source for these enzymes (Xia et al., 2021). High tolerance of isoamylase

towards high temperature makes these enzymes to be favoured compared to pullulanase (Li et al., 2017).

2.3 Amylase in Industry

2.3.1 Sources of Amylase

Living organisms are the major source of enzymes (Sharma et al., 2021) where most industrial enzymes are derived from microbes (Illanes, 2019). Microorganisms gained attention in the production of enzymes due to the inability of animals and plants in fulfilling the requirements of industries (El-Gendi et al., 2021). For more than 50 years, humankind has been applying the knowledge that naturally occurring microbes produce the most enzymes (Patel et al., 2017). Referred to as microbial enzymes, these enzymes that are produced by microbes are currently marketed under well supplies and standardisation by various companies around the world. Microbial enzymes are occupying more than 2 billion of the global market (Singh et al., 2019).

Fungi and yeasts gained the status as notable sources of enzymes by producing more than half of industrial enzymes, and a third of production is currently occupied by bacteria (Sharma et al., 2021). Market share of fungal enzymes with a value of 50% is mainly contributed by the enzymes produced from members of *Aspergillus*, *Trichoderma*, *Rhizopus* and *Penicillium* genera (El-Gendi et al., 2021). Microbial enzymes are preferred over non-microbial enzymes in some industries due to several reasons, including the ease in obtaining raw materials, and cellular metabolism of microbes are clearly defined (Sharma et al., 2021). As compared to microbes, plant and animal tissues may contain or produce more substances that are potentially dangerous. Qualities of enzymes derived from microbes are also higher than similar products from plant and animals (Yoo et al., 2017).

Simple production and control process with low costs are observed in obtaining microbial enzymes (Illanes, 2019). Production cost is lower due to the requirement of only small production facilities as microbial enzymes require shorter production time (Deckers et al., 2020). It is also easier to perform modification and optimisation operations of microbial enzymes (Tiwari et al., 2015). To produce enzymes in industrial scale, bacteria and fungi are the most suitable organisms as they do not require light to grow, simple handling techniques and they grow at a very high rate (Patel et al., 2017). Microbial enzymes are also stable under heat, and they are active under a wider pH range (Lee & deMan, 2018).

Industrial amylases could be obtained from plants, α -amylase could be acquired from cereal grain while β -amylase is obtained from sweet potato (Collados et al., 2020). Most amylase applied in industries are obtained from microorganisms, and they are also applied in scientific research (Gopinath et al., 2017). Members of *Bacillus* genus are the main contributor of bacterial amylase and *Aspergillus* species are involved in producing fungal amylase (Liu & Kokare, 2017). Members of *Bacillus* are considered as the workforce of enzyme production due to their capability to produce numerous enzymes, including amylase (Patel et al., 2017). Bacterial amylases are more stable under heat; therefore, they are being applied in brewing, alcohol, textiles, detergent and industries starch degrading processes (Garske et al., 2017).

Filamentous fungi are significant producers of enzymes and fungal amylases are more favourable than amylases from other microbial sources (Tiwari et al., 2015). This is due to several advantages fungi have, where the production of enzymes from fungi are reported to have high production potential and the enzymes produced possess required stability to function under severe state (El-Gendi et al., 2021). The ability of fungi to penetrate solid substrate and extracellular secretion of amylase by fungi made them

especially suitable for solid based fermentation (Gopinath et al., 2017). Requirements in the purification and separation of produced enzymes are simpler when fungi are being used as the source, particularly filamentous fungi (El-Gendi et al., 2021). Alpha-amylases obtained from fungi are also reported to be favoured over other sources as their status as generally considered as safe (GRAS) is more accepted (Tiwari et al., 2015). One disadvantage of fungal amylases is that they are less stable under heat and being mostly applied in the baking industry to act as supplement to amylases that are already present in the wheat flour (Garske et al., 2017).

2.3.2 Production of Amylase

Amylases, similar to other enzymes, could be produced through submerged fermentation (SmF), and solid-state fermentation (SSF). Submerged fermentation is historically used to produce enzymes, where a liquid nutrient broth will act as growth media for microbes, while in SSF, a solid substrate with minimal amount of moisture is involved in growing the microorganisms (Paul et al., 2021). Soluble sugars, fruit and vegetable juices, wastewaters and molasses are among the substrates that have been used in SmF (Paul et al., 2021). To produce enzymes through SSF, agro-industrial residues are regarded as the best substrates (Karim & Tasnim, 2018). Natural substrates, including agricultural residues, are suitable as they mimic the state under which the fungi grow (Liu & Kokare, 2017).

The fed batch SmF is the most practised approach in the production of industrial enzymes, where the substrates are supplemented at random time intervals and the enzymes will be harvested when the reactor is full or fermentation has been completed (Kumar et al., 2016). In the production of glucoamylase (γ -amylase), Submerged fermentation had been applied traditionally, but recently the popularity of using SSF in the production of this enzyme had been growing (Karim & Tasnim, 2018). A similar situation could be observed

in the production of α -amylase, where SSF is currently being widely applied to overcome several drawbacks of SmF (Paul et al., 2021).

The involvement of high moisture level in SmF possesses various advantages, where it is easier for sterilisation, production, purification, and enabling easier temperature, pH, and nutrient control (Far et al., 2020). Submerged fermentation is easier to handle, and this approach provides better aeration and dispersion for microbial growth (Yoo et al., 2017). This technology is practised by most large-scale enzyme producing facilities due to the ability of SmF to be scaled-up. However, there are several drawbacks in practising SmF for enzyme production, one of it being causing substrates degradation as the enzymes produced by microbes are released into the liquid culture medium (Karim & Tasnim, 2018). The concentration of enzymes produced through SmF is also relatively low (Yoo et al., 2017).

Solid state fermentation possesses several advantages against SmF, where the process is easier, high production efficiency, requiring simple fermentation media, while the requirement for capital and energy is lower (Karim & Tasnim, 2018). As SSF requires the usage of lesser water, the process will also produce less wastewater. Besides that, the control for bacterial contamination in SSF is easier than SmF, and the cost for downstream processing is lower. Monitoring of pH is not necessary when performing SSF, as most of the substrates in this approach possess remarkable buffering capacity and could maintain a favourable pH for the growth of microbes (Sindhu et al., 2017). Nevertheless, application of this technique has problems in management and scale-up (Yoo et al., 2017).

The enzymes will be extracted after fermentation, with centrifuging the medium in SmF and retaining the supernatant, and in SmF, water or buffers will be added to the fermented matter before obtaining the filtrate after filtration (Sindhu et al., 2017). The crude

enzymes obtained after extraction will undergo the purification steps. Although not all commercial applications of amylase require purified enzymes, they are required in the pharmaceutical sector (Sindhu et al., 2017), where there are three main purification methods as mentioned (Liu & Kokare, 2017). Enzymes are purified through their ionic properties, their capability to be adsorbed, and the differences in molecular size. There are also several other special strategies which are crystallisation, electrophoresis and chromatography (Liu & Kokare, 2017). Some examples of chromatography include affinity chromatography, gel exclusion chromatography and ion-exchange chromatography (Yoo et al., 2017). The market, cost of processing, final quality and the technology altogether will determine the purification process in the downstream processing step (Saini et al., 2017).

2.3.3 Importance of Amylase in Industry

There are various industries which applied amylases in the production processes, where one of them is the food processing industry. Amylase is one of the important enzymes in the bakery industry, where it is present in flour to retain the moisture (Sharma et al., 2021). Dextrin produced after starch hydrolysis could reduce the hardness of bread and could also yield an adequate amount of fermentable sugar for production of lively doughs (Balakrishnan et al., 2019). Browning of crust and appealing baked flavour could be improved through the enhancement of Millard reactions (Hashim, 2019). Stalling will occur during the storage of baked products where moisture migrates from the starch granules and enters the interstitial spaces, which causes amylose and amylopectin to realign (Fernandes & Carvalho, 2017). Therefore, amylases are used to control amylopectin recrystallisation (Balakrishnan et al., 2019).

Apart from that, the production of beverages also requires the incorporation of amylases. In the production of juice, amylases are involved to clarify fruit and vegetable

juice (Patel et al., 2017). Amylases could also increase the production of juice (Sharma et al., 2021). Amylases are also important enzymes in the beer industry, where the application of enzymes is observed in the malting step (Ray et al., 2016). Malting involves drying of the grain into malt for hydrolysing carbohydrates into dextrin and maltose (Gomaa, 2018) where the rate and quantity of product could be enhanced through the application of amylase (Gudynaite-Savitch & White, 2016). Fermentable sugar for beer production will also be produced through starch hydrolysis by amylase (Balakrishnan et al., 2019). Amylase could reduce starch germination temperature (Drożdowska, 2018) and also lower the viscosity of beer as well as improving quality (Nigam et al., 2018).

Conversion of starch into glucose through action of amylase is important in starch processing industry (Prasad & Roy, 2017). There are three steps in starch conversion which are gelatinisation, liquefaction and saccharification (Balakrishnan et al., 2019). The enzymes are added after gelatinisation in which starch slurry had been produced to initiate liquefaction (Hashim, 2019), where viscosity will be reduced through incomplete hydrolysis and further hydrolysis will be carried out in the saccharification step to yield simple sugars (Balakrishnan et al., 2019). Alpha-amylases are added in the liquefaction process, while glucoamylase is involved in the saccharification process, with the assistance of pullulanase and isoamylase (Karim & Tasnim, 2018).

Glucose and fructose syrups of high concentration which are important additives and sweeteners, are produced through processing starch (Singh et al., 2019). High-maltose corn syrup (HMCS) and high-fructose corn syrup (HFCS) are two examples of sugar syrups produced through amylolytic hydrolysis of starch (Balakrishnan et al., 2019). Amylases are also important in the chocolate industry, as they prevent the syrup from becoming too thick, thus producing syrups that are stable and have high flow properties (Savaner & Sohani,

2020). Amylases are also used to modify rice starch, due to the wide application of this ingredient in manufacturing food (Sanromán & Deive, 2017). The enzymes are also involved in the recovery of sugars from candy scraps (Ray et al., 2016).

In the manufacture of textile, breaking of yarn during weaving is prevented by applying a layer of starch known as sizer (Gudynaite-Savitch & White, 2016). The sizer needs to be removed for the fabric to absorb water or the finishing agents (Hashim, 2019). Amylases are therefore important as a desizing agent (Mojsov et al., 2018). Amylases are also being used to increase the stiffness of the products (Ahmad et al., 2020). Starch is also used as a sizer and surface coating material in the paper and pulp industry (Hashim, 2019) to prevent mechanical damage of papers (Far et al., 2020). Amylases are important to maintain the viscosity of starch paste (Hashim, 2019). Feasibility of papers are also enhanced through the usage of amylases (Far et al., 2020). Amylases also aid in cleaning and improving drainage, while help to produce papers that are smooth and could provide higher writing quality (May, 2019).

Amylases are also the most applied enzyme in the detergent industry (Singh et al., 2016) to remove insoluble starch residues (Thapa et al., 2019) and to enhance starch solubility (Gürkök, 2019). Amylase is also being applied in the pharmaceutical industry as digestive aid (Paul, 2016), as it takes part in the production of cyclodextrin (Gudynaite-Savitch & White, 2016). Usage of amylases as feed enzymes is also another application of amylases where efficiency of animal feed could be enhanced through feed enzymes (Thapa et al., 2019). In the leather processing process, α -amylases are efficient in facilitating splitting of fibre in goatskins (Hashim, 2019). Fermentable sugars required for bioethanol production are also produced through the action of α -amylase (Paul et al., 2021).

2.4 Factors Affecting Enzyme Production

The most common technique for enzyme production is through fermentation (Paul et al., 2021), where it is affected by several factors. Incubation temperature is one of the important factors where it will affect the growth of microbes (Sindhu et al., 2017), thus playing a role in determining the amount of enzyme produced (Paul et al., 2021). This happens as the development of metabolic heat in the production media will inhibit the growth of microbes and the production of enzymes (Karim & Tasnim, 2018). That viability of microbial cells will also decrease at high temperature, while at low temperature, metabolism of microbes will be suppressed, both leading to reduction in enzyme production (Paul et al., 2021). Alpha-amylase production through both SmF and SSF is usually performed under temperature between 25 °C and 37 °C (Sindhu et al., 2017).

The pH of the production media is another factor where it will affect the metabolism of the microbes and affect enzyme production (Paul et al., 2021). The probable reason for the important role that pH plays is that the distribution of the ion of both substrate and enzyme will determine the binding of substrate and its catalysis. Morphological changes will also be induced by the pH, as microbes are sensitive towards hydrogen ion concentration in the media (Sindhu et al., 2017). Fungi and yeasts are active at an acidic environment (Karim & Tasnim, 2018).

Fermentation needs to be performed for an optimal duration to maximise the yield of enzyme (Farooq et al., 2021), as the activity of enzymes will increase with the incubation time until the optimum duration is reached (Paul et al., 2021). On the contrary, prolonged fermentation will cause reduction in enzyme production due to the build-up of toxic materials and the depletion of nutrients (Paul et al., 2021). An optimum duration for fermentation needs to be determined before the production process is carried out as the lag

and log phase of growth differs among organisms (Karim & Tasnim, 2018). Apart from that, moisture level is another crucial factor where the optimum level of moisture varies within different microbes (Farooq et al., 2021). A low level of moisture is sufficient for fungi to grow and produce enzymes, while bacteria require a high moisture level.

The content of the nutrient media is also important in enzyme production, as the rate of substrate being metabolised will determine the product formation, where the choice of carbon source depends on the main product of fermentation (Stanbury et al., 2017). Nitrogen also plays a significant role in affecting the growth of microbes (Far et al., 2020), and it also determines enzyme production (Karim & Tasnim, 2018). In the production of α -amylase, maltose, glucose and sucrose are the most studied carbon source, while most applied organic nitrogen source are yeast extracts, soybean and peptone, as well as several types of inorganic nitrogen such as ammonium sulphate and ammonium chloride (Far et al., 2020).

2.5 Fungi from Waste

2.5.1 Background

Organic wastes are largely produced due to agroindustry (Brojanigo et al., 2020) and consumption of starchy food (Sanabria et al., 2021). For instance, Brojanigo et al. (2020) mentioned that the byproducts of rice milling consisted of unqualified rice and lignocellulosic waste of rice husk and straw were discarded by millions of tons annually. They also reported that in 2018 alone, 23% of the product produced out of 92 million tons of sweet potatoes was discarded. Food waste is also a type organic waste, where the vast production of this waste is equivalent to 3.3 billion tonnes of carbon dioxide emission (Yu et al., 2017). Yu et al. (2017) also explained the differences between food wastes, where post-consumer food wastes are heterogenous while pre-consumer wastes are homogenous. Wastes are also generated through the spoilage of food, where one of the major causes of

food spoilage is due to microbial attack that causes chemical and physical changes (Sharma & Sharma, 2024).

Generation of vast amount of organic wastes is an issue worldwide. Nevertheless, they serve as important source of potential fungi, where the feasibility of organic wastes could be proven from numerous studies working on isolating fungi from various wastes. As mentioned by Ja'afaru (2013), isolation and screening of potential fungi from natural environment is still considered important in biotechnology despite the advancement in various techniques such as genetic engineering in this field. Researchers isolating potential fungi from different wastes are also looking into the potential of these fungi for waste management due to the huge amount of organic waste produced, apart from investigating the industrial application of the fungi.

There were numerous reports on isolating indigenous fungi from wastes. A type of waste from the agricultural sector, oil palm empty fruit branches was shown to serve as a substrate for several isolates that were laccase producers (Fithri et al., 2020). Kitchen wastes consisting of pre-consumer food waste such as vegetables peeling and potato cutlets yielded fungal isolates which possess biodegrading ability from several genera such as *Aspergillus* and *Trichoderma* (Ashraf et al., 2017). Obagbemisoye (2024) worked on isolating fungi from kitchen waste water and revealed several amylolytic *Aspergillus* species such as *A. flavus*. The fungus isolated from fruit and vegetable waste had the ability to produce several important enzymes, including protease, amylase, lipase and cellulase (Suresh et al., 2021). This potential fungus displayed a high degradation rate of 95% when it was used to compost fruit and vegetable peels. All of the research showed that wastes served as an important source of fungi.

2.5.2 Amylolytic Fungi from Waste

Amylolytic fungi could be obtained from starchy organic wastes, as Sanabria et al. (2021) mentioned that these wastes still possess a considerable amount of starch that could be utilise and high carbohydrate content was also found in food waste (Taha et al., 2019). This indicated that the wastes are suitable for colonisation of amylolytic microbes and the wastes also had the potential to be reused. The ability of fungi, especially filamentous fungi, to produce enzymes, enabled these microbes to grow on these solid materials and use them as substrates (Ogbonna et al., 2014). Previous studies around the world had supported the feasibility of using organic waste as a source of fungi, where for example, Okunwaye et al. (2021) also successfully isolated various amylolytic fungi, for instance *Aspergillus* sp., *Penicillium* sp. and *Geotrichum* sp. from agro-industrial waste and food waste such as yam waste, Irish potato waste and parboiled water from noodles. Nuhu and Yusuf (2023) also successfully isolated several *Aspergillus* species from mouldy bread, while Al-Rajhi et al. (2023) isolated both members of *Aspergillus* and *Penicillium* from similar source.

2.6 Studies of Amylolytic Fungi in Local Context

Although there were many reports on amylolytic fungi from waste, it is also necessary to study the fungal diversity on local waste as climate is one of the factors that affect fungal diversity and distribution (Větrovský et al., 2019). Factors such as temperature, humidity and wind speed played an important role in fungal diversity, where for example, positive correlation had been revealed between the genus *Alternaria* and factors which are temperature and humidity (Lu et al., 2022). Fungi from varied environments may have different characteristics as rapid changes in genome organisation enables a fungus to adapt quickly to the environment (Naranjo-Ortiz & Gabaldón, 2020), and this might be reflected in their biochemical activities.

In Malaysia, amylolytic fungi were obtained from various sources. From living plants, Marlida et al. (2000) had reported that *Acremonium* sp. was isolated from forest trees while Azuddin et al. (2023) found thirty-four isolates from spines of rattan plant, where ten of them are amylolytic fungi. From the environment such as soil, *Aspergillus sydowii* and *Aspergillus versicolor* was isolated (Mohammad & Ariffin, 2020). Previously, researchers also isolated amylolytic isolates, *Rhizopus* sp. and *Geomyces* sp. from river catfish (Husin & Iberahim, 2022). However, fungi from different sources might have different amylolytic properties. Xiong et al. (2017) mentioned that fungi are capable of adjusting their metabolism based on availability of nutrients in the environment, resulting in variation of metabolic activities even in similar species when they are isolated from different sources. This might be due to horizontal gene transfer which enables fungal adaptation to novel niches, for example *Penicillium* sp. growing on cheese developed genes that favour this environment (Naranjo-Ortiz & Gabaldón, 2020).

Sarawak is the main exporter of sago starch in Malaysia, where this industry generates three main types of wastes, which are sago pith waste, bark and wastewater (Amin et al., 2019). Sago pith is a starchy lignocellulosic waste still containing 60% to 70% of starch per gram dry weight after the extraction process, where the starch granules are trapped within the lignocellulosic matrix (Singhal et al., 2008). Lai et al. (2013) also reported that the sago hampas has high moisture content of 82% by dry weight. The waste serves as a potential substrate for fermentation for production of simple sugars. Amin et al. (2019) mentioned that hydrolysates from sago pith waste yielded higher monosaccharides concentrations, especially glucose, compared to oil palm trunk waste. Sago starch manufactures used to discharge sago pith waste into rivers along with the wastewater (Lai et al., 2013), but management of this waste is currently getting more attention of researchers.

However, most studies on this waste focused on exploring the potential of this residue as a substrate for non-indigenous fungi of the waste. For example, Jalil et al., (2015) used *Rhizopus oligosporus* to enhance nutrient content of sago pith waste before utilising it as poultry feed while Vincent et al. (2015) used sago pith waste for the production of bioethanol through SSF with *Saccharomyces cerevisiae*.

Malaysian was reported to generate 0.76 kg of wastes per day, where food waste was the main waste occupying 45% of the generated waste (Adam et al., 2022). Therefore, food waste management is crucial. However, researchers working on reutilising these wastes mostly through fermentation by fungal isolates not indigenous to the sources, rather than exploring indigenous microbes on the wastes. For example, Adebayo et al. (2011) used four fungal isolates from different source around the country for production of compost through decomposing food wastes. On the other hand, Tang and Wong (2024) used rice flour-based food waste, kuih talam and kuih lapis as the substrate for yeast fermentation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials used for this study include 1 kb DNA ladder (Thermo Scientific, USA), 30% acrylamide/bis solution (Bio-Rad, USA), β -mercaptoethanol (Amresco, USA), acetic acid (J. T. Baker, USA), agarose gel (Vivantis, Malaysia), ammonium persulphate (Bendosen), ammonium sulphate (Merck, Germany), bovine serum albumin (Sigma-Aldrich, USA), chloramphenicol (Nacalai Tesque, Japan), chromatein prestained protein ladder (10.5-175 kDa) (Vivantis, Malaysia), citric acid (R & M Chemicals, Malaysia), coomassie Brilliant Blue R-250 (Bio-Rad, USA), DNS powder (Sigma Aldrich, USA), Dream Taq PCR Master Mix 2X (Thermo Fisher Scientific, USA), ethidium bromide (Promega, USA), glycine (Vivantis, Malaysia), hydrochloric acid (Nacalai Tesque, Japan), iodine (System, Malaysia), ITS1 and ITS4 primer (Integrated DNA Technologies, USA), lactophenol cotton blue solution (Merck, Germany), methanol (LOBA Chemie, India), potassium phosphate dibasic (Mallinckrodt, Mexico), potassium phosphate monobasic (Amresco, USA), potato dextrose agar (HiMedia, India), potato dextrose broth (HiMedia, India), Rochelle salt/potassium sodium tartrate (R & M Chemicals, Malaysia), SDS (Nacalai Tesque, Japan), sodium acetate (Amresco, USA), sodium citrate (BDH, UK), sodium hydroxide (LOBA Chemie, India), sodium phosphate dibasic (System, Malaysia), sodium phosphate monobasic monohydrate (Acros Organics, India), starch powder (Sigma, Canada), tetramethylethylenediamine (TEMED) (Bio Rad, USA) Tris-base (Fisher Scientific, USA), Triton X-100 (BDH, UK), Tween 80 (R & M Chemicals, Malaysia) and Vivantis GF-1 Plant DNA Extraction Kit (Vivantis, Malaysia).

3.2 Methods

3.2.1 Sample Collection and Fungal Isolation

A total of six starchy waste samples were collected, namely sago pith waste, potato, bread, banana cake, glutinous rice cake and washed rice water. Sago pith waste was obtained from the Molecular Genetic Lab (MGL), Faculty of Resource Science and Technology (FRST), Universiti Malaysia Sarawak (UNIMAS), Sarawak, Malaysia. One gram of the waste was added to 9 ml saline solution, shaken thoroughly, serially diluted to a fifth dilution to reduce microbial load and isolate specific fungal species (Ayansina et al., 2017). Potato dextrose agar (PDA) supplemented with chloramphenicol was used to isolate the fungi, where the antibiotic was added in a concentration of 50 µg/ml (Hawar, 2022) to minimise the growth of bacteria (Twumasi et al., 2014). Each dilution was plated on the agar in 0.5 ml and the plates were incubated for 5 days at 27 °C (Kumari et al., 2021).

All the other samples were obtained locally in Kuching, Sarawak. Washed rice water was the water obtained from the first rinsing of white rice, collected from household. The water was fermented for 3 days without the addition of other components for fungi proliferation as a previous study found the highest fungi population after 3 days of fermentation (Nabayi et al., 2022). Fungi were isolated from washed rice water was through serial dilution to the fifth dilution before plating. While for the potato, bread, banana cake and glutinous rice cake waste, the samples were left on PDA for the fungi to grow (Nuhu & Yusuf, 2023). Subculturing was performed to obtain pure fungal cultures from each sample.

3.2.2 Morphological Identification of Fungi

3.2.2.1 Macroscopic Identification

The colour of the colony, colour of mycelium, texture, elevation, colour of colony reverse, margin, sporulation pattern and other distinct characteristics were observed to for macroscopic identification of the fungi (Samson et al., 2014).

3.2.2.2 Microscopic Identification

The lactophenol cotton blue method was performed for microscopic identification of the isolates. A drop of lactophenol cotton blue stain was placed on a clean microscopic slide (Kumari et al., 2021). A fragment of fungal mycelia was cut from the colony with a scalpel, placed on glass slides, minced into smaller sizes, and covered with a cover slip. Fruiting bodies and structure of fungi were observed with a compound microscope. The hyphae, conidiophores, presence of vesicles, phialides and characteristics of conidia (Samson et al., 2014) were observed for species identification. Pictures of magnified fruiting bodies and hyphae were taken with a smartphone.

3.2.3 Molecular Identification of Fungi

3.2.3.1 Genomic DNA Extraction

Genomic DNA of the fungi was extracted using Vivantis GF-1 Plant DNA Extraction Kit following the protocols provided by the manufacturer. Fungal mycelium was ground in liquid nitrogen into fine powder and 280 µl of lysis buffer (Buffer PL) was added to the sample. The sample was vortexed for 30 seconds and the samples were incubated at 65 °C for 1 hour 30 minutes after 20 µl of Proteinase K was added. The sample was centrifuged at 13,000 x g for 5 minutes and the supernatant was transferred to a new microcentrifuge tube. Two volumes of Binding buffer (Buffer PB) were added based on the volume of the supernatant and the samples were then incubated at 65 °C for 10 minutes after mixing. Next,

200 µl of absolute ethanol was added and the sample was loaded into a spin column after mixing. The sample was centrifuged at 10,000 x g for 1 minute, and the flow through was discarded. The column was then washed with 650 µl of Wash Buffer and centrifuged at 10,000 x g for 1 minute. The flow through was also discarded and centrifugation was repeated to ensure complete removal of wash buffer. The extracted DNA was eluted by adding 50 µl Elution Buffer to the column, and the samples were left to stand for 2 minutes and later centrifuged at 10,000 x g for 1 minute. The extracted DNA was stored at -20 °C.

3.2.3.2 Polymerase Chain Reaction

The extracted DNA was used as the DNA template for PCR. ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') primer was used as the primer pair for the PCR (Jainlabdin et al., 2018). Components of PCR mixture were 2X PCR Master Mix, ITS1 forward primer, ITS4 reverse primer, nuclease-free water and DNA template. The volume of PCR reaction mixture was 20 µl and volume of each component was added according to Takam et al. (2019), as shown in Appendix B. Polymerase chain reaction was performed with a thermal cycler, with protocols adapted and modified from Scibetta et al. (2020), shown in Appendix C.

3.2.3.3 Agarose Gel Electrophoresis

After PCR, the amplified products were resolved using agarose gel electrophoresis. Agarose gel with concentration of 1.0% (Siddique et al., 2022) and containing ethidium bromide was prepared for electrophoresis under 90 V for 25 minutes. The gel was viewed under UV transilluminator, and the sizes of the amplicon will be estimated by comparing with the 1 kb DNA ladder (Douksouna et al., 2019).

3.2.3.4 DNA Sequencing

The product of PCR was sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing and identification of fungi species was performed through the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST).

3.2.4 Screening for Amylase Production

The fungal isolates were studied for their ability to produce amylase. The solid-based method, where screening was performed on nutrient agar (Gopinath et al., 2017), was applied to screen for amylase production. Starch agar was prepared by adding 2% soluble starch to potato dextrose agar (Famotemi et al., 2015; Swamy & Pratima, 2022). The isolated species was cultured on starch agar by inoculating spores scraped off from 5-day-old stock cultures. The inoculated plates were incubated for 5 days at 27 °C (Ekedegba et al., 2022). After the incubation period, 10 ml of 1% iodine solution was poured onto the agar plates for staining. Iodine solution was allowed to react for 10 minutes (Das et al., 2020). Excess iodine solution was discarded and the formation of clear zones, which is the indication of amylase production was observed (Das et al., 2020). Screening assays were performed in duplicates.

3.2.5 Amylase Activity of Fungi through Solid-Based Method

Amylase-producing species were studied to compare their amylase activity. The spores from stock cultures of each species were inoculated on 2% starch agar through touching the inoculation loop once on the spores or mycelia and transferring the spores or mycelia to the agar. Inoculation was performed in triplicates for each species, and the fungi were incubated for 5 days at 27 °C (Ekedegba et al., 2022). The staining procedure was performed as described in 3.3.4 before discarding excess iodine solution (Das et al., 2020). The diameter of the fungus colony prior to the staining and the halo zone formed after

staining on each plate were measured both horizontally and vertically. The enzymatic activity index (EAI) was calculated as the ratio of the fungus colony diameter to the halo zone diameter (Goldbeck et al., 2012), as shown in Equation 3.1. Highest amylase producers were selected based on the index.

$$\text{Enzymatic activity index} = \frac{\text{Diameter of halo zone (cm)}}{\text{Diameter of colony (cm)}} \quad \text{Equation 3.1}$$

3.2.6 Amylase Production through Submerged Fermentation

For subsequent study, submerged fermentation (SmF) was applied as the approach of producing amylase from selected fungi. Spore suspensions were prepared as inoculum for SmF. Tween 80 solution was added to sterile distilled water to achieve a concentration of 0.01% (Hashimoto et al., 2022) and the solution was sterilised with 0.45 µm syringe filter. The selected fungi were cultured on PDA at 27 °C for 5 days (Bellaouchi et al., 2021) and 10 ml of 0.01% Tween 80 was poured onto the plates (Sala et al., 2020). The spores were dislodged, and suspensions were collected for spores counting with an improved Neubauer haemocytometer under light microscope of 400X magnification (Awan et al., 2021). All suspensions were diluted to a concentration of 6.5×10^5 spores/ml where all liquid cultures were inoculated with 10 ml/l spore suspension of this concentration (Shahryari et al., 2019). Submerged fermentation was performed with potato dextrose broth (PDB) supplemented with 2% soluble starch (Veerana et al., 2022) in a volume of 50 ml for 5 days at 27 °C (Karim et al., 2018) on rotary shakers with 140 rpm (Peraza-Jiménez et al., 2021).

3.2.7 Amylase Assay

Amylase assay was carried out through the DNS (3,5-dinitrosalicylic acid) method, with preparation described by Lam et al. (2021) and shown in Appendix D. To prepare the DNS reagent, 1 g of DNS was added in 20 ml of 2.0 N sodium hydroxide solution and 30 g

of potassium sodium tartrate (Rochelle salt) was added in 50 ml of distilled water. The solution was mixed and diluted to 100 ml (Lam et al., 2021). The DNS method was adapted from the protocols of Nisa et al. (2020) with slight modification. Half a millilitre of crude amylase was added to 0.5 ml of 1% starch in 0.2 M phosphate buffer solution (pH 7.0) and the solution was incubated for 10 minutes at 40 °C. The DNS solution was added in 2 ml to stop the reaction after incubation before boiling the solution for 5 minutes. The final volume was adjusted to 10 ml by adding distilled water after the solution cooled down. The absorbance was measured at 540 nm with a spectrophotometer after the solution cooled. Amylase activity was defined as the amount of enzyme that releases 1 µmol of glucose per minute and calculated by the formula shown in Equation 3.2.

$$\text{Amylase activity (IU/ml)} = \frac{\text{Glucose concentration} \times \text{reaction volume (enzyme+substrate)}}{\text{MW of glucose} \times \text{reaction time} \times \text{volume of enzyme}} \quad \text{Equation 3.2}$$

A standard curve was prepared for the amylase assay according to methods described by Nisa et al. (2020) with slight modifications. Glucose stock solution of concentration 10 mg/ml was diluted into concentrations ranging from 100 to 1000 µg/ml with the addition of distilled water. DNS reagent was added in 1 ml to 1 ml of the glucose solution and the solution was boiled for 5 minutes before the addition of 1 ml 40% Rochelle salt. The final volume of the solution was also adjusted to 10 ml by adding distilled water to the solution after it had cooled down. The absorbance was read at 540 nm with a spectrophotometer. The glucose standard curve plotted was shown in Appendix E.

3.2.8 Optimisation of Amylase Production in Selected Species

Species of *Penicillium* and *Aspergillus* were targeted for further studies, with two highest amylase producers from each genus were selected. The selected species were *Penicillium* sp. BB3, *P. citrinum* KBA4, *A. europaeus* BNN1 and *A. niger* KBA2.

Optimisation studies were performed using the one-factor-at-a-time approach (Batista et al., 2021) with triplicates, focusing on the optimisation of pH and incubation period for amylase production via SmF. The effect of pH on amylase production was investigated from the range of pH 3 to pH 8 (Bellaouchi et al., 2021), while fermentation was carried out for 5 days at 27 °C and shaking of 140 rpm. The pH of fermentation broth was adjusted using 1N HCl and 1N NaOH (Abdulsalam, 2022). The effect of incubation time on amylase production was studied with the incubation period range described by Naher et al. (2021) with slight modification, where fermentation was carried out for 3, 5, 7 and 9 days. Fermentation was performed with PDB supplemented with 2% soluble starch as the medium (Veerana et al., 2022), under 27 °C for 5 days with shaking at 140 rpm and pH of the medium was maintained at pH 6.9 for study of incubation period (Ire et al., 2020).

After fermentation, the growth of fungi under different parameters were investigated through examining the fungal biomass. Fungal mycelia were removed with filter paper (Whatman No. 1) and the mycelia were washed with distilled water (Ire et al., 2020). The mycelia were dried in an oven until constant mass before determining the weight (Ire et al., 2020). Crude amylase was obtained through centrifugation with 10,000 x g and was used for amylase assay (Adejuwon & Tsygankova, 2019; Iqbalsyah et al., 2018).

3.2.9 Partial Purification of Amylase

3.2.9.1 Ammonium Sulphate Precipitation

Two species that produced the highest amylase during optimisation studies, *A. niger* KBA2 and *Penicillium* sp. BB3, were selected for amylase characterisation. Submerged fermentation (SmF) was used for amylase production from the selected species with spore suspension as the inoculum as described in Section 3.2.6. Submerged fermentation was conducted with PDB medium supplemented with starch in a volume of 50 ml at 27 °C on

rotary shakers with 140 rpm under respective optimum pH and incubation period of the fungi (Karim et al., 2018; Peraza-Jiménez et al., 2021). Crude amylase was obtained through filtering the fungal mycelia with filter paper, followed by centrifugation at 10,000 x g (Adejuwon & Tsygankova, 2019; Iqbalsyah et al., 2018).

Ammonium sulphate precipitation was used to partial purify the amylase where ammonium sulphate was sequentially added to the crude amylase in concentrations of 30%, 50% and 80% (Karim et al., 2018). The solution was left for 1 hour on ice with continuous stirring (Kalia et al., 2021) and was later centrifuged at 10,000 x g at 4 °C for 10 minutes (Ahmed et al., 2020; Iqbalsyah et al., 2018). The pellets obtained after each concentration were dissolved in 50 mM phosphate buffer (pH 7) (Salem et al., 2021), and ammonium sulphate salt was removed with spin columns (Amicon Ultra-15 Centrifugal Filter Units, MWCO 10 kDa) as described by Sidar et al. (2023). Centrifugation was performed at 5,000 x g for 20 minutes at 4 °C as recommended by the manufacturer and desalting was repeated twice. The partial purified protein was and stored at 4 °C (Salem et al., 2021). Amylase assay and Bradford assay were performed to determine amylase activity and protein content.

3.2.9.2 Bradford Assay

Protein concentration of the samples was determined using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as the protein standard where the protein solution was prepared in a concentration from 0 µg/ml to 100 µg/ml. A volume of 100 µl from each concentration was pipetted into test tubes and Bradford reagent was added in 1 ml to each test tubes before mixing through inversion (Taghipour et al., 2018). The solution was left for 10 minutes for the colour to develop before reading the absorbance at 595 nm. Absorbance of each concentration was determined through subtracting the absorbance of a blank and a standard curve of absorbance against protein concentration was

plotted. Assay of partially purified amylase was similar with protocols of the standard. The plotted protein standard curve is shown in Appendix F.

3.2.9.3 SDS-PAGE

The molecular weight of the purified protein was determined through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970) and Gallagher (2012). A stacking gel of 5% and resolving gel of 12% was used, with the composition shown in Appendix G and H. The running buffer was consisting of Tris-base, glycine and SDS, with the exact amount for 1X buffer shown in Appendix I. The sample buffer consisted of Tris-HCl buffer, SDS, glycerol and bromophenol blue dye as shown in Appendix J. Sample buffer was added in 9.5 μ l in a PCR tube, followed by the addition of 0.5 μ l β -mercaptoethanol for a final concentration of 335 mM and 5 μ l of sample. The samples were heated for 4 minutes at 95 °C on a heating block and electrophoresis was carried out until the bromophenol blue marker reached the bottom of the gel. Electrophoresis was conducted under 100V for 15 minutes for protein stacking process and 200V for 45 minutes for resolving the protein. After electrophoresis, the gel was stained by 0.1% Coomassie brilliant blue solution overnight, where the staining solution was prepared according to Appendix K. The gel was later repeatedly washed with destaining solution. Ethanol, glacial acetic acid and distilled water was added in 300 ml, 100 ml and 600 ml respectively to prepare 1 l of destaining solution. Protein bands were later viewed and sizes of the partial purified amylase were compared with the protein ladder. Pictures were taken with a smartphone.

3.2.9.4 Native-PAGE

Native-PAGE was performed through protocols described by Karim et al. (2018). The process was similar to SDS-PAGE, with the exception of addition of β -mercaptoethanol

in sample buffer and sample heating. Sodium dodecyl sulphate was not added in the running buffer, and the running buffer of Native-PAGE was prepared with components shown in Appendix L. Electrophoresis was performed at 120V for 1 hour 30 minutes. After electrophoresis, SDS in the gel was removed by washing the gel with 0.2% Triton X-100 prepared in sodium acetate buffer (0.1 M, pH 5). The gel was then incubated in sodium acetate buffer (0.1M, pH 5) containing 1% soluble starch for 30 minutes at 50 °C. Iodine solution was used to stain the gel after incubation and the gel was observed for the formation of clear bands on a dark bluish-black background which indicated presence of amylolytic activity.

3.2.10 Characterisation of Partial Purified Amylase

3.2.10.1 Effect of Temperature on Amylase Activity

Amylase assay with various incubating temperatures was conducted to determine optimum temperature for the partially purified amylase. The experimented temperature ranged from 30 °C to 90 °C (Karim et al., 2018). Substrate concentration, pH and incubation period was maintained at 1%, pH 7 and 10 minutes, respectively. The alpha-amylase assay was performed in triplicate to evaluate enzyme activity.

3.2.10.2 Effect of pH on Amylase Activity

The effect of pH on amylase activity was investigated through standard amylase assay. Reaction mixtures were prepared with different pH ranging from pH 3 to 9. The buffer systems used included: 0.1 M sodium citrate buffer (pH 3), 0.1 M sodium acetate buffer (pH 4 and 5), 0.1 M potassium phosphate buffer (pH 6 and 7) and 0.1 M Tris-HCl buffer (pH 8 and 9) (Karim et al., 2018). Substrate concentration, temperature and incubation period was maintained at 1%, 40 °C and 10 minutes respectively. Amylase assay was performed in

triplicates to ensure accuracy. The buffers were prepared according to components shown in Appendix M.

3.2.11 Statistical Analysis

Statistical analysis was conducted using IBM SPSS software. One-Way ANOVA was used to compare mean enzyme activities, followed by Tukey's post hoc test to identify significant differences. The General Linear Model (Multivariate) was applied to evaluate interaction between an independent factor (pH, temperature) and multiple dependent variables (amylase production and fungal growth).

CHAPTER 4

RESULTS

4.1 Fungal Isolation and Identification

Forty-one fungal isolates were isolated from six starchy waste. Sago pith waste yielded eight isolates, washed rice water yielded eleven, potato yielded seven, eight isolates were from bread, while glutinous rice cake and banana cake recorded four and three isolates respectively. Macroscopic and microscopic characteristics of the isolates, observed as mentioned by Samson et al. (2014) are presented in Table 4.1 and Table 4.2, while Appendix N presents the images of all the isolates.

Table 4.1: Macroscopic observation of fungal isolates

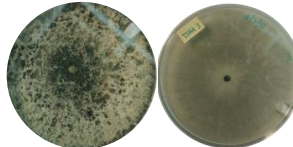
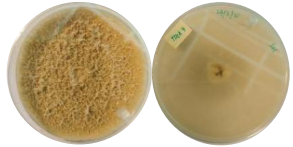
Isolate	Characteristics						
	Colony Colour	Reverse Colour	Texture	Elevation	Margin	Sporulation Pattern	Distinct Characteristics
TSHA1  F R	Black	Black	Smooth	Flat	-	-	Fruity smell
TSHA2 / RWA1 / RWB2  F R	Green and white	Grey	Floccose	Crateriform	-	-	-
TSHA3 / TSHB2  F R	Yellowish brown	Creamy	Floccose	Flat	-	-	-
TSHB1  F R	Brown	Brown	Floccose	Flat	White	-	Layer of white mycelium covering the colony

Table 4.1 continued

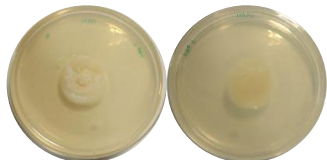
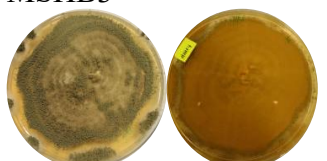
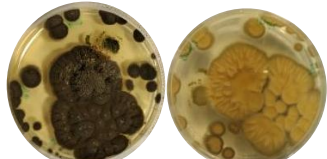
TSHB3 / RWA2 / RWB7  F R	White and light pink	Orange with dark orange rings	Granular	Flat	White	Sporulation rings in green	Mycelium rings and black centre present
MSHB2  F R	White	Creamy with white margin	Smooth	Umbonate	-	-	-
MSHB3  F R	Green	Yellowish orange	Granular	Umbonate	-	Sporulation rings in green	White mycelium
RWA3 / PA2 / PB4 / BA5 KBA4 / BNN3  F R	Dark green	Yellowish creamy	Velvety	Umbonate	-	-	Sulcations on the mycelium

Table 4.1 continued

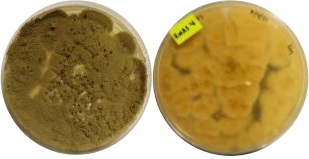
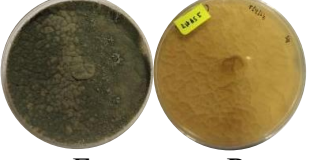
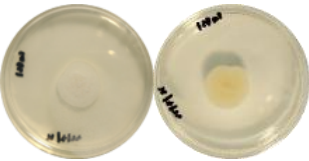
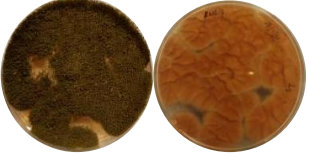
RWA4  F R	Olive-green	Yellowish creamy	Granular	Umbonate	-	-	Sulcations on the mycelium
RWA5 / RWB5 / BA3  F R	Greyish green	Creamy	Powdery	Flat	-	-	Sulcations on the mycelium
RWB1 / PA3 / BA1 / BB1  F R	White	Creamy	Smooth	Flat	-	-	-
RWB4  F R	Dark green	Reddish orange	Granular	Umbonate	-	-	Sulcations on the mycelium

Table 4.1 continued

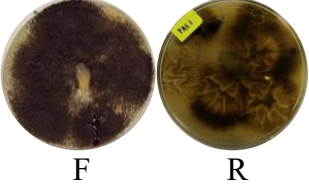
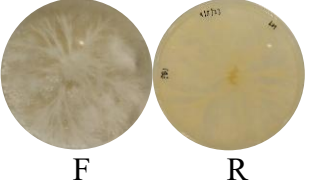
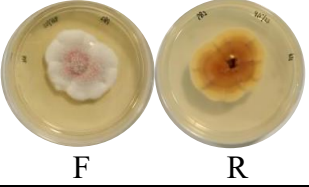
RWB8  F R	Black	Creamy	Granular	Umbonate	White	-	Creamy reverse blackens as colony develops and sulcations on the mycelium
PA1 / PB3 / BA4 / KBA2 BNN2  F R	Black	Creamy	Granular	Umbonate	-	-	Creamy reverse blackens as colony develops
PB1  F R	White	Creamy	Cottony	Flat	-	-	Aerial hyphae and sclerotia present
PB2  F R	Light pink	Orange with black centre	Cottony	Umbonate	White	-	Sulcations on the mycelium

Table 4.1 continued

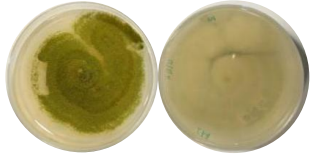
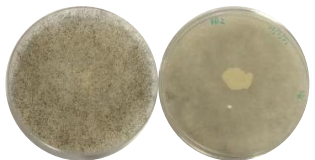
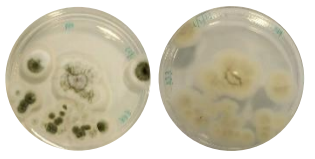
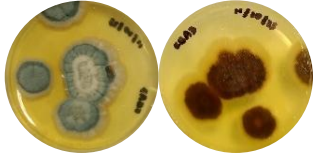
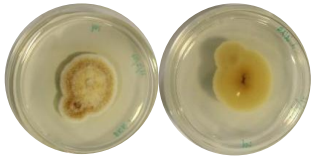
BA2  F R	Green	Creamy	Granular	Umbonate	-	Sporulation rings in green	-
BB2  F R	Grey	Greyish white	Woolly	Aerial growth	-	-	Fast growing and sporangia found among mycelium
BB3  F R	Greyish green	Creamy	Velvety	Flat	White	-	Sulcations on the mycelium
KBA1  F R	Light brown	Yellowish orange	Floccose	Raised	White	-	Sulcations on the mycelium

Table 4.1 continued

KBA3  F R	Greyish green	Reddish brown	Velvety	Flat	White	-	Sulcations on the mycelium
BNN1  F R	Brownish yellow	Yellowish creamy with dark centre	Cottony	Convex	White	-	-

Note: F= Front view of colony, R= Reverse view of colony

Table 4.2: Microscopic observation of fungal isolates

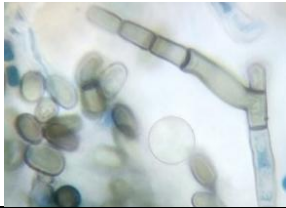
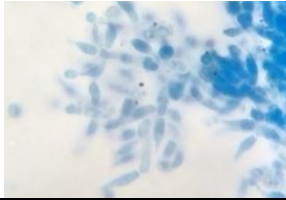
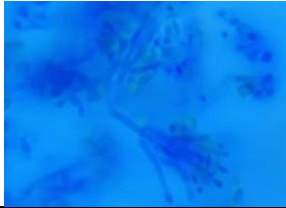
Isolate	Characteristics				
	Hyphae	Conidiophore Structure	Phialides	Conidia	Distinct Structures
TSHA1 	Septate	-	Arising from hyphae	Oval and barrel shaped aleurioconidia	Conidia formed in chains
TSHA2 / RWA1 / RWB2 	Septate	Tree-like and paired branching	Ampuliform	Round	-
TSHA3 / TSHB2 	Septate	Irregular branching	Brush-like	Ellipsoidal	-

Table 4.2 continued

TSHB1 	Septate	Monophialidic	Adelophialides and ampulliform phialides present	Subglobose and short	-
TSHB3 / RWA2 / RWB7 	Septate	Biverticillate branching	Brush-like	Subglobose	-
MSHB2 	Septate	-	Sporothrix-like	Round	-
MSHB3 	Septate	Round vesicle	Covering entire vesicle	Green and round	Hyaline conidiophore stalk

Table 4.2 continued

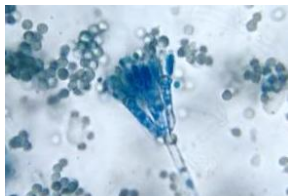
RWA3 / PA2 / PB4 / BA5 KBA4 / BNN3 	Septate	Biverticillate branching	Brush-like	Round	-
RWA4 	Septate	Round vesicle	Covering entire vesicle	Green and round	Hyaline conidiophore stalk
RWA5 / RWB5 / BA3 	Septate	Pear-shaped vesicle	Covering two-third of the vesicle	Green and round	Hyaline conidiophore stalk
RWB1 / PA3 / BA1 / BB1 	Septate	-	-	Barrel-shaped arthroconidia bud from corner of cells and	Dichotomous branching of the hyphae

Table 4.2 continued

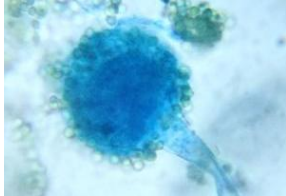
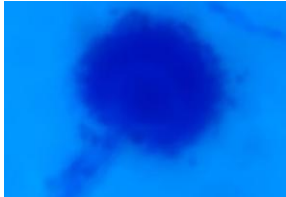
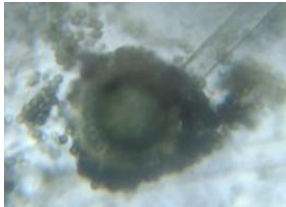
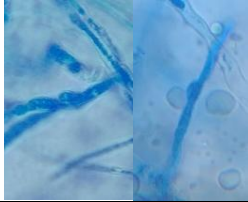
RWB4 	Septate	Round vesicle	Covering entire vesicle	Green and round	Hyaline conidiophore stalk
RWB8 	Septate	Round vesicle	Covering entire vesicle	Black and round	Hyaline conidiophore stalk
PA1 / PB3 / BA4 / KBA2 BNN2 	Septate	Round vesicle	Covering entire vesicle	Black and round	Hyaline conidiophore stalk
PB1 	Septate	-	-	-	Clamp connections in the hyphae

Table 4.2 continued

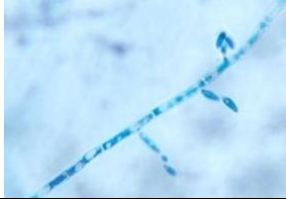
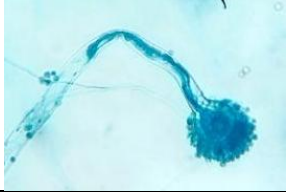
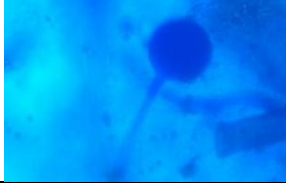
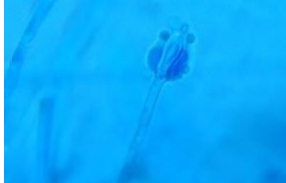
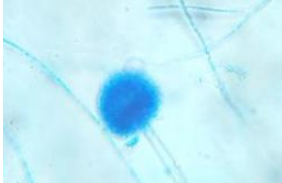
PB2 	Septate	-	Short phialides	Elongated microconidia and form in chains	-
BA2 	Septate	Round vesicle	Covering entire vesicle	Green and round	Hyaline conidiophore stalk
BB2 	Aseptate	-	-	Sporangiospores are formed	Columella observed and extended to sporangia
BB3 	Septate	Monoverticillate branching	Brush-like	Round conidia	-

Table 4.2 continued

KBA1 	Septate	Round vesicle	Covering entire vesicle	Round	Hyaline conidiophore stalk
KBA3 	Septate	Tertverticillate branching	Brush-like	Round conidia	-
BNN1 	Septate	Round vesicle	Covering entire vesicle	Round	Hyaline conidiophore stalk

Genomic DNA from all isolates were extracted and PCR was performed before sequencing. Identification of fungal species using Internal Transcribed Spacers (ITS) gene sequencing and BLAST analysis revealed a total of twenty-three species from twelve genera among the forty-one isolates. Nine species were identified from the genus *Aspergillus*, while three belonged to *Penicillium*. Additionally, one species each was identified from the genera *Trichoderma*, *Geotrichum*, *Talaromyces*, *Paecilomyces*, *Ceratocystis*, *Pleurostoma*, *Ophiostoma*, *Agroathelia*, *Fusarium*, and *Rhizopus*. Fungal identity and their GenBank accession numbers are shown in Table 4.3.

Table 4.3: Molecular identification of fungal isolates

Source	Code	Identity of Fungi	GenBank Accession Number	Sequence Similarity (%)
Sago Pith Waste	TSHA1	<i>Thielaviopsis paradoxa</i>	PV562478	99.40
	TSHA2	<i>Trichoderma asperellum</i>	PV596148	100.00
	TSHA3	<i>Paecilomyces maximus</i>	PV562474	96.65
	TSHB1	<i>Pleurostoma richardsiae</i>	PV598161	93.76
	TSHB2	<i>Paecilomyces maximus</i>	PV562475	100.00
	TSHB3	<i>Talaromyces siamensis</i>	PV596152	99.63
	MSHB2	<i>Ophiostoma valdivianum</i>	PV598160	86.54
	MSHB3	<i>Aspergillus nomiae</i>	PV598163	100.00
Washed Rice Water	RWA1	<i>Trichoderma asperellum</i>	PV596150	100.00
	RWA2	<i>Talaromyces siamensis</i>	PV596151	99.63
	RWA3	<i>Penicillium citrinum</i>	PV596162	98.43
	RWA4	<i>Aspergillus flavus</i>	PV598168	99.82

Table 4.3 continued

Washed Rice Water	RWA5	<i>Aspergillus fumigatus</i>	PV596155	100.00
	RWB1	<i>Geotrichum candidum</i>	PV554880	95.77
	RWB2	<i>Trichoderma asperellum</i>	PV596149	99.82
	RWB4	<i>Aspergillus oryzae</i>	PV598158	95.27
	RWB5	<i>Aspergillus fumigatus</i>	PV596154	99.64
	RWB7	<i>Talaromyces siamensis</i>	PV596153	99.63
	RWB8	<i>Aspergillus tubingensis</i>	PV562477	99.82
Potato	PA1	<i>Aspergillus niger</i>	PV596159	100.00
	PA2	<i>Penicillium citrinum</i>	PV596167	97.67
	PA3	<i>Geotrichum candidum</i>	PV554879	97.20
	PB1	<i>Agroathelia delphinii</i>	PV598165	92.19
	PB2	<i>Fusarium verticillioides</i>	PV598159	95.79
	PB3	<i>Aspergillus niger</i>	PV596161	99.26
	PB4	<i>Penicillium citrinum</i>	PV596164	100.00
Bread	BA1	<i>Geotrichum silvicola</i>	PV562473	99.10
	BA2	<i>Aspergillus</i> sp.	PV598167	100.00
	BA3	<i>Aspergillus fumigatus</i>	PV596156	99.64
	BA4	<i>Aspergillus niger</i>	PV596158	99.82
	BA5	<i>Penicillium citrinum</i>	PV596166	99.42
	BB1	<i>Geotrichum candidum</i>	PV554881	98.52
	BB2	<i>Rhizopus arrhizus</i>	PV562476	100.00
	BB3	<i>Penicillium</i> sp.	PV598166	99.82

Table 4.3 continued

Glutinous Rice Cake	KBA1	<i>Aspergillus flocculosus</i>	PV598157	99.83
	KBA2	<i>Aspergillus niger</i>	PV596160	100.00
	KBA3	<i>Penicilium expansum</i>	PV598164	99.82
	KBA4	<i>Penicillium citrinum</i>	PV596163	99.61
Banana Cake	BNN1	<i>Aspergillus europaeus</i>	PV598162	100.00
	BNN2	<i>Aspergillus niger</i>	PV596157	100.00
	BNN3	<i>Penicillium citrinum</i>	PV596165	100.00

The genus *Aspergillus* was the most prevalent and diverse, with a total of fifteen out of forty-one isolates (36.59%) and comprised of nine different species. The next most abundant was *Penicillium*, with eight out of forty-one isolates (19.51%) with three species. The prevalence of fungi species across six sources is displayed in Table 4.4. *Penicillium citrinum* was the most prevalent species, found in five sources except sago pith waste. This is followed by *Aspergillus niger* found in four sources, except from sago pith waste and washed rice water. Sago pith waste exhibited the highest diversity of isolates, with a total of six genera, followed by washed rice water and potato, both yielded isolates from five different genera. Bread samples included isolates from four genera, while isolates from glutinous rice cake and banana cake are sole members of *Aspergillus* and *Penicillium*.

Table 4.4: Prevalence of fungal species across six starchy wastes

Fungi Isolates	Source						Total
	SH	RW	P	B	RC	BC	
<i>Aspergillus nomiae</i>	1	-	-	-	-	-	1
<i>Aspergillus flavus</i>	-	1	-	-	-	-	1
<i>Aspergillus fumigatus</i>	-	2	-	1	-	-	3
<i>Aspergillus oryzae</i>	-	1	-	-	-	-	1
<i>Aspergillus tubingensis</i>	-	1	-	-	-	-	1
<i>Aspergillus niger</i>	-	-	2	1	1	1	5
<i>Aspergillus flocculosus</i>	-	-	-	-	1	-	1
<i>Aspergillus europaeus</i>	-	-	-	-	-	1	1
<i>Aspergillus</i> sp.	-	-	-	1	-	-	1
<i>Penicillium citrinum</i>	-	1	2	1	1	1	6
<i>Penicillium expansum</i>	-	-	-	-	1	-	1
<i>Penicillium</i> sp.	-	-	-	1	-	-	1
<i>Trichoderma asperellum</i>	1	2	-	-	-	-	3
<i>Geotrichum silvicola</i>	-	-	-	1	-	-	1
<i>Geotrichum candidum</i>	-	1	1	1	-	-	3
<i>Talaromyces siamensis</i>	1	2	-	-	-	-	3
<i>Paecilomyces maximus</i>	2	-	-	-	-	-	2
<i>Thielaviopsis paradoxa</i>	1	-	-	-	-	-	1
<i>Pleurostoma richardsiae</i>	1	-	-	-	-	-	1
<i>Ophiostoma valdivianum</i>	1	-	-	-	-	-	1

Table 4.4 continued

<i>Agroathelia delphinii</i>	-	-	1	-	-	-	1
<i>Fusarium verticillioides</i>	-	-	1	-	-	-	1
<i>Rhizopus arrhizus</i>	-	-	-	1	-	-	1
Total	8	11	7	8	4	3	41

Note: SH= Sago pith waste, RW= Washed Rice water, P= Potato, B= Bread, RC= Glutinous rice cake, BC= Banana cake, (-) = Absent

4.2 Screening for Amylase Production

All isolates were able to grow on starch agar and screening for amylolytic activity through iodine staining revealed a total of thirty-four isolates out of forty-one isolates (82.93%) produced amylase (Table 4.5). Clear zones are formed by the amylase producer when iodine was poured and spread gently over the fungal mycelia, as shown in Appendix O. All isolates derived from glutinous rice cake and banana cake were amylase producers, while bread waste yielded the most isolates without amylase activity. The isolates that did not produce amylase were *Thielaviopsis paradoxa*, *Geotrichum candidum*, *Fusarium verticillioides*, *Geotrichum silvicola* and *Rhizopus arrhizus*.

Table 4.5: Results of amylase screening in fungal isolated from starchy waste

Source	Isolate Code	Species	Growth on Starch Agar	Amylase Production
Sago Pith Waste	TSHA1	<i>Thielaviopsis paradoxa</i>	+	-
	TSHA2	<i>Trichoderma asperellum</i>	+	+
	TSHA3	<i>Paecilomyces maximus</i>	+	+

Table 4.5 continued

Sago Pith Waste	TSHB1	<i>Pleurostoma richardsiae</i>	+	+
	TSHB2	<i>Paecilomyces maximus</i>	+	+
	TSHB3	<i>Talaromyces siamensis</i>	+	+
	MSHB2	<i>Ophiostoma valdivianum</i>	+	+
	MSHB3	<i>Aspergillus nomiae</i>	+	+
Washed Rice Water	RWA1	<i>Trichoderma asperellum</i>	+	+
	RWA2	<i>Talaromyces siamensis</i>	+	+
	RWA3	<i>Penicillium citrinum</i>	+	+
	RWA4	<i>Aspergillus flavus</i>	+	+
	RWA5	<i>Aspergillus fumigatus</i>	+	+
	RWB1	<i>Geotrichum candidum</i>	+	-
	RWB2	<i>Trichoderma asperellum</i>	+	+
	RWB4	<i>Aspergillus oryzae</i>	+	+
	RWB5	<i>Aspergillus fumigatus</i>	+	+
	RWB7	<i>Talaromyces siamensis</i>	+	+
	RWB8	<i>Aspergillus tubingensis</i>	+	+
Potato	PA1	<i>Aspergillus niger</i>	+	+
	PA2	<i>Penicillium citrinum</i>	+	+
	PA3	<i>Geotrichum candidum</i>	+	-
	PB1	<i>Agroathelia delphinii</i>	+	+
	PB2	<i>Fusarium verticillioides</i>	+	-
	PB3	<i>Aspergillus niger</i>	+	+

Table 4.5 continued

Potato	PB4	<i>Penicillium citrinum</i>	+	+
Bread	BA1	<i>Geotrichum silvicola</i>	+	-
	BA2	<i>Aspergillus</i> sp.	+	+
	BA3	<i>Aspergillus fumigatus</i>	+	+
	BA4	<i>Aspergillus niger</i>	+	+
	BA5	<i>Penicillium citrinum</i>	+	+
	BB1	<i>Geotrichum candidum</i>	+	-
	BB2	<i>Rhizopus arrhizus</i>	+	-
	BB3	<i>Penicillium</i> sp.	+	+
Glutinous Rice Cake	KBA1	<i>Aspergillus flocculosus</i>	+	+
	KBA2	<i>Aspergillus niger</i>	+	+
	KBA3	<i>Penicilium expansum</i>	+	+
	KBA4	<i>Penicillium citrinum</i>	+	+
Banana Cake	BNN1	<i>Aspergillus europaeus</i>	+	+
	BNN2	<i>Aspergillus niger</i>	+	+
	BNN3	<i>Penicillium citrinum</i>	+	+

Note: (+) = Presence of fungal growth/positive result of amylase production, (-) = Absence of fungal growth/negative result of amylase production

4.3 Amylase Activity of Fungi through Solid-Based Method

Amylase activity was investigated through solid-based methods, revealing variation in amylase production among amylase producers, as shown in Figure 4.1. Among the amylase producers, *Penicillium sp.* BB3 isolated from bread was found to have the highest Enzymatic Activity Index (EAI) of 1.45 ± 0.10 , followed by *P. citrinum* KBA4 (1.36 ± 0.02). All *Penicillium* species demonstrated strong amylase production ability in the solid-based study on starch agar, with EAI ranging from 0.99 ± 0.05 (*P. expansum*) to 1.45 ± 0.10 (*Penicillium sp.*). *Aspergillus* species also showed good results with seven isolates having EAI exceeding 1.00. The highest amylase activity was observed in *A. europaeus* BNN1 (1.19 ± 0.04), followed by *A. niger* KBA2 isolated from glutinous rice cake (1.16 ± 0.02). Statistical analysis using One-Way ANOVA at a 95% level of confidence showed significant differences among the amylase activity of isolates. Activity of strain BB3 was found to be significantly different from most isolates, except for several *P. citrinum* isolates ($p \leq 0.05$; Tukey HSD). Tabular results of the study of amylase activity and One Way ANOVA analysis are shown in Appendix P.

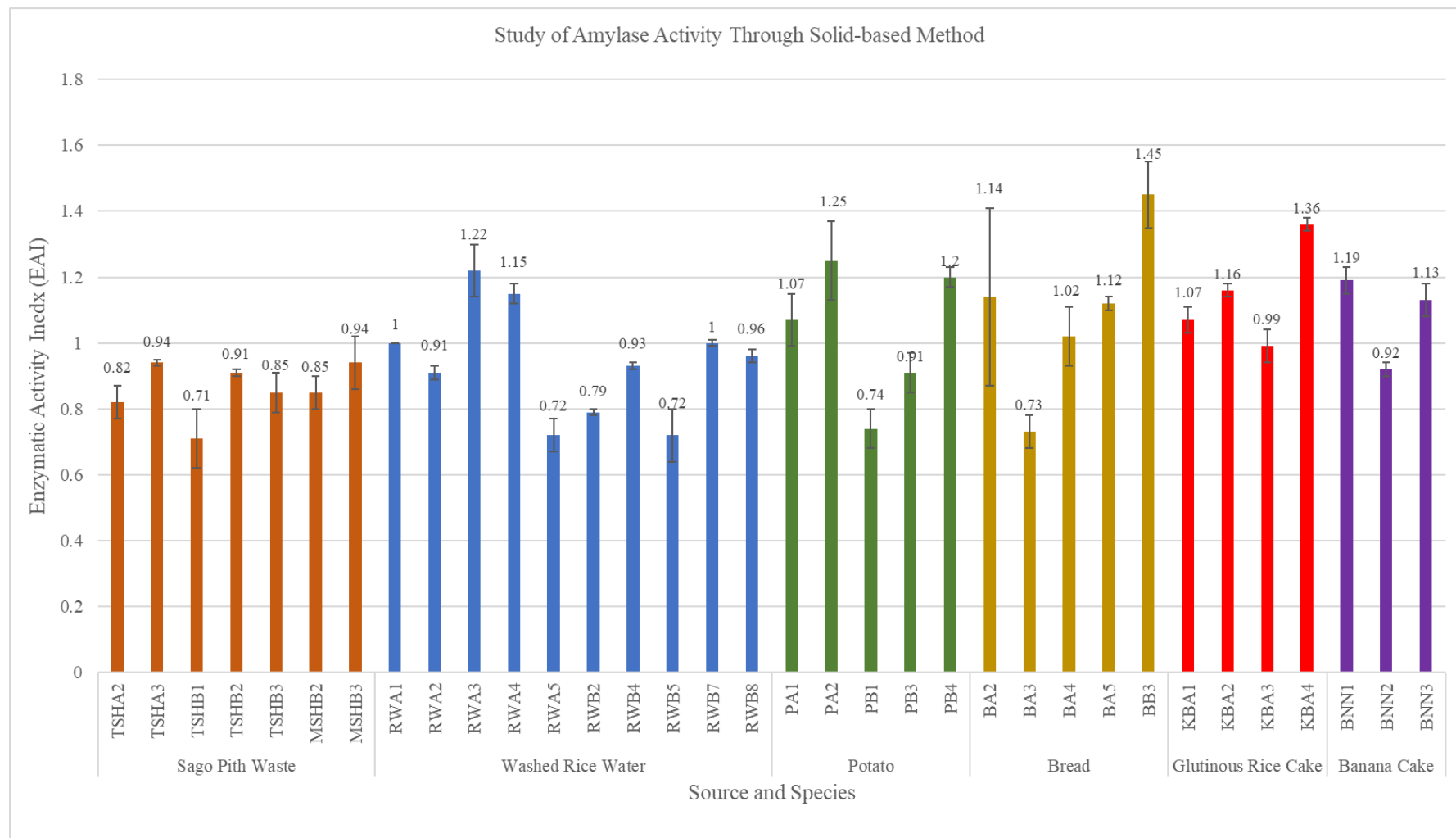


Figure 4.1: Study of amylolytic activity of isolates through solid-based method

4.4 Optimisation of Amylase Production in Selected Species

4.4.1 Optimisation of pH

The *Aspergillus* and *Penicillium* genera were selected for further studies. *Penicillium* sp. BB3, *P. citrinum* KBA4, *A. europaeus* BNN1 and *A. niger* KBA2 were selected for the optimisation studies as the isolates showed highest amylase production. It was found that the optimum pH and fermentation period for amylase production differed across the species, where results of statistical analysis were presented in Appendix Q and R. Amylase production of strain BB3 was significantly higher with an activity of 1.11 ± 0.01 IU/mL when cultured under a neutral pH (pH 7) compared to other pH except pH 4 ($p \leq 0.05$; Tukey HSD) as shown in Figure 4.2. Fungal biomass showed a steady increase with rising pH, reaching the peak of 0.54 ± 0.03 g at pH 8. On the other hand, amylase production of strain KBA4 increased with the pH and peaked at pH 5 with a significantly highest activity of 0.59 ± 0.03 IU/ml ($p \leq 0.05$; Tukey HSD). Although enzyme activity decreased at pH 6, it rose again at the subsequent pH before declining at higher pH values. The growth of this fungus generally followed a bell-shaped curve, where strain KBA4 grew best at pH 5, similar to its amylase production level, yielding a mass of 0.58 ± 0.01 g, as shown in Figure 4.3.

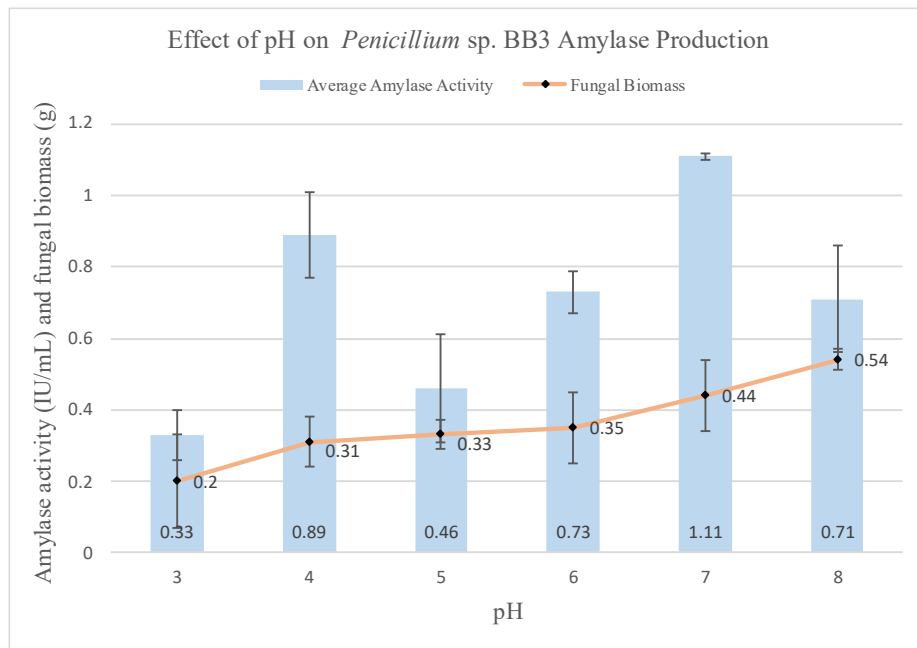


Figure 4.2: Effect of pH on amylase production and growth of *Penicillium* sp. BB3, with error bars showing standard deviation among triplicates

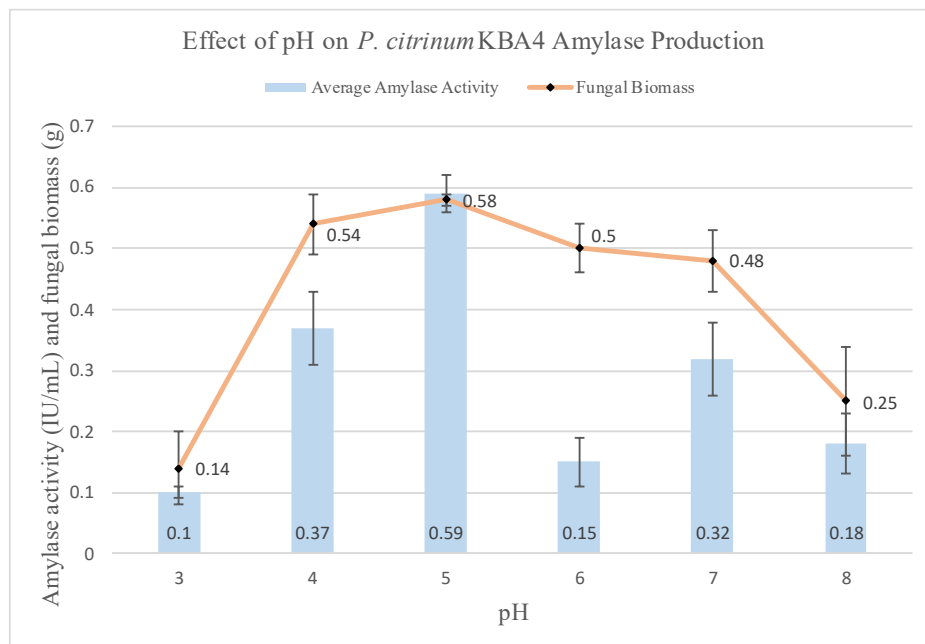


Figure 4.3: Effect of pH on amylase production and growth of *P. citrinum* KBA4, with error bars showing standard deviation among triplicates

Similar to strain BB3, strain BNN1 also exhibited biphasic response to pH, with two peaks in amylase production, as shown in Figure 4.4. Amylase activity of the fungus increased sharply at pH 4 (0.57 ± 0.09 IU/ml), declined significantly at the subsequent pH, then showed an increasing trend until a second peak at pH 8 (0.58 ± 0.02 IU/ml). These values were significantly higher than the other pH ($p \leq 0.05$; Tukey HSD). The best pH for strain BNN1 growth was observed at pH 6 (0.35 ± 0.06 g), although a lower peak was also found at pH 4 (0.25 ± 0.09 g).

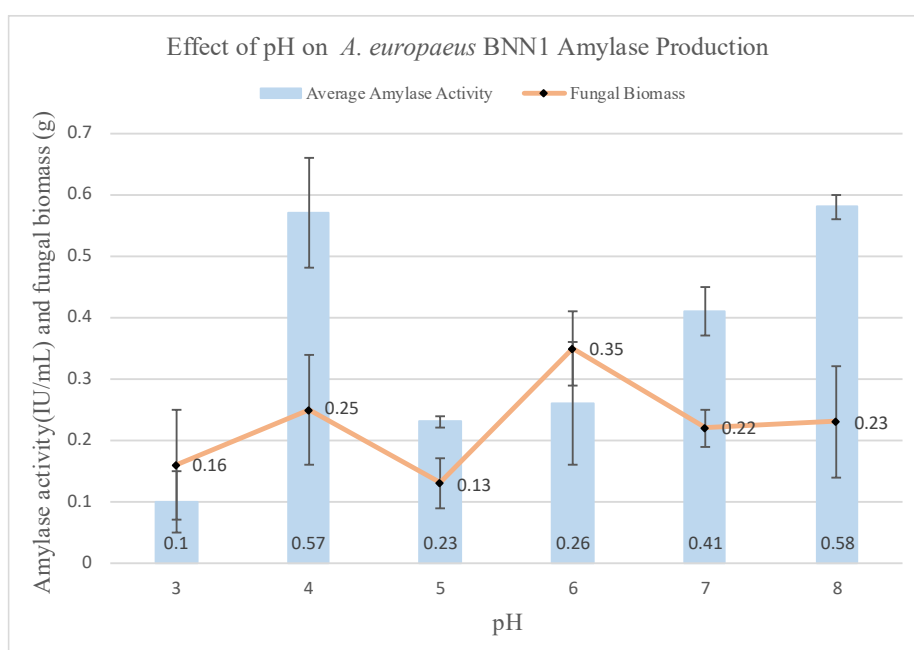


Figure 4.4: Effect of pH on amylase production and growth of *A. europaeus* BNN1, with error bars showing standard deviation among triplicates

Amylase production of another *Aspergillus* species, *A. niger* KBA2, showed optimum pH ranging from pH 5 to pH 7 (Figure 4.5), and the highest activity at pH 5 (1.35 ± 0.11 IU/ml). The enzyme activity remained similarly high at these three pH and statistical analysis at 95% level of confidence revealed no significant differences among them. The optimum pH for fungus growth was observed at pH 4 (0.79 ± 0.20 g), however, amylase production at this pH was the lowest (0.38 ± 0.19 IU/ml). Generally, the selected

fungi preferred conditions ranging from acidic (pH 4) to slightly basic (pH 8) for amylase production. Additionally, all species, except *Penicillium* sp., also favoured a slightly acidic environment for growth.

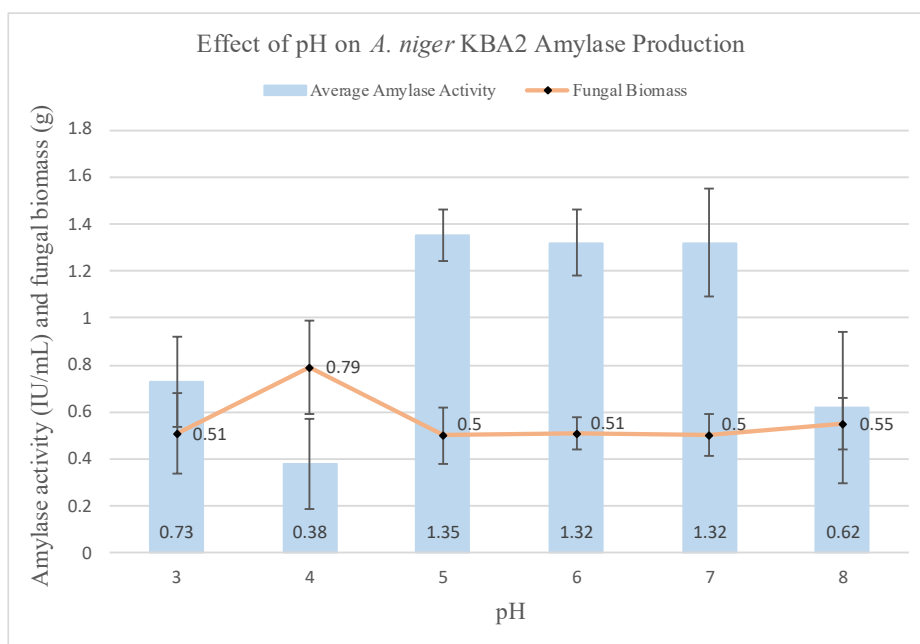


Figure 4.5: Effect of pH on amylase production and growth of *A. niger* KBA2, with error bars showing standard deviation among triplicates

4.4.2 Optimisation of Incubation Period

In optimising incubation period for amylase production, most species showed an optimum incubation period of more than 3 days, except for *A. europaeus* BNN1. Despite this, all fungi displayed an increasing trend in growth with extended incubation period. In strain BB3, enzyme production peaked significantly after one week of fermentation (0.36 ± 0.11 IU/ml, $p \leq 0.05$; Tukey HSD) as seen in Figure 4.6, and decreased sharply under prolonged fermentation beyond 9 days. However, highest fungal growth was observed after 9 days of fermentation, with 0.72 ± 0.11 g. On the other hand, strain KBA4 secreted the most amylase after fermentation of 9 days (Figure 4.7). with an activity of 0.24 ± 0.06 IU/ml. An

increasing trend in amylase activity as the fermentation period increased could be observed if not for the sudden decline in the activity after 5 days of fermentation. Similar to strain BB3, growth of strain KBA4 peaked after a period of 9-day fermentation with a biomass of 0.56 ± 0.04 g.

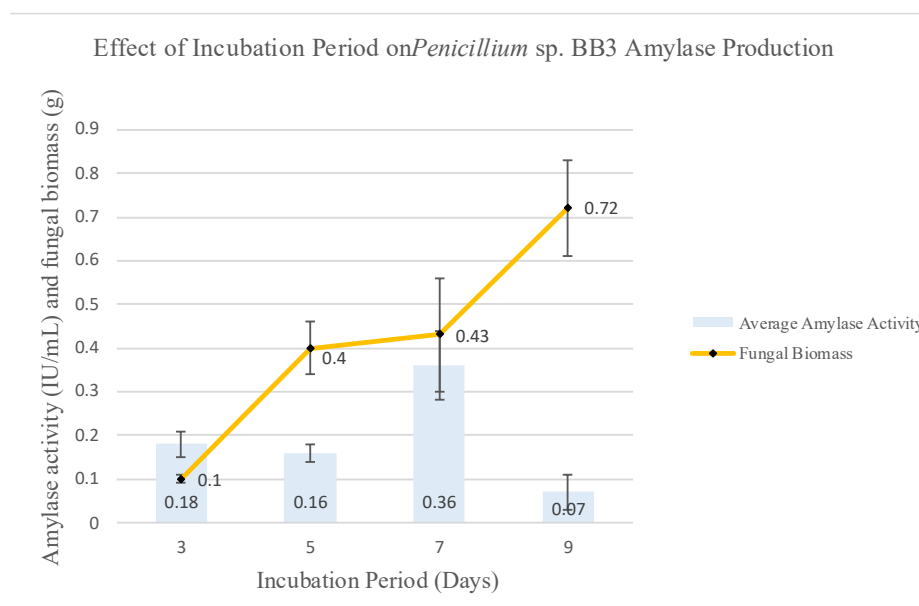


Figure 4.6: Effect of incubation period on amylase production and growth of *Penicillium* sp. BB3, with error bars showing standard deviation among triplicates

A distinctive result was observed in strain BNN1 as the fungus achieved optimal amylase production after incubation period of 3 days (0.58 ± 0.04 U/ml), as observed in Figure 4.8. Enzyme production decreased after 5 days of fermentation but reached another peak under an incubation period of 7 days with activity of 0.51 ± 0.06 IU/ml. Amylase production after 3 and 7 days of incubation are similarly high and both were significantly higher than at other incubation period ($p \leq 0.05$; Tukey HSD). Growth of the fungus was recorded as highest (0.8 ± 0.02 g) after 9 days of incubation. For strain KBA2, maximum amylase production was after 5 days of incubation (0.43 ± 0.12 IU/ml) which was

significantly higher than other incubation periods ($p \leq 0.05$; Tukey HSD). The highest fungal growth for strain KBA2 was observed after 9 days of incubation, with a value of 0.84 ± 0.06 g. Amylase production declined when fermentation was carried out for periods longer than 5-days as shown in Figure 4.9.

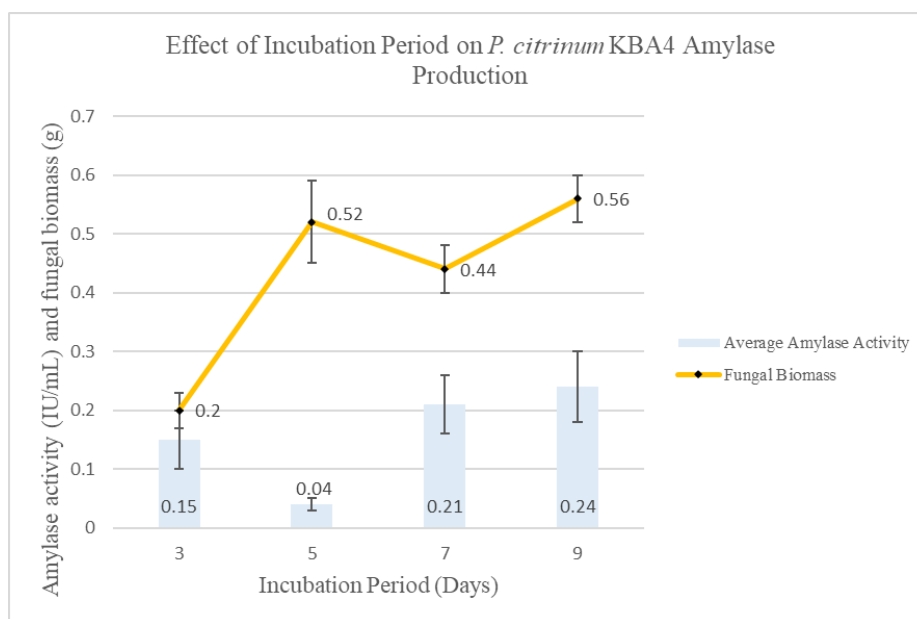


Figure 4.7: Effect of incubation period on amylase production and growth of *P. citrinum* KBA4, with error bars showing standard deviation among triplicates

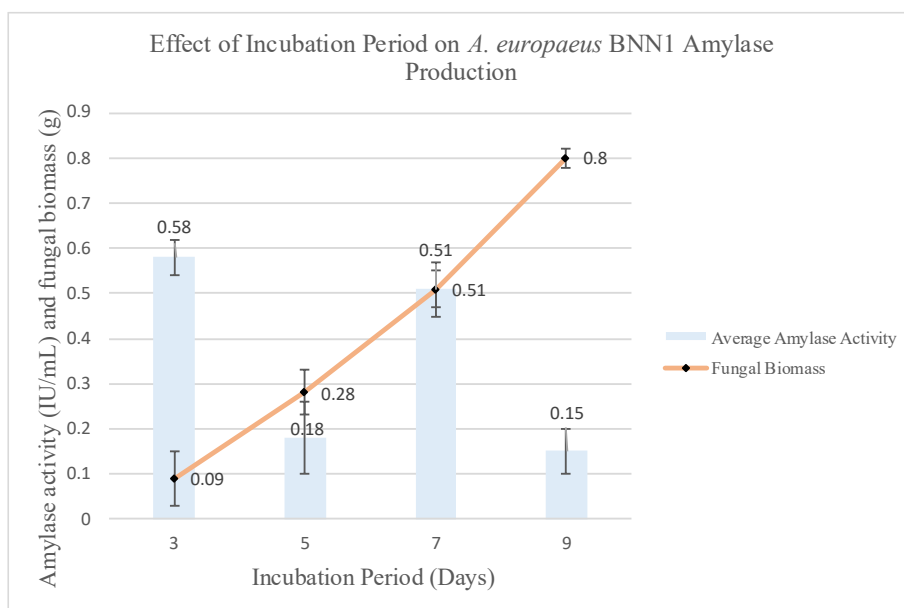


Figure 4.8: Effect of incubation period on amylase production and growth of *A. europaeus* BNN1, with error bars showing standard deviation among triplicates

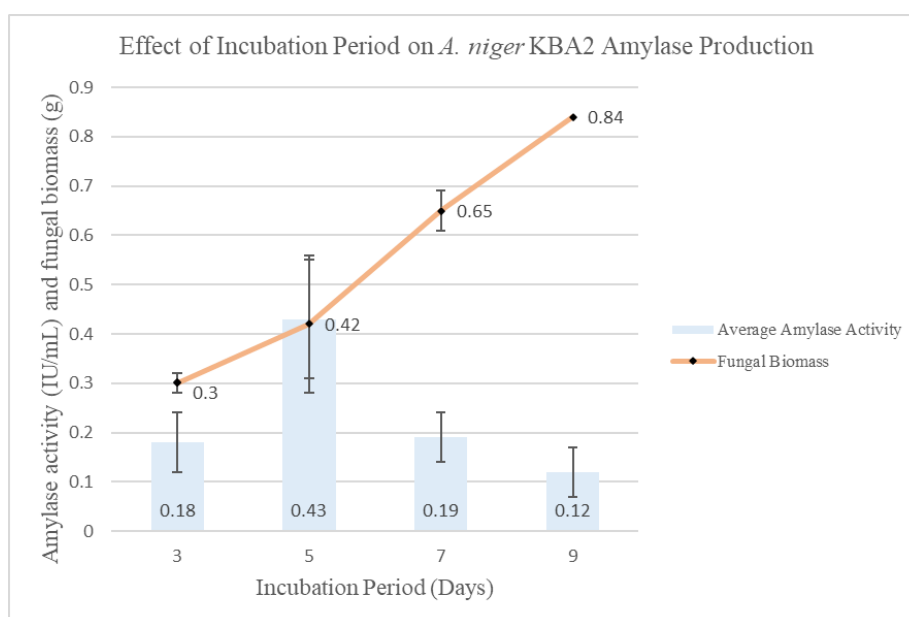


Figure 4.9: Effect of incubation period on amylase production and growth of *A. niger* KBA2, with error bars showing standard deviation among triplicates

4.5 Partial Purification of Amylase

Ammonium sulphate precipitation was performed to fractionally precipitate amylase from crude supernatant. It was discovered that amylase from strain KBA2 and strain BB3 precipitated at different concentrations of ammonium sulphate. Amylolytic activity was highest in protein fragment from 80% ammonium sulphate from strain BB3 crude extract. After purification, specific activity of amylase was 0.21 IU/mg, with a purification fold of 0.51 and yield of 26.47% as presented in Table 4.6. For strain KBA2, the protein fraction obtained from 50% ammonium sulphate precipitation exhibited the highest activity. Amylase from this fungus was successfully purified at 2.08-fold purification, with specific activity of 0.25 IU/mg and a yield of 28.85% as shown in Table 4.7.

Table 4.6: Purification of amylase from *Penicillium* sp. BB3

Purification Step	Volume (ml)	Activity (IU/ml)	Total Activity (IU)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (IU/mg)	Purification Fold	Yield (%)
Crude Extract	300	0.17	51	0.41	123	0.41	1	100
80% (NH ₄) ₂ SO ₄	150	0.09	13.5	0.42	63	0.21	0.51	26.47

Table 4.7: Purification of amylase from *A. niger* KBA2

Purification Step	Volume (ml)	Activity (IU/ml)	Total Activity (IU)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (IU/mg)	Purification Fold	Yield (%)
Crude Extract	400	0.13	52	1.12	448	0.12	1	100
50% (NH ₄) ₂ SO ₄	100	0.15	15	0.59	59	0.25	2.08	28.85

Amylases from strain BB3 were present in several isoforms or types, with molecular weights of approximately 80 kDa, 65 kDa, 42 kDa, 40 kDa and 35 kDa (analysed on SDS-PAGE and native-PAGE, as showed in Figure 4.10). In contrast to strain BB3 which secreted multiple amylases, strain KBA2 only produce a single amylase type with an estimated molecular weight of 130 kDa (Figure 4.11).

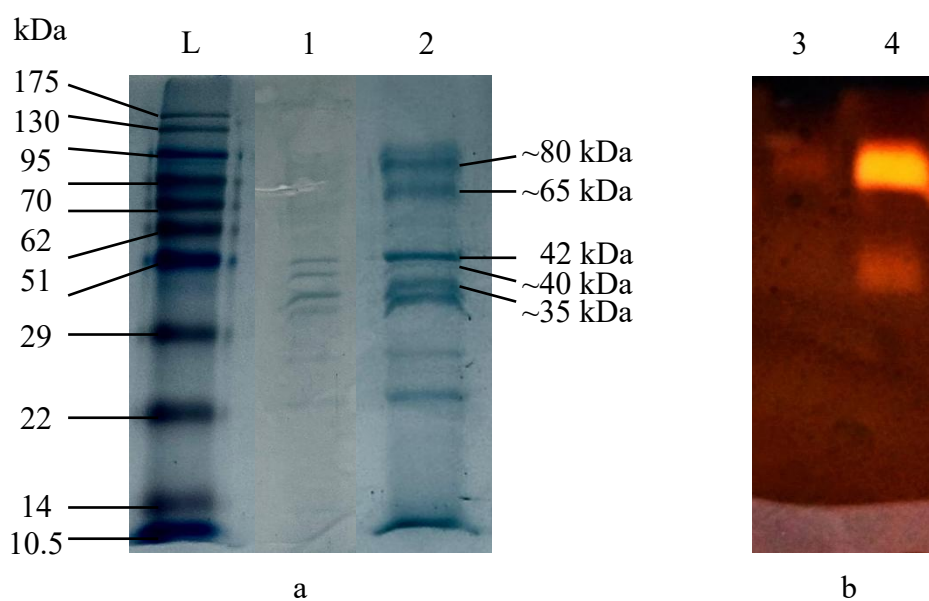


Figure 4.10: SDS-PAGE (**a**) and native-PAGE (**b**) of crude extract and partial purified amylase from *Penicillium* sp. BB3, L: Chromatein prestained protein ladder (175-10 kDa);
1: Crude extract; 2: Partially purified amylase with 80% ammonium sulphate precipitation. 3: Amylase activity of crude extract; 4: Amylase activity of partial purified amylase

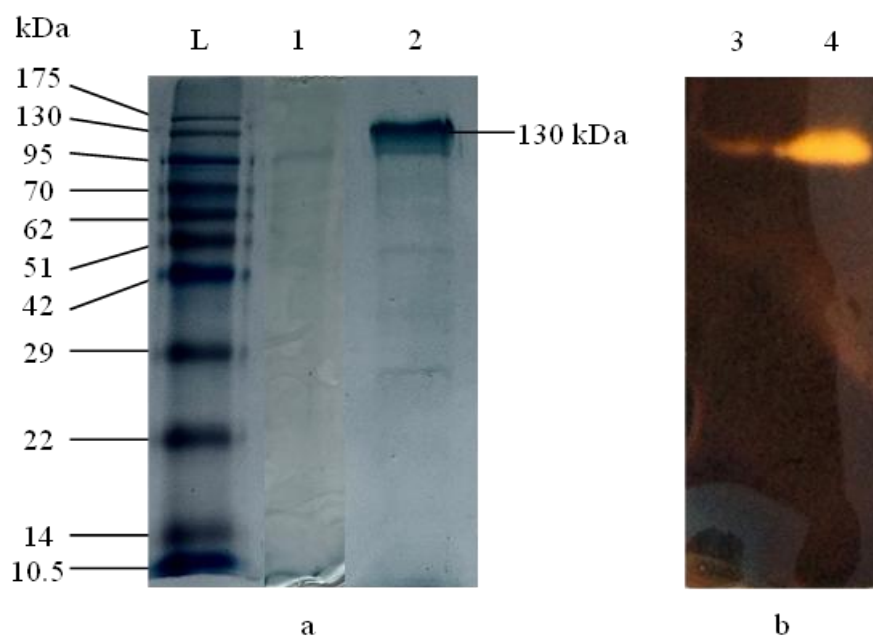


Figure 4.11: SDS-PAGE (a) and native-PAGE (b) of crude extract and partial purified amylase from *A. niger* KBA2. L: Chromatein prestained protein ladder (175-10 kDa); 1: Crude extract; 2: Partially purified amylase with 50% ammonium sulphate precipitation. 3: Amylase activity of crude extract; 4: Amylase activity of partial purified amylase

4.6 Characterisation of Partial Purified Amylase

4.6.1 Effect of Temperature on Amylase Activity

The partial purified amylase from both strain BB3 and KBA2 were sensitive towards temperature variations, with results of statistical analysis shown in Appendix S. For strain BB3, the optimum temperature for enzyme was 40 °C with activity of 3.23 ± 0.21 IU/ml, which was significantly higher than other temperatures at 95% level of confidence levels, in Tukey HSD as shown in Figure 4.12. Enzyme activity peaked at this temperature and declined as the temperature increased, and the activity was almost zero (0.04 ± 0.04 IU/ml) at extreme temperature (90 °C). However, it should be noted that 40 °C was the best temperature for catalytic actions of all amylases present.

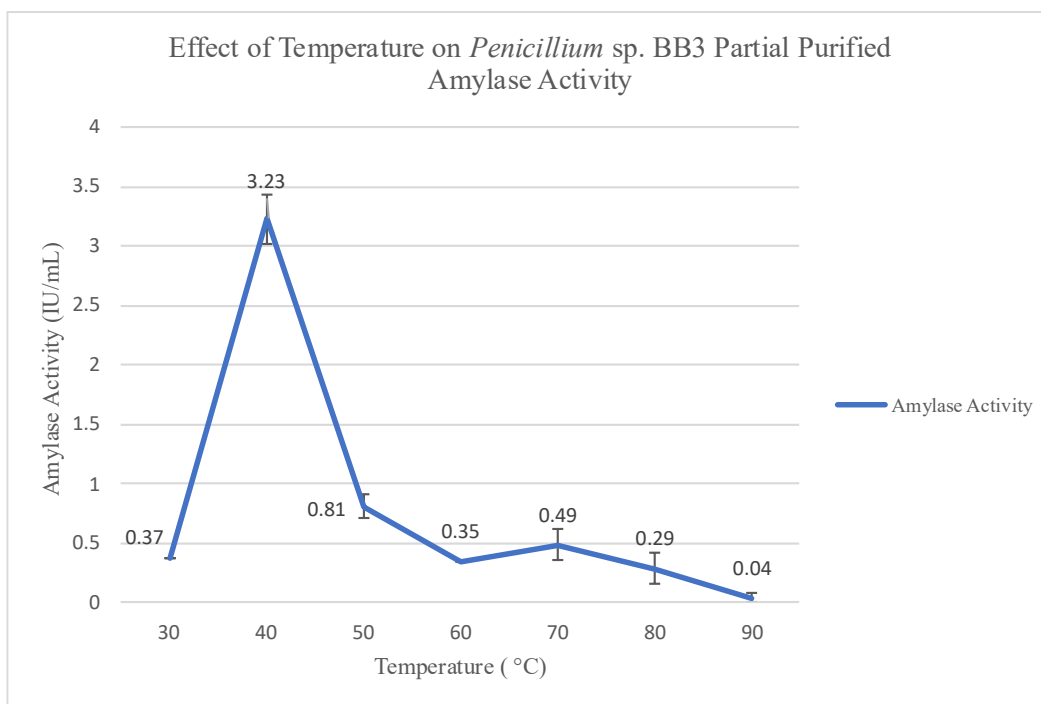


Figure 4.12: Effect of temperature on activity of partial purified amylase from *Penicillium* sp. BB3, with error bars showing standard deviation among triplicates

On the other hand, amylase from strain KBA2 had a higher optimum temperature for amylase action compared to strain BB2 as shown in Figure 4.13. When assayed at 50 °C, amylase activity peaked at 1.31 ± 0.17 IU/ml, significantly higher than at other temperatures (95% confidence levels, Tukey HSD). Amylase activity initially increased with temperature until peaking at 50 °C, but declined until the activity significantly declined to 0.04 ± 0.04 IU/ml at an extreme temperature of 90 °C (95% confidence levels, One-Way ANOVA and Tukey HSD), a trend similar to strain BB3.

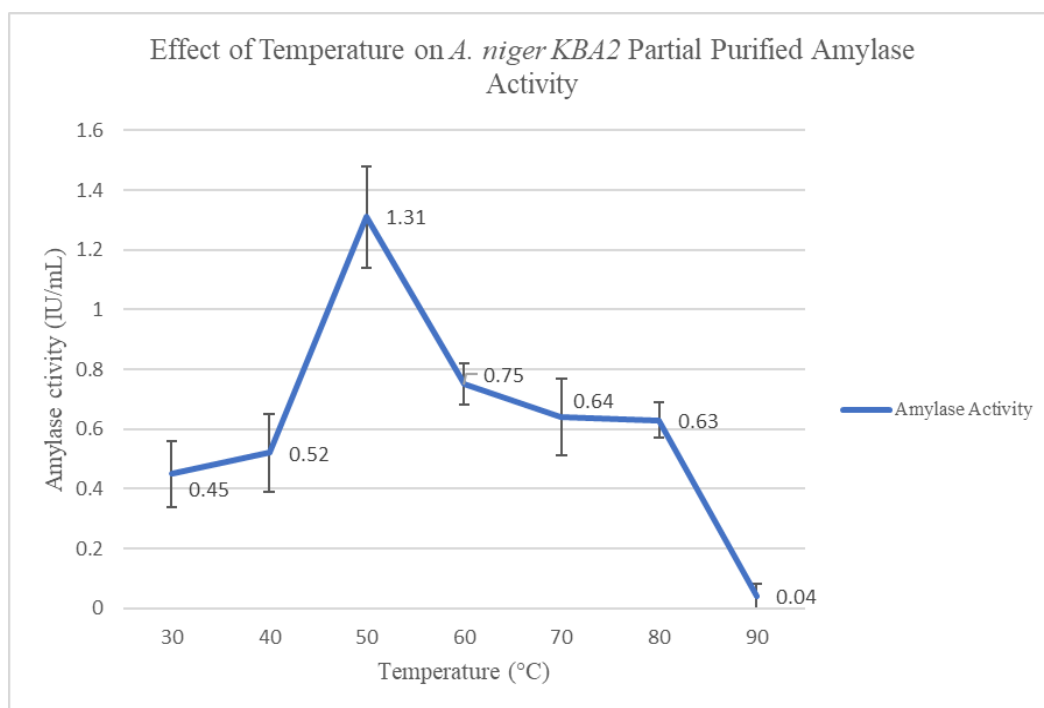


Figure 4.13: Effect of temperature on activity of partial purified amylase from *A. niger* KBA2, with error bars showing standard deviation among triplicates

4.6.2 Effect of pH on Amylase Activity

Amylase activity of strain BB2 and strain KBA2 was found to differ between one another, but interestingly similar to their respective optimum pH for amylase production. Amylase activity of strain BB3 peaked at pH 7 (0.93 ± 0.00 IU/ml), which is significantly higher than both acidic (pH 3) and alkaline pH (pH 8 and 9), proven through Tukey HSD test. However, amylase activity assayed under pH ranging from 4 to 7 did not exhibit any significant difference, indicating that the enzyme worked well under these pH range. Nevertheless, it should be noted that the results reflect the activity of all amylases produced by the fungus. Figure 4.14 presents the result of pH optimisation for partial purified amylase of strain BB3.

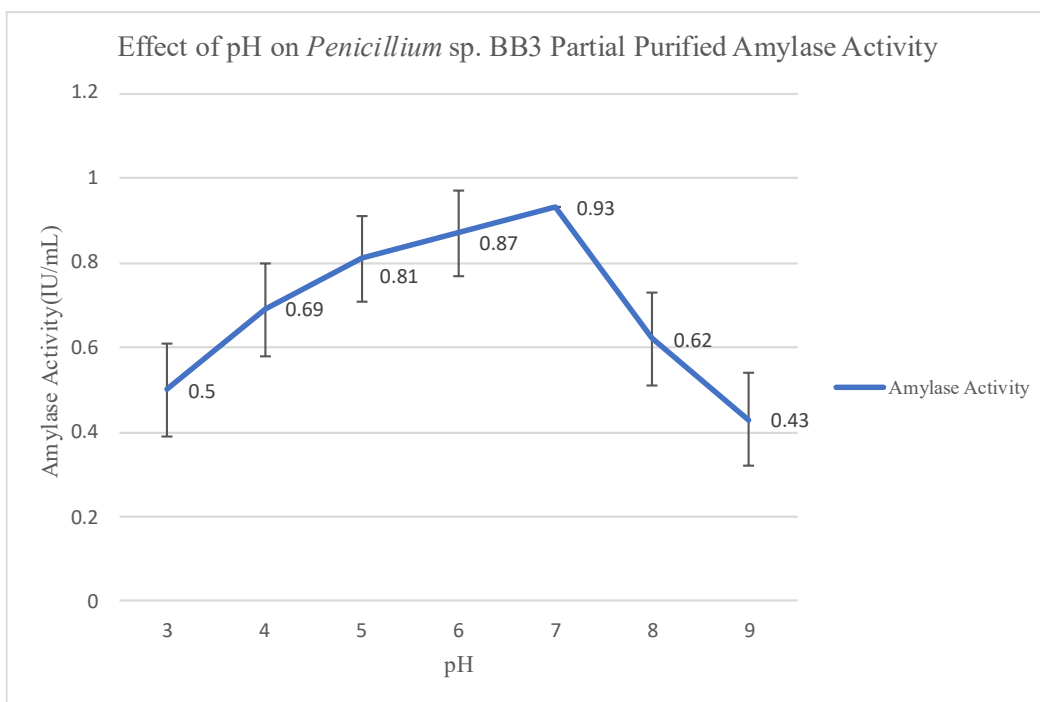


Figure 4.14: Effect of pH on activity of partial purified amylase from *Penicillium* sp. BB3, with error bars showing standard deviation among triplicates

The partial purified amylase of strain KBA2 had the significant highest activity of 10.99 ± 0.19 IU/ml at pH 5 (95% confidence levels, Tukey HSD) as observed in Figure 4.15. The amylase activity was similarly low at both pH 3 (0.31 ± 0.10 IU/mL) and pH 9 (0.56 ± 0.00 IU/ml), and was significantly low compared to other pH in between (95% confidence levels, Tukey HSD). Results of statistical analysis for both species were presented in Appendix T.

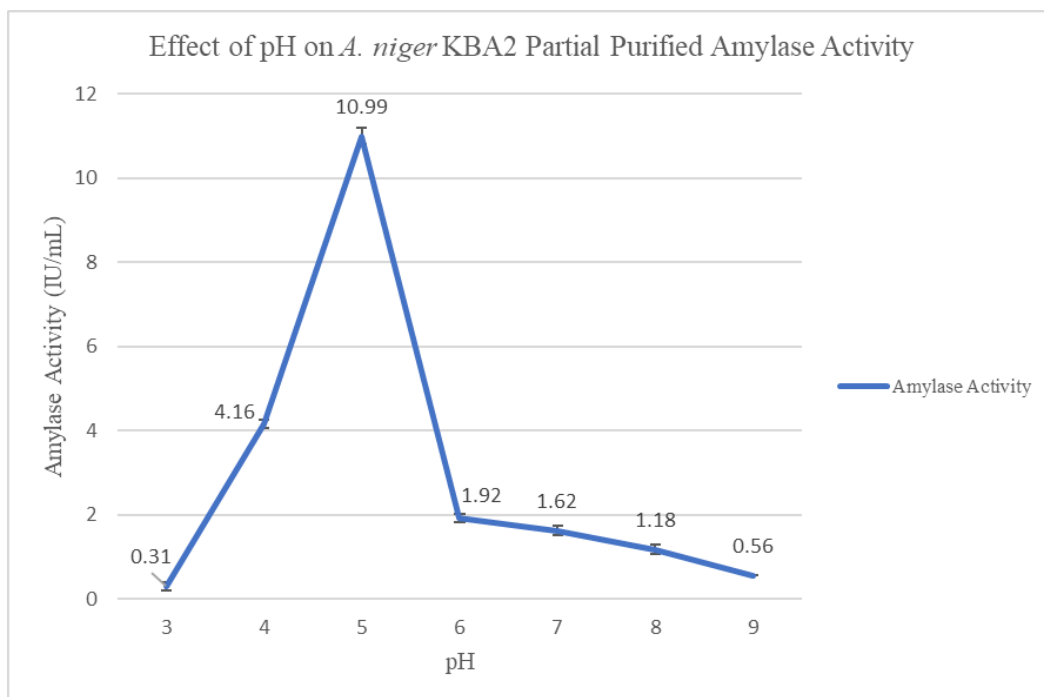


Figure 4.15: Effect of pH on activity of partial purified amylase from *A. niger* KBA2, with error bars showing standard deviation among triplicates

CHAPTER 5

DISCUSSION

5.1 Fungal Isolation and Identification

Fungal isolates were successfully retrieved from the samples with the addition of chloramphenicol, an antibiotic effective against both Gram-positive and Gram-negative bacteria (Blachowicz et al., 2022). This bacteriostatic antibiotic binds to the 50S ribosomal subunit, suppressing the synthesis of protein (Oong & Tadi, 2024). The presence of bacteria and fungi will affect the growth of one another due to competition for limited resources or the production of growth-suppressing substances (Black, 2020). Therefore, addition of antibiotic could minimise the interference of bacteria during fungal isolation process.

Although the primer pair of ITS1 and ITS4 successfully identified all isolates apart from *Aspergillus sp.* and *Penicillium sp.*, nevertheless ITS remains an effective standard and widely accepted fungal DNA barcode (Schoch et al., 2012). All isolates had high sequence similarity with NCBI database except for the species *Ophiostoma valdivianum* MSHB2 where the percentage identity is 86.54% and the alignment covered 83% of the sequences. However, the E-value of this alignment is $4e-117$, which is a very low value. Low E-value is favoured in BLAST, where this value is the most used measure in determining the quality of alignment, which also reflects the degree of sequence similarity (Choudhuri, 2014). Low E-value also showed that authentic homolog was detected, indicating that the hit is statistically significant, where query coverage above 50% to 80% and percent identity higher than 50% could support the results (Nestor et al., 2023).

The high prevalence of *Aspergillus* genus in this study highlights the adaptability of this genus to diverse environments and resilience of spores under extreme conditions (Douksouna et al., 2019). *Aspergillus* conidia are ubiquitous and easily spread in the environment (Sabino et al., 2019). Both *Aspergillus* and *Penicillium* are also significant food spoilage fungi (Taniwaki et al., 2018), explaining the prevalence of these fungi on spoiled food waste. It was also reported that the members of *Aspergillus* were prevalent in food (Fendiyanto & Satrio, 2020). On the other hand, *Penicillium* isolates are the main causal agents of food spoilage in temperate environments and could even colonise refrigerated food (Shukla & Mishra, 2020). The most frequently isolated fungus, *P. citrinum* is one of the most common and widespread filamentous fungus, with presence reported from diverse substrates (Houbraken et al., 2010), justifying the prevalence of this fungus in the current study. The second most prevalent isolate, *A. niger* is also a ubiquitous environmental fungus, generally found on stored crops and decaying plants (Mahmood et al., 2018).

There were few studies focusing on investigating fungi that colonise sago pith waste. A previous study reported the isolation of a mesophilic fungus, *Chalara* sp. from sago pith (Vikineswary et al., 1995). Another study identified four fungi genera in sago pith waste, which are *Gliocladium* (also known as *Trichoderma artaviridae*), *Giotrichum*, *Aspergillus* and *Rhizopus* (Asben & Irawadi, 2013). Similarly, the fungus *C. paradoxa* was previously found in sago pith waste (Nuk Mustaffa, 2015). On the other hand, various studies have examined sago pith bacteria. One study studied bacteria from sago pith from Pusa, Sarawak and revealed 52 Gram-positive and 32 Gram-negative bacteria (Apun et al., 2000). Another study identified indigenous *Bacillus*, *Serratia* and *Acinetobacter* species from sago waste (Hastuti et al., 2014), while subsequent research reported the

isolation of 21 cellulolytic bacteria (Faizah et al., 2020) and a bacterial load of 1.65×10^7 cfu/g bacteria from the waste (Nurmiati et al., 2022).

Several fungi isolated from sago pith waste were found to be plant pathogens. Members of *Ophiostoma* are associated with both biotic and abiotic plant diseases, but most cause blue-stain on wood instead of serious diseases (Zapata et al., 2018), as in the case of *O. valdivianum* (Gutiérrez et al., 2011). *Pleurostoma richardsiae* (*Pl. richardsiae*) showed positive results in pathogenicity tests on grapevines (Carlucci et al., 2015) and is commonly associated with Petri disease in Italian grapevines (Carlucci et al., 2017). Other studies revealed the ability of this fungus to cause infection in grapevines (Varela et al., 2016) and olive trees (Calvo-Peña et al., 2022) in Spain. Additionally, this species was identified to infect cedar trunks that caused dieback (Chauiyakh et al., 2022). *Thielaviopsis paradoxa*, or also known as *Ceratocystis paradoxa* is a fungus found all over the world and they are soilborne plant pathogens with a wide host range (Mbenoun et al., 2014). The black fungus was reported to infect harvested coconuts in Taiwan, causing black rot disease (Tzeng & Sun, 2009). In Malaysia, the fungus has been isolated from various sources including decaying palm leaves, sweet potato, oil palm, pineapple and coconut (Lee et al., 2012).

Paecilomyces maximus has been identified as one of the stain fungi infecting Indonesian rubberwood, causing severe wood discolouration (Nandika et al., 2021). The fungus also caused dieback and canker disease in pistachio tree in Turkey (Ozan et al., 2022), meanwhile researchers in Morocco have associated it with dieback in cedar trunks and twigs (Chauiyakh et al., 2023). *Tichoderma asperellum* stands out as a unique isolate among the plant-associated pathogens due to its role as a beneficial fungus. For seven decades, this fungus has been recognised and applied as biocontrol agents against various

pathogens (Podder & Ghosh, 2019). The species effectively controls *Fusarium* infection on Cavendish banana cultivars (Syd Abdul Rahman et al., 2021), and tomato plants (Sehim et al., 2023), while in rice plants it controls both *Rhizoctonia solani* and *Sclerotium oryzae* infection (Chinnaswami et al., 2021). However, *T. asperellum* is also a potential post-harvest pathogen as it was reported to cause green mould disease in sweet potato (Yang et al., 2021). Sago pith waste, or sago hampas is a starchy lignocellulosic residue of the starch extraction process (Amin et al., 2019), therefore enabling the survival and growth and lignivorous fungi. However, the isolation of these pathogens from sago pith waste does not necessarily indicate that the sago palms were infected before harvest.

Aspergillus was the most prevalent genus among isolates from washed rice water. This genus was previously reported in rice directly obtained from field, in the processing stage, and from markets sources (Katsurayama et al., 2020). A previous study revealed *Aspergillus* to be the most frequently isolated fungi although both *Aspergillus* and *Penicillium* were prevalent on stored rice (Oh et al., 2007). This result aligns with a study in Thailand that reported 80% of their rice isolates belong to *Aspergillus*, while 20% were categorised under *Penicillium* and *Talaromyces* (Laut et al., 2023). *Geotrichum candidum* was among the isolates found from Egyptian rice grains along with the dominant *Aspergillus* species, as well as other less prevalent genus such as *Penicillium*, *Cladosporium*, *Fusarium*, *Trichoderma*, and *Paecilomyces* (Moharram et al., 2019). Isolates from the genera *Fusarium*, *Rhizopus*, *Tilletia* and *Gibberella* were also reported to be prevalent in stored rice apart from *Aspergillus* and *Penicillium* (Li et al., 2022). Members of *Aspergillus*, *Fusarium* sp., *Trichoderma* sp., *Curvularia* sp., *Mucor* sp., *Pyrocularia* sp. and *Collectotricum* sp. were reported in Ghanaian rice (Ackaah et al., 2023). Both geographical factors and differences in processing methods might explain

the variation in fungal species associated with rice (Laut et al., 2023). However, all studies agreed on the prevalence of *Aspergillus* genus.

Makun et al. (2011) reported *A. flavus* and *A. fumigatus* were dominant isolates of Nigerian rice, while *A. oryzae* was a less common storage fungi and *A. tubingensis* was only found in field rice. However, *A. tubingensis* was isolated from commercial rice in Thailand in a recent study by Laut et al. (2023), suggesting the species is also a storage fungus. Both *A. flavus* and *A. fumigatus* were also reported in Indian rice grains, where Gorai et al. (2022) also isolated *P. citrinum*. Some indigenous rice fungi are toxigenic, for example *A. flavus* and *P. citrinum*, where storage would contribute to the prevalence of these isolates as rice is a suitable substrate for fungi (Katsurayama et al., 2020). Washing of rice helps to remove contaminants such as dust (Abba et al., 2021), where the fungi present in stored rice may be washed out during this process. A previous study focusing on investigating microbes in fermented rice after-wash water isolated *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. similar to this study, also species from other genus such as *Rhizopus*, *Saccharomyces*, *Paecilomyces* and *Pestalotiopsis* (Sairi et al., 2018). Another study isolated *Penicillium* sp. and *Fusarium* sp. from parboiled rice water (Okunwaye et al., 2021).

This study identified *Aspergillus* and *Penicillium* species as prevalent bread fungi, where these genera were associated with rapid spoilage of bakery products (Garcia et al., 2019). Both of these genera were also reported as bread spoilage fungi in a previous study, together with *Mucor* sp. and *Rhizopus* sp. (Ravimannan et al., 2016). The fact that *Aspergillus* members are common bread mould (Chaste et al., 2019) was also supported by Nuhu and Yusuf (2023), where they isolated *Aspergillus* sp. and *A. niger* from bread. *Aspergillus fumigatus* and *A. niger* were previously reported as the dominating species in

mouldy white bread (Al-Rajhi et al., 2023), along with the isolation of *P. chrysogenum* and *P. expansum*. Garba et al. (2022) also reported the isolation of *Penicillium* sp. and *Aspergillus* sp. from bread samples.

Aspergillus pseudoglaucus, *Cladosporium* sp., *H. burtonii*, *P. brevicompactum*, *P. roqueforti* (Garcia, Bernardi, Parussolo, et al., 2019), and *P. citrinum* (Liu et al., 2011), were previously isolated from mouldy loaf bread. Plain bread with a shelf life of seven days was dominated by *S. cerevisiae*, *C. sphaerospermum*, *H. burtonii* and *A. tamarii*, while samples with shelf life of thirty days yielded *A. flavus* and *E. chevalieri* apart from the previous mentioned isolates (Fendiyanto & Satrio, 2020). To the date of writing, there is no report of isolating this fungus from bread, but the species was previously isolated from sugarcane (Koricha et al., 2019) and acidic gruel produced from fermented millet (Wang et al., 2020).

The isolation of *A. niger* and *F. verticillioides* from potatoes in this study were similar with a previous study, although the authors reported some other isolates such as *F. oxysporium*, *A. flavus* and *Trichoderma* sp. (Ogunsola & Aduramigba-Modupe, 2014). A similar study also revealed the presence of several other *Fusarium* species including *F. verticillioides*, *F. solani* and *F. oxysporium*, apart from other isolates such as *Alternaria* sp. and *Geotrichum* sp. (García-Ávila et al., 2018). *Geotrichum candidum* is a commensal fungus in human, but it is also found in fruit, soils and plants (Bennetot et al., 2023). This fungus is an opportunistic saprophyte that cause rubbery rot in potato (Termorshuizen, 2007), and is also a postharvest pathogen causing rotting in crops such as tomatoes and onions (Keene et al., 2019). Several members of *Penicillium* including *P. citrinum*, *P. polonicum* and *P. solitum* were also prevalent pathogens in potatoes (Shah et al., 2023). *Agroathelia delphinii* (GenBank JN241550.1), is also known as *Sclerotium delphinii* or

Sclerotium rolsii var. *delphinii*, where the species of *Sclerotium* are soilborne plant pathogens (Chen et al., 2022). This species was found to infect cotton (Mukherjee et al., 2015) and black pepper plants (Severo et al., 2021). Pathogenicity of the fungus towards potato was also previously identified where the infection could cause death of potato plant (Obee, 1937).

Contamination of fungal spores is unavoidable in the post-processing steps of food manufacture although the ingredients are heated, where *Penicillium*, *Aspergillus* and *Eurotium* species are among the fungi that would colonise food surface (Ji et al., 2007). Glutinous rice cake is a traditional Chinese dessert made from steamed glutinous rice flour (Lyu et al., 2024). A previous experiment on Mi Gao, a cake made from mixing glutinous rice and rice powder, found colonisation of *P. citreoviride* and *P. citrinum* on the food (Ji et al., 2007). A study focusing on sweet dumpling, another product of glutinous rice flour, found prevalence of *Apiotrichum*, *Aspergillus* and *Geotrichum* species (Suo et al., 2023). The isolation of fungi from another product of sticky rice, lemang reported the presence of *A. niger*, *A. ustus*, *Aspergillus* sp., *M. racemosus* and *P. expansum* (Fifendy et al., 2020). Similarly, cakes are substantially susceptible to fungal spoilage due to their intermediate-moisture content (Samsudin et al., 2019). Samsudin et al. also reported that fungal loads in different types of banana cakes which were stored separately exhibited an increasing trend throughout the storage period. From different cake samples, species of *Aspergillus* such as *A. niger* and *A. flavus* were isolated, as well as other species such as *P. glabrum* (Abdelhameed & Khalifa, 2024).

Two interesting *Aspergillus* species were isolated from the cakes, namely *A. flocculosus* and *A. europaeus*. *A. flocculosus* was previously isolated from several saline environment, including from an alga, *Padina* sp. in Vietnam (Choi et al., 2021), also from

saltern surface in both China (Zheng et al., 2013) and Slovenia (Butinar et al., 2011). At the point of writing, there is no report of this fungus isolated directly from food source. However, *A. flocculosus* had been isolated from indoor environment of hospital laboratories (Atya et al., 2019), suggesting that this fungus was airborne where the spores possibly came into contact with the cake and contaminate the food. On the other hand, *A. europaeus* was a novel fungus first reported as a soilborne species isolated from European caves (Hubka et al., 2016). However, recently there were reports of this fungus from various food. *Aspergillus europaeus* was a pathogen causing bulb rot in garlic (Spadoni et al., 2020), also isolated from yerba mate (Silva et al., 2019) and corn grain (Nguyen et al., 2020). This species is also an airborne fungus as it was present in the surrounding air of apple warehouses (Smiri et al., 2021). As a member of section *Cremeri* in the *Aspergillus* genera, the species resembles *A. wentii*, and could be identified as *A. wentii* or *A. tamaris* morphologically, which indicates frequent morphological misidentification (Hubka et al., 2016).

5.2 Screening for Amylase Production

Extracellular amylase production by fungal isolates was assessed by observing halo zone formed on starch plates, where the formation of halo zone indicates that amylose in starch is being hydrolysed as amylose binds with iodine (a staining agent) to form deep blue complex (Brust et al., 2020). All forty-one isolates in this study were able to grow on starch agar, but not all isolates produced extracellular amylase. However, the prevalence of amylase-producing isolates across six starchy wastes types further justified the results of fungal isolation, where they are able to grow on the waste due their ability to utilise starch as substrate. The result that all *Aspergillus*, *Penicillium* and *Trichoderma* species were amylase producers is supported by the fact that these three genera are previously reported as sources of amylase (Okunwaye et al., 2021). Notably, while all

species of these genera produced amylase in this study, previous findings have recorded non-amylolytic activity in some isolates of these genera, including *Aspergillus* sp., *A. sydowii*, *Penicillium* sp., *P. citrinum* and *T. viride* (Verma & Verma, 2018).

All isolates apart from *Thielaviopsis paradoxa* derived from sago pith waste demonstrated extracellular amylase secretion. Previous reports showed that *Aspergillus* sp. isolated from sago pith waste was an amylase producer, while *Rhizopus* sp. showed no amylase activity and *Geotrichum* sp. showed ambiguous results due unclear halo zones formation (Asben & Irawadi, 2013). Apart from indigenous fungi, previous research had reported the isolation of *B. amyloliquefaciens* from sago pith waste, where this bacterium is a potent producer of amylase (Apun et al., 2000).

For rice water isolates, all but *Geotrichum candidum* produced amylase. Previous study reported that *Fusarium* sp. and *Penicillium* sp. isolated from parboiled rice water are amylase producers (Okunwaye et al., 2021). Additionally, an unidentified mesophilic fungus isolated from cooked rice displayed an amylase activity of 79.4 mg/ml (Sidkey et al., 2010). Another study that isolated fungi from spoiled cooked rice reported *A. awamori* as the potent amylase producing fungus with activity of 44.82 U/ml (Kalaiarasi & Parvatham, 2015). Among potato waste isolates, *Geotrichum candidum* and *Fusarium verticillioides* did not show amylase activity on starch agar. However, other studies indicated that fungi from Irish potatoes, namely *Rhizopus oryzae*, *Fusarium redolens*, *Botrydiplodia theobromae*, *Fusarium oxysporum* and *Penicillium* sp. were all found to be amylase producers (Salami & Popoola, 2007). Another research in isolating fungus from potato peels also reported positive result for amylase production in all isolates (Akponah & Akpomie, 2011). Additionally, Yasser et al. (2019) reported in their studies that all

isolates from potato, including members of *Aspergillus*, *Penicillium* and *Fusarium* were amylase producers.

All isolates from bread, but *Geotrichum silvicola* and *Rhizopus arrhizus* produced amylase. *Penicillium* sp. and *Aspergillus* sp. isolated from bread in the study of Garba et al. (2022) also demonstrated the ability to degrade starch. Al-Rajhi et al. (2023) also reported that members of *Aspergillus* and *Penicillium* isolated from bread were producing amylase although they isolated some other amylolytic fungi from the genus *Rhizopus*, *Mucor*, *Monilia* and *Alternaria*. However, both studies reported on amylase activity of *R. arrhizus*, which was different to the current finding. This study found that all *Aspergillus* and *Penicillium* species from glutinous rice cake and banana cake are amylase producers. A previous study also found that all isolates from lemang, including members of *Aspergillus*, *M. racemosus* and *P. expansum* were amylase producers (Fifendy et al., 2020). Another study on different cake samples reported that all isolates apart from *P. glabrum* were producing amylase and most *Aspergillus* species were moderate and high producers (Abdelhameed & Khalifa, 2024).

To the date of writing, there was little information on the amylolytic activity of *Paecilomyces maximus*, *Pleurostoma richardsiae*, *Talaromyces siamensis*, *Ophiostoma valdivianum* and *Agroathelia delphinii*. Apart from *P. maximus*, another species of *Paecilomyces*, *P. variotii* was an amylase producer (Michelin et al., 2010). Similarly, apart from *A. delphinii* (*Sclerotium delphinii*), *S. rolfsii*, another member of the *Sclerotium* genus was also able to secrete amylase (Valdez et al., 2019). Although not been isolated from starchy sources thus far, the ability of *A. flocculosus* to produce amylase had been observed by Kithaku et al. (2019) through a strain isolated from fungal contaminated banana cultures in banana tissue culture laboratory. The novel species, *A.*

europaeus was also reported as an amylase producer by Chamekh et al. (2019) working on an isolate obtained from soil samples.

This study discovered that *Thielaviopsis paradoxa*, *Geotrichum candidum*, *Fusarium verticillioides*, *Geotrichum silvicola* and *Rhizopus arrhizus* are not producing extracellular amylase. In contrast to the current study, a previous work on four *C. paradoxa* strains isolated worldwide revealed the ability of this species to produce amylolytic enzymes such as α -amylase, malt limit-dextrinase and pullulanase (Barišić, 2023). However, amylolytic activity of the strains varied from good to high unlimited activity, thus suggesting the activity is subject to the strains. The ability of *Geotrichum candidum* in producing amylase was found to differ across studies. Similar to the current study, *G. candidum* isolated from cassava garbage landfill site soil in a previous study failed to form starch hydrolysis zone after staining, indicating the absence of extracellular amylase production (Abdulsalam, 2022). On the other hand, *G. candidum* isolated from cassava macerated soil was producing amylase and was the best amylase producer compared to *Aspergillus* sp. and *Mucor* sp. obtained from potato (Silva et al., 2009). *Geotrichum* sp. obtained from Irish potato waste by Okunwaye et al. (2021) also had the ability of producing amylase.

Different to this study, *F. verticillioides* isolated from wheat displayed amylase activity ranging between 38.7 U/g and 76.13 U/g (Bakri et al., 2014). Another study in Egypt isolated *F. verticillioides* from potato and the fungus was a good amylase producer with clear zones exceeding 9 mm (Gherbawy et al., 2019). Previous bioinformatics analysis of *G. silvicola* revealed that the species was strongly correlated with raw amylase activity, indicating the possibility of this species in producing amylase (Tian et al., 2025). Previously, Dolatabadi et al. (2014) observed that amylolytic activity varied among *R.*

arrhizus, where some strains were potent amylase producers while several exhibited weak amylolytic activity. *R. arrhizus* from stored tuber revealed weak amylolytic activity while isolate from sorghum malt did not produce amylase (Dolatabadi et al., 2014).

Nevertheless, the absence of halo zone formation does not always indicate the inability of a fungus to produce amylase, as the enzyme might not be secreted extracellularly, or inhibition of detection by the medium might occur (Verma & Verma, 2018). As extracellular amylase production was not detected in seven isolates in the current study, further investigation could be performed to determine whether these fungi produce intracellular amylase. Apart from having the possibility of producing intracellular amylase, growth of these isolates on starch agar might be attributed to the capability of utilising other substrates present in the medium (Abdel-Raheem & Shearer, 2002). Similarly, their ability to grow on starchy wastes without producing amylase may also indicate that the fungi were relying on other substrates for survival. There is also a lack of established correlation between the ability of a fungus to grow on a certain test medium and to secrete the corresponding enzyme for the substrate in the medium (Abdel-Raheem & Shearer, 2002). Besides, amylase genes might be present in the genomes of the fungi but are not expressed, thus showing a negative result for extracellular amylase detection. There is possibility where gene induction failed to occur due to the absence of inducers and carbon starvation (Zhang et al., 2020). For future work, different media could be applied for the seven isolates in order to investigate whether they only secrete amylase under certain media and compounds.

5.3 Amylase Activity of Fungi through Solid-Based Method

Current study identified *Aspergillus* and *Penicillium* as the top amylase producers. This result aligns with prior reports indicating that these two genera are the most widely

used fungal genera in the production of polymer-degrading enzymes (Sethi et al., 2017), indicating that members of these genera are also strong amylase producers to degrade starch. Additionally, the ease of cultivation and excellent extracellular enzyme production in industrial scale also contributed to the interest in studying these genera for industrial enzyme production (Khokhar et al., 2011). Consequently, this study focused on *Aspergillus* and *Penicillium* for further analysis. Similar observations of *Aspergillus* and *Penicillium* producing more amylase than members of *Trichoderma* and *Paecilomyces* were also observed in the study by Mukunda et al. (2012). A similar trend was observed by Chamekh et al. (2019), who reported that these genera were generally superior amylase producers compared to other isolates.

While this study revealed that *Penicillium* isolates generally produced more amylase than *Aspergillus* species, a previous screening on starch agar found that several unidentified *Aspergillus* members outperformed *Penicillium* isolates, and other identified *Aspergillus* species such as *A. niger*, *A. flavus* and *A. fumigatus* (Mukunda et al., 2012). Nevertheless, another study on soil samples reported similar observation with the current results where *Penicillium* species has higher amylase activity than *Aspergillus* isolates when cultured on starch agar (Ugoh & Ijigbade, 2013). Chamekh et al. (2019) also reported a similar trend of most *Penicillium* isolates exhibiting stronger starch-degrading performance than most *Aspergillus* species. However, a study on endophytic fungi from potato plants revealed that all *Penicillium* species and most *Aspergillus* isolates excluding *A. flavus* cultured on glucose-peptone medium (GYP) agar supplemented with starch were weak amylase producers (Yasser et al., 2019).

In this study, *Penicillium* sp. BB3 exhibited highest amylase activity among all isolates. This species was also a potent amylase producer among fungi isolated from iron

ore mined overburden soil, with an activity index lower than the current study which was 1.224 (Verma & Verma, 2018). In contrast, this species was not the best amylase producer as reported by Chamekh et al. (2019) with indexes of 0.25 and 0.5, yet still outperformed *A. europaeus* consistent with this study. Mukunda et al. (2012) reported an isolate of this species as moderate amylase producer, with quantitative assays confirming it as the lowest producer (2.01 ± 0.03 $\mu\text{mol/ml/min}$) compared to *A. niger* and *A. flavus*. Like the current study, Shruthi et al. (2020) also found that this species as one of the best amylase producers among their isolates. Okunwaye et al. (2021) reported variability in amylase production among *Penicillium* isolates from different sources, with clearance zones ranging from 2.2 cm to 8.9 cm. The species was also the best amylase producer among isolates obtained from stagnant water with hydrolysis zone of 12.5 mm on starch agar (Priya & Renu, 2018).

The most prevalent species, *P. citrinum* are generally good producers of amylase. *Penicillium citrinum* from mangrove sediment had an activity index of 5.2, higher than the current study (Sahoo et al., 2014). Sahoo et al. (2014) also reported that this species (5.2) in their study had higher activity index compared to *A. niger* (3.09), *T. viride* (2.68) and *P. variotii* (3.63). The amylolytic index of this fungus ranged from 1.25 to 1.43 when cultured on Czapek-Dox agar supplemented with starch under pH ranged from pH 4 to pH 7 (Aydoğdu et al., 2012). When amylolytic activity was studied by means of hydrolysis zone, *P. citrinum* had a mean of 4.0 ± 0.0 mm (Omemu et al., 2015). Amylolytic activity of *P. citrinum* (31 mm) from seed samples was observed to outperform most *Aspergillus* fungi apart from *A. flavus* (33 mm) and *A. niger* (36 mm) (Saleem & Ebrahim, 2014). This species also showed excellent amylase activity during primary screening and

Shruthi et al. (2020) later reported in secondary screening that *P. citrinum* had an activity of 0.27 ± 0.015 U/ml.

In this study, *A. europaeus* BNN1 exhibited highest amylase activity among *Aspergillus* isolates but there were limited papers reporting on amylolytic activity of this fungus. A previous study reported that the fungus had an activity index of 0.30, which was lower compared to this study (Chamekh et al., 2019). *Aspergillus niger* KBA2 was the second highest amylase producer and most prevalent isolate from *Aspergillus* genus. *Aspergillus niger* isolated from *Aloe vera* gel in the study of Khokhar et al. (2011) has an activity index of 1.00 ± 0.0 when cultured on minimal salt agar containing starch, and lower than half of *A. niger* isolates in the current study. In the work of Fifendy et al. (2020), *A. niger* had activity indexes of 1.25 and 0.60, with an average of 0.93 on rice flour agar. On the other hand, *A. niger* cultured on cassava peels demonstrated excellent amylase activity where a substrate degrading distance of 42.70 ± 3.2 mm was recorded (Aisien & Igbinosa, 2020). Isolates of *A. niger* obtained from different cake samples had amylase activity ranging from 11.1 ± 0.1 mm to 28.9 ± 0.61 mm (Abdelhameed & Khalifa, 2024).

Similar to this study, the degrading performance of *A. niger* (42.70 ± 3.2 mm) was higher than *P. expansum* (37.10 ± 5.0) in a previous study (Aisien & Igbinosa, 2020). However, *A. niger* (1.25 and 0.60) was also previously found to have lower indexes than *P. expansum* (0.61 and 1.50), which was in contrast with the current study (Fifendy et al., 2020). Also different to the current result, Ekedegba et al. (2022) reported that *A. niger* was the best starch degrader with a halo zone diameter of 67.00 ± 1.81 mm, higher than *P. citrinum* (50.25 ± 1.30 mm). There was also a study that reported *A. niger* (36 mm) as the highest amylase producers among *Aspergillus* isolates and has higher activity than *P.*

citrinum (31 mm) (Saleem & Ebrahim, 2014). *Aspergillus niger* was reported as the most potent amylase producer among fungal isolates from bread, with an amylase activity of 7.67 U/ml when starch yeast was used as the substrate (Al-Rajhi et al., 2023). However, when white bread flour was used as the substrate, amylase activity of *A. fumigatus* (5.76 U/ml) surpassed *A. niger* (5.12 U/ml), therefore indicating that substrate specificity of isolates is different and will determine amylase production.

Several other *Aspergillus* species in this study had been previously reported as potent amylase producers. *Aspergillus flavus* was ranked the second highest in amylase activity among all isolates in the work by Idres et al. (2021), determined based on the clear zones (24 mm). All isolates of *A. flavus* were also exhibiting excellent amylolytic activity in another study, with width of clear zones ranging from 44.4 ± 0.08 mm to 60.8 ± 0.34 mm (Abdelhameed & Khalifa, 2024). *Aspergillus oryzae* in the study of Aydoğdu et al. (2012) had a highest index of 2.63 growing under an optimal pH of 4.5, while the second-best amylase producer, *Aspergillus* sp., recorded a value of 2.58 at its optimal pH of 5.5. While the current study reported *A. fumigatus* had the lowest amylase activity, Singh et al. (2014) observed that this species was a promising amylase producer with a clear zone of 4.0 mm and activity of 164.1 ± 1.7 U/ml.

By comparing the current work with others, it is observed that differences in amylolytic activity within similar species are caused by several factors including but not limited to fungal source, strain, medium used and incubation condition. The enzymatic activity index is an indication of the rate of substrate utilisation by fungi, where potent amylase producers could be selected based on this value (Sahoo et al., 2014). Higher zone of hydrolysis also reflects higher amylase activity of the microbes (Yassin et al., 2021).

However, study of amylase activity on starch plate did not quantify the amount of amylase produced by the fungi, therefore quantitative studies are recommended.

5.4 Optimisation of Amylase Production

5.4.1 Optimisation of pH

Penicillium sp. BB3 in this study had an optimum pH of 7 for amylase production, which was similar to the report of Jakheng et al. (2020). Lower optimum pH was also observed in several other works. For example, Abdullah et al. (2017) reported their strain of *Penicillium* sp. secreted maximum amylase under pH 6 while Sana et al. (2018) observed that pH 6 was optimal for soil-isolated *Penicillium* sp. on both maize flour and wheat bran. Priya and Renu (2018) observed that the *Penicillium* species isolated from stagnant water had an optimum pH of 6 when cultured on jackfruit seeds. In contrast, a more acidic environment of pH 5 was required for maximum amylase production in *Penicillium* sp. as reported by Lin et al. (2011). *Penicillium citrinum* KBA4 in this study require a medium with pH 5 for maximum amylase production. In another study, Aydoğdu et al. (2012) reported pH 5.5 was the optimum pH for amylase production. While this study and other previous works indicate that *P. citrinum* preferred slightly acidic environments, other work such as by Ekedegba et al. (2022) reported a basic media of pH 11 was best for amylase production in this species.

In this study, *A. europaeus* BNN1 showed highest amylase production at pH levels of 4 and 8. While there is limited data on the amylolytic activity of *A. europaeus*, a closely related species, *A. wentii*, favoured pH 4.5 for amylase production (Aydoğdu et al., 2012), which is consistent with the current result. Basic medium of pH 8 was also reported best for amylase production of *A. niger* (Unal, 2015) and *A. ochraceus* that was isolated from saltpan sediment, in minimal salt medium (MSM) for both amylase secretion and fungal

growth in the study by Devi et al. (2022). Different optimum pH was observed in other studies for other *Aspergillus* species. In another study, maximum amylase production and fungal growth of *A. wentii* was recorded at pH 6 (Lago et al., 2021) while Banjo (2020) observed that immobilised *A. flavus* in *A. digitata* matrix produced the highest amylase level at pH 4, similar to this finding. However, pH 6 was reported as the optimum pH for *A. flavus* free cells.

The current study revealed that *A. niger* KBA2 produces high amount of amylase within a pH range of 5 to 7. Mahmood et al. (2016) found pH 5 was the optimal initial pH for amylase production in their study, which is consistent with *A. niger* isolated from Iraqi soil (Mezeal & Alwaan, 2023). Other studies have reported maximum amylase production at pH 6 (Chakraborty et al., 2018; Saleem & Ebrahim, 2014) and pH 6.5 (Piyarathne & Weerasooriya, 2020). However, Monga et al. (2011) reported a slightly more acidic pH of 4.5 for maximum amylase production. Aydoğdu et al. (2012) also found *A. niger* had highest amylase activity when cultured at pH 4.5 on Czapek Dox agar supplemented with 2% starch. The wide pH range for amylase production in *A. niger* KBA2 is a promising feature as this could be applied in processes requiring pH ranging from slightly acidic to slightly basic environment (Piyarathne & Weerasooriya, 2020).

The fungal mycelium growth and enzyme production are largely affected by the changes of H⁺ concentration in the medium (Aydoğdu et al., 2012). This is due to the impact of pH on the transport of molecules across cell membranes and on enzymatic processes (Racheal & Alhassan, 2019). Nutrient availability would be affected by pH (Abubakar et al., 2013), as pH gradients across membranes regulate the import of nutrients together with ions and various solutes (Kane, 2016). For example, in the case of *Saccharomyces cerevisiae*, when extracellular pH draws nearer to cytosolic pH, the pH

gradient will be lost, thus affecting nutrient uptake (Kane, 2016). Changes in pH could also affect the expression of genes coding for starch hydrolysis, as Farh et al. (2021) found in their study where acidic pH would downregulate genes responsible for these enzymes in *Saccharomycopsis fibuligera*.

The observation of fungi preferring an acidic environment is attributed to the fact that fungal growth and production of amylase are supported under an acidic pH (Abdullah et al., 2017). *Penicillium citrinum* KBA4, *A. europaeus* and *A. niger* KBA2 had optimum pH for growth ranged from 4 to 6, which is within the suitable pH range of 3 to 7 for fungal growth (Ire et al., 2020). The growth of *Penicillium* sp. increased with pH, where Abubakar et al. (2013) previously mentioned that pH requirement for fungal growth might also reach more than pH 8. Fungi could grow under pH outside of the normal range of 3 to 7, but denaturation of extracellular enzymes might occur (Ire et al., 2020). Fungi also prefer a mild range of initial pH due to the characteristic of fungal fermentation in secreting and accumulating organic acids that will result in a decrease in pH (Napitupulu et al., 2020).

Multiple peaks were observed in several species, indicating two optimum pH in both *Penicillium* sp. and *A. europaeus*, while three in *A. niger* KBA2. The observation of two peaks was also reported by Zaferanloo et al. (2014), where amylase activity of *Preussia minima* had a lower peak at pH 5 and a second higher peak was recorded at pH 9. Zymography showed halo bands for the amylase secreted at both pHs, with the enzyme secreted under pH 9 produced brighter bands. Sethi et al. (2016) also found two peaks in their study on optimising amylase production from *A. terreus*, where a lower peak was observed at pH 5 and highest amylase production was at pH 7.

There might be several possible reasons for the observation of two peaks. Enzymes secretion is controlled by a pH homeostatic system within the fungi to make sure that they are produced at a range of pH that they could function (Peñalva et al., 2008). The fungi secrete high enzyme at more than one pH as there is likely more than one functional amino acids in its active site that contribute to activity at multiple pH values. Optimum pH for amylase production is likely an indication of the original environment where the fungi was isolated (Zaferanloo et al., 2014). As environment conditions of where fungi cultivate are constantly under fluctuations, the ability of them to react to changes in pH is crucial for their survival (Cornet & Gaillardin, 2014). Therefore, the fungi in this work might possess the ability to produce amylase under multiple pH to adapt to the changing environment, also to ensure their survival and competitiveness with other microbes. Apart from that, the genes coding for amylase in these species might be pH-dependent and favour several pH values. Wallis et al. (2001) revealed in their study that production and secretion of glucoamylase in *A. niger* was regulated by pH, and pH was also responsible for glycosylation, a post-translational modification process of the enzyme. However, this assumption was not proven in this study, suggesting possibilities of expanding the work.

This study observed that the optimum pH for fungal growth was not necessarily the best pH for amylase production, except in *P. citrinum* KBA4. In *A. niger* KBA2, highest fungal growth was accompanied by lowest amylase production. This coincides with the report of Benassi et al. (2012). They found that after 3 days of fermentation in Machado Benassi (MB) medium supplemented with glucose, mycelial mass of *Aspergillus phoenicis* reached a peak of 2.01 ± 0.067 g, but both intracellular (146.43 ± 0.012 U) and extracellular (685.54 ± 0.012 U) amylase production was the lowest. Similarly in the work of Sethi et al. (2016), maximum fungal growth was at pH 6, but

amylase production under that pH was low (~1.0 U/ml). This might be due to secretion of other proteins that could utilise available nutrients in the medium for fungal development when pH is not suitable for amylase production. Wallis et al. (2001) found that when glucoamylase production by *A. niger* declined at pH 5.4, production of extracellular proteins did not show significant decrease, indicating other more useful proteins were secreted.

5.4.2 Optimisation of Incubation Period

A 7-day fermentation period was found to be sufficient for amylase production by *Penicillium* sp. BB3. A similar observation was reported by Priya and Renu (2018) that isolated *Penicillium* sp. from stagnant water and grown with jackfruit seeds as substrates. Contrast to the current study, Abdullah et al. (2017) showed highest amylase production in this species was observed after 3 days (72 hours) of incubation with wheat bran and distilled water. A shorter period of 2 days was observed by Jakheng et al. (2020), where amylase production peaked after 2 and 3 days of submerged fermentation. When grown on wheat bran, the fungus required only 36 hours for maximum amylase production (Sun et al., 2007). Apart from the reports of shorter optimal incubation time, there were also studies that revealed a longer fermentation period. Selim et al. (2017) reported fermentation of 9 days was required for maximum amylase production of two strains of *Penicillium* sp. when grown on rice bran and corn bran. *Penicillium* sp. cultured in soluble starch medium also showed a similar increasing trend in growth as with this study, that gained fungal dry weight of 200 mg by day 10 (Salami & Popoola, 2007).

This study also showed that a 9 days fermentation period was required for *P. citrinum* KBA4 to produce maximum amount of amylase, similar to the work by Selim et al. (2017). However, several other studies reported shorter optimum incubation periods

than 9 days. For example, Carvalho et al. (2014) reported that *P. citrinum* produced highest amylase on day 7, coinciding with the decline of fungal growth on day 6. A shorter period of 4 days was observed by Shruthi et al. (2020). An optimum period of 96 hours for amylase production was also observed when *P. citrinum* was mix-cultured with *Mucor racemosus* and *Rhizopus stolonifer* on *A. digitata* (Racheal & Alhassan, 2019). A relatively short optimum incubation period of 24 hours was observed by Ekedegba et al. (2022) for *P. citrinum* obtained from soil samples. However, Metin et al. (2010) reported that amylase production of *P. citrinum* was at the highest on day 12 of fermentation, longer than this study.

For *A. europaeus* BNN1 in this study, 3 days of incubation yielded maximum amylase production. Studies on other *Aspergillus* species showed varied optimal incubation periods, Mahmood et al. (2018) reported a similar 3-day optimal period for *A. niger* while *A. flavus* required 4 days (Ire et al., 2020). Similar to the current study, *A. wentii* grown on medium supplemented with yeast extract, peptone and starch also favoured a fermentation period of 72 hours (3 days) for both growth and amylase production (Lago et al., 2021). *A. tamaraii*, a fungus morphologically similar to *A. europaeus*, reached maximum amylase production after 4 days of fermentation with inorganic salt solution supplemented with starch (Mohan et al., 2019). Longer incubation of 6 days was required for *A. oryzae* to produce the most amylase with soybean husk as the substrate (Melnichuk et al., 2020).

Aspergillus niger KBA2 in the current study had an optimum period of 5 days for amylase production, similar to the observation of Monga et al. (2011) under both shake flask and stationary fermentation with Czapek Dox medium supplemented with 3% soluble starch. Adebare et al. (2021) fermented *A. niger* on cassava peel and also reported

a similar optimum incubation time of 5 days. However, a shorter optimum incubation period of 3 days was reported in the work of Mahmood et al. (2016) when *A. niger* was cultured on raw potato peel. Longer incubation period was also observed, where *A. niger* from legume seeds had an optimal incubation of 6 days (Saleem & Ebrahim, 2014). Unal (2015) observed that *A. niger* achieved maximum production in one week across various media types such as mycological medium, stock basal medium and starch yeast medium. A similar incubation period of 7 days was also required for *A. niger* isolated from Iraqi soil to produce the highest amount of enzyme (Mezeal & Alwaan, 2023). Al-Rajhi et al. (2023) observed a longer period of 9 days for maximise amylase production on starch yeast medium, while 12 days were needed when *A. niger* was cultured in white flour and black flour media.

Optimum incubation time varied among species as it depends upon culture characteristics as well as the growth rate of fungi (Racheal & Alhassan, 2019). Fungus with shorter optimum incubation period is significant as it contributes to the economical aspect (Abdullah et al., 2017), through reducing multiple requirements and costs. Amylase production happens in the primary growth phase of fungi, where prolonged incubation will cause amylase degradation and reduction in amylase activity (Chaurasia et al., 2015), as observed in *Penicillium* sp. BB3, *A. europaeus* BNN1 and *A. niger* KBA2. Ekedegba et al. (2022) mentioned that enzyme production might also decrease with incubation because of hindrance in fungal growth caused by nutrient depletion or secretion of other byproducts.

However, in this study, hindrance of fungal growth was not the reason of declining amylase production under prolonged incubation. The growth of all isolates was increasing although the incubation time surpassed the optimal period for amylase production from

Penicillium sp. BB3, *A. europaeus* BNN1 and *A. niger* KBA2. The possible explanation is the occurrence of catabolite repression (Bellaouchi et al., 2021), where the activity of secreted amylase had produced sufficient glucose or other simple sugars under prolonged incubation, thus enzyme production had been repressed. This was supported by a previous study which revealed that glucose or other nutrients was repressing amylase production of the yeast *Saccharomycopsis fibuligera* at transcription level (Choo et al., 2016). This may explain why the fungi had high growth although low enzyme production was recorded, as the isolates could still utilise the available simple sugars or other nutrients. Low amylase production after 5 days of fermentation in *P. citrinum* KBA4 might be due to similar reason of catabolic repression or other enzymes more useful to the fungus were produced.

The observation of two possible optimum incubation periods in strain BNN1 could be attributed to the changes in pH of the medium. Yahya et al. (2021) working on *A. niger* MS and *A. tubingensis* SY reported that initial pH of the medium rises from pH 6 to pH 7 approximately after a day of fermentation and gradually declined with incubation. The pH of the medium was lowest after 3 days of fermentation in both species, but started to increase again as incubation progressed. In fermentation of *A. niger* MS, pH rose from approximately pH 3.5 (day 3) to pH 4 (day 5), while pH of *A. tubingensis* SY medium rose from around pH 3.5 (day 3) to approximately pH 4.5 (day 5). As *A. europaeus* had two optimum pH of 4 and 8, it therefore could be deduced that similar situation happened in the fermentation, where the initial pH might rise from pH 6.9 to around pH 8 during the early stage of fermentation, and dropped under longer incubation to pH 4. Nonetheless, pH needed to be checked throughout the fermentation process in order to validate this possible explanation.

Similar to the case of pH optimisation, it was also discovered that the optimum incubation period for maximum fungal growth varied with the fermentation time for highest amylase production. This observation was in agreement with Naili et al. (2016), where *A. oryzae* exhibited maximum growth after nearly 50 hours in batch fermentation, but amylase production peaked after approximately 100 hours of fermentation. Ire et al. (2020) also recorded that maximum growth and amylase production of *A. flavus* occurred after 48 hours and 96 hours of fermentation respectively. An increasing trend in growth with incubation period was observed by Sethi et al. (2016), where fungal biomass continues to increase post optimal amylase production period of 96 hours, up to 144 hours of incubation. In another study on *A. flavus* growing on potato peels, the researchers found that amylase activity was highest after 3 days of incubation, but fungal growth maximised upon 5 days of fermentation (Olakusehin & Oyedele, 2022). A reason for this might be due to the fungi is still experiencing the exponential stage of growth (Olakusehin & Oyedele, 2022).

5.5 Partial Purification of Amylase

Purification of a target enzyme from crude supernatant is crucial as it could reveal information on whether the degradation of substrates is due to a single enzyme or a multi-enzymes system (Sethi et al., 2016). Ammonium sulphate is often used for enzyme precipitation due to its high solubility, cost effective, and has minimal effect on enzyme pH and stability (Jebor et al., 2014). The salt promotes native conformation of the proteins by facilitating “salting-out” proteins from supernatant (Wingfield, 2016). According to Wingfield (2016), the use of salts such as ammonium sulphate act to decrease solubility of proteins without denaturing them, and precipitated protein could be redissolved using buffers. The required concentration of ammonium sulphate varied with the number of charges on the surface of protein, where the salt disrupts water molecules surrounding the

protein and causes precipitation of enzymes (Jebor et al., 2014). Precipitation occurs due to proteins folding and forming more compact conformation under increasing hydrophobic interaction with water (Wingfield, 2016).

The concentration of ammonium sulphate required for maximum amylase precipitation from crude supernatant differed between the two species, with amylase from *Penicillium* sp. BB3 requiring 80% saturation while enzyme from *A. niger* KBA2 required 50%. Shruthi et al. (2020) reported that amylase activity was at its peak in enzyme fractions obtained between 50% to 80% ammonium sulphate precipitation. In this study, an 80% ammonium sulphate concentration yielded proteins with the highest amylase activity from strain BB3 crude supernatant, an observation consistent with Ahmed et al. (2020).

Amylase from *Penicillium* sp. BB3 was partially purified to 0.51-fold with a specific activity of 0.21 IU/mg with 26.47% of yield. Previously, Sun et al. (2007) partial purified amylase from *Penicillium* sp. X-1 using 65% ammonium sulphate and produced 4.3±0.1-fold purification with a yield of 91% and 58.7±0.3 U/mg specific activity. Other studies focused on different *Penicillium* species, reported on different purification approaches and results. A study using *P. oxalicum* applied 67% ethanol precipitation, reported amylase achieved a 6.67-fold increase with specific activity of 1.20 U/mg and a yield of 53.51% (Xu et al., 2016). In the work by Shruthi et al. (2020), amylase from *P. citrinum* achieved 3.34-fold purity, and a specific activity of 11.86 U/mg and yield of 39%. Meanwhile, amylase from *P. javanicum* was 2.2-fold purified at 80% ammonium sulphate precipitation with a specific activity of 81.6 U/mg and 51.11% of the activity was recovered after purification (El-Gendy & Alzahrani, 2020).

This study partial purified amylase from *A. niger* KBA2 with a specific activity of 0.25 IU/mg, a yield of 28.85% and 2.08-fold purification. In comparison, a previous study reported a lower specific activity of 5.5-fold purified amylase with 0.066 U/mg and high yield of 82.96% from *A. niger* after precipitation with 90% ammonium sulphate (Aisien & Igbinosa, 2020). Adebare et al. (2021) observed a lower yield and specific activity for amylase purification from *A. niger* with 65% ammonium sulphate, achieving 0.0392-fold purification with specific activity of 0.0157 U/mg and yield of 3.68%. On the other hand, amylase after 90% ammonium sulphate concentration in the work of Obafemi et al. (2018) had a yield of 63% and a specific activity of 9.33 U/mg, higher than the current findings. However, the amylase was only 1.86-fold purified, lower than the current work. In the work reported by Jebor et al. (2014), a higher specific activity of 3.626 U/mg after 80% ammonium sulphate precipitation was achieved, but the enzyme was only 1.241-fold purified and yield was only 0.38%. Higher yield of 36.95% along with a high specific activity of 15.24 U/mg was reported by Sethi et al. (2016) after gradually purifying crude supernatant from *A. terreus* with 40-80% ammonium sulphate. The 2.305-fold purification achieved in their work is similar to the results of this study.

Specific activity of an enzyme after purification is expected to increase (Sethi et al., 2016) as observed in purification of amylase from *A. niger* KBA2. However, specific activity along with purification fold decreased in purification of amylase from *Penicillium* sp. BB3, where the enzyme was 0.51-fold purified. A similar observation was reported by Adebare et al. (2021), where they observed that the amylase was 0.0392-fold purified after 65% ammonium sulphate precipitation. Martgrita et al. (2023) purified amylase from *R. oryzae* and reported that although application of 60% ammonium sulphate resulted in a high yield of 97.51%, amylase was only 0.653-fold purified. Similarly, Abo-Kamer et

al. (2023) partially purified amylase from *B. cereus* with 40-60% ammonium sulphate and produced a low 0.224-fold purification and specific activity of 33.7 U/mg.

Specific activity is subject to changes in condition of purification steps (Zaferanloo et al., 2014), Mishandling of amylase from strain BB3 during purification may lead to loss of enzyme, causing decrease in specific activity and purification fold. A possible mistake is foaming during the addition of ammonium sulphate salt, as this would cause oxidation of proteins and denaturation might occur at the air-water interface (Ryan & Kinsella, 2017). Purification fold may also decrease due to precipitation of contaminant proteins together with the targeted proteins (Arshad et al., 2017), as proteins without amylolytic activity was observed in native-PAGE of strain BB3. Arshad et al. (2017) reported a similar observation of obtaining contaminant proteins, causing the targeted protein, bromelain, to be only 0.05-fold purified. This suggested the importance of further purification steps to obtain pure target proteins. In this study, the yield of partial purified enzymes was low, which was less than 30% in both species. High loss of enzyme might be the reason of getting low purification yield, as mentioned by Zaferanloo et al. (2014). Lower purification yield of amylase from strain BB3 than strain KBA2 might due to lower initial protein concentration in the crude supernatant, an observation also reported by Sahnoun et al. (2012).

It was found that amylase from *Penicillium* sp. BB3 had multiple isoforms or types with estimated molecular weight ranging from 35 to 80 kDa. Amylase from *A. wentii* had a similar weight of 86 kDa after purification with column chromatography (Lago et al., 2021). Metin et al. (2010) working on *P. citrinum* reported that amylase from this fungus had a size of 65 kDa as observed in this study. Another previous research found that amylase from *Penicillium* sp. isolated from stagnant water formed a single

band on SDS-PAGE and had a molecular weight of 57 kDa after gel filtration chromatography (Priya & Renu, 2018). Amylase with molecular weight of 42 kDa was observed by Sahnoun et al. (2012) while Carrillo-Sancen et al. (2016) reported purified amylase with 40 kDa. A previous study working on *A. flavus* also revealed amylase produced by the fungus had a molecular weight of 35 kDa similar to this study (Ayinde et al., 2023). Researchers working with different *Penicillium* species reported different size of amylase, for example, raw starch degrading enzyme from *P. oxalicum* was revealed to have a molecular weight of 75.4 kDa (Xu et al., 2016).

The current work found that amylase from *A. niger* KBA2 had a molecular weight of 130 kDa, similar to amylase from *A. clavatus* of 132 kDa in size (Shruthi et al., 2020). Slivinsk et al. (2011) reported that glucoamylase from *A. niger* had a size of 118.17 kDa, slightly smaller than this study. Amylase with smaller size was also reported, for example, Angelia et al. (2019) reported partial purified amylase from *A. niger* had a molecular weight of 60 kDa. In contrast to the current work, *A. niger* was previously reported to produce amylase isoforms. Partial purified amylase from *A. niger* in the work of Jebor et al. (2014) had two isoforms with sizes of 66 kDa and 52 kDa. Obafemi et al. (2018) also reported the observation of two bands with amylolytic activity after SDS-PAGE and native-PAGE, with molecular weight of 55 kDa and 35 kDa.

Multiple bands on both SDS-PAGE and native-PAGE suggested the presence of isoforms or multiple amylase types in *Penicillium* sp. BB3. Members of *Penicillium* were reported to produce isoforms, for example *P. citrinum* in the work of Shruthi et al. (2020) produced three isoforms of cellulase. Among *Aspergillus* species, Sahnoun et al. (2012) observed that *A. oryzae* isolated from sweet soy sauce secreted extracellular α -amylase with molecular weight of 50 kDa (AmyA) and 42 kDa (AmyB). Shruthi et al. (2020) also

found that amylase partial purified from *A. clavatus* exhibited three protein bands of 132 kDa, 76 kDa and 62 kDa with amylolytic activity. Amylases possess the ability of coexisting as isoenzymes, where the production of isoforms are subject to parameters such as protease action, as Sahnoun et al. (2012) found that AmyA was degraded by protease to derive AmyB. Post-translational modifications and expression of multigene families are also the possible reasons for the presence of isoforms (Sahnoun et al., 2012). The number of isozymes is determined by the original application of the secreted amylase by the organism (Sahnoun et al., 2012). Production of isoform indicates that the fungus is capable of tailoring its amylase production based on the nutrient content of substrate, for example Carrillo-Sancen et al. (2016) found that *A. niger* produced amylase isoform only when starch is the sole carbon source and glucose concentration will affect the secretion of isoforms. This enabled the fungus to adapt and survive under varying environment.

Apart from being isoforms, presence of multiple bands might also suggest the production of various types of amylases by strain BB3. Starch is the main substrate for a total of 10 types of enzymes, as mentioned by van der Maarel et al. (2002), including several amylases including α -amylase, glucoamylase and maltopentaose-forming amylase. Thus, halo bands on native-PAGE indicating starch hydrolysis may not entirely due to a single amylase and there is possibility of cross-activity by multiple amylases (Carrillo-Sancen et al., 2016). Presence of isoforms also complex the purification process for each amylase (Sahnoun et al., 2012), causing difficulties in the characterisation. Further work of purifying individual band and peptide sequencing are important to identify the produced amylase.

5.6 Characterisation of Partial Purified Amylase

5.6.1 Effect of Temperature on Amylase Activity

A temperature of 40 °C was found to favour the activity of the partial purified amylase from *Penicillium* sp. BB3. This is consistent with analysis results of amylase produced by *P. chrysogenum* isolated from air which also exhibited maximum activity at 40 °C (Balkan & Ertan, 2005). In contrast, partial purified amylase from *Penicillium* sp. isolated from decayed raw corn in the study of Sun et al. (2007) had a higher optimum temperature of 65 °C. Arora et al. (2017) showed that crude amylase of *Penicillium* sp. growing on different substrates had varying optimum temperature for its activity. Amylase produced with wheat bran as the substrate worked best at 55 °C while the enzyme produced with rice bran had an optimum temperature of 90 °C, indicating the difference in secreted amylase or enzyme characteristics depended upon the substrates used. On the other hand, Lin et al. (2011) reported a milder optimum temperature of 50 °C for the action of their crude starch degrading enzyme.

The study on optimum temperature for activity of partial purified amylase from *A. niger* KBA2 found that the enzyme performed best under 50 °C, where a similar result was observed by Aisien and Igbinosa (2020). Similarly, Lago et al. (2021) reported optimum temperature for purified amylase from *A. wentii* was 50 °C when incubated under pH 7. Additionally, they found that optimum temperature for amylase activity increased (60 °C) when hydrolysis was carried out under its optimum pH (pH 5). This suggested that pH and temperature might affect one another. A lower optimum temperature was reported by Obafemi et al. (2018), where the activity of purified amylase peaked under incubation at 30 °C. There were also reports of purified amylase from *A. niger* that preferred higher temperature of 60°C (Slivinsk et al., 2011) while Rodríguez et al. (2023) reported an optimum temperature of 70 °C.

Temperature is an important factor in enzymatic activity. As observed in both species, amylase activity showed an increasing trend before reaching the peak at the optimum temperature and declined under higher temperature. This is attributed to the fact that rate of molecular motion increases with temperature and extreme high temperature causes protein denaturation which severely impact reaction stability (Martgrita et al., 2023). Temperature that causes denaturation differs across enzymes, where denaturation becomes more significant at temperatures above 40 °C (Robinson, 2015). Declination of amylase activity to almost zero at 90 °C in both species suggested that enzyme denaturation happened at this temperature.

As mentioned before, optimum temperature for partial purified amylase of strain BB3 was an environment most favourable for a combination of isoforms or different types of enzymes. Differences in optimum temperature might exist among isoforms, as mentioned by Jebor et al. (2014) where glucoamylase A favoured 40 °C while glucoamylase B work best under 30 °C. However, there was also report on isoforms with similar characteristic in temperature, for example Sahnoun et al. (2012) reported that both isoforms of amylase favoured 50 °C for their activity. The amylases of strain BB3 might also be of different types with different characteristics or they might be a combination of distinct enzymes, where 40 °C was a temperature that generally support enzymatic activity. Therefore, isolation and characterisation of each amylase are required for better understanding.

5.6.2 Effect of pH on Amylase Activity

This study revealed a neutral pH of 7 was optimal for maximising the activity of partially purified amylase from *Penicillium* sp. BB3. Similarly, Saber et al. (2021) revealed purified amylase of *P. citrinum* isolated from soil worked best at pH 7.1. Sun et

al. (2007) reported a slightly acidic pH of 6.5 for *Penicillium* sp. More acidic conditions were also reported by Lin and co-workers, where crude amylase from *Penicillium* sp. grown under basal fermentation medium exhibited maximum activity at pH 4.5 (Lin et al., 2011), like observation of Xu et al. (2016) on amylase from *P. oxalicum*. When cultured on wheat bran and rice bran, *Penicillium* sp. produce amylase that had the highest activity under a basic environment of pH 9 (Arora et al., 2017).

For *A. niger* KBA2, the partially purified amylase exhibited highest activity at pH 5, like a previous study that worked on purified amylase from *A. niger* grown on potato processing residue (Slivinsk et al., 2011). Another research culturing *A. niger* on cassava peel also reported optimum pH 5 for activity of purified amylase (Aisien & Igbinosa, 2020). Purified amylase from *A. wentii* also had an optimum pH 5 for hydrolysing the substrate (Lago et al., 2021). Angelia et al. (2019) studied the effect of pH on amylase activity of *A. niger* crude extract and revealed that the enzyme worked best between in the range of pH 5 and 6. On the other hand, study by Piyyarathne and Weerasooriya (2020) reported an optimal pH 6. As reported by Obafemi et al. (2018), partially purified amylase of *A. niger* isolated from deteriorated tomato worked best under pH 6. In the study by Rodríguez et al. (2023), they found that purified amylase required a much more acidic environment of pH 3.

Significant decrease in amylase activity under both pH flanking the optimum value indicates that the enzyme is very sensitive to changes in pH and has a limited range of pH for its activity (Abdullah et al., 2017). Changes in pH values will cause alteration in bonding, which will in turn affect conformation of enzyme active site (Jakheng et al., 2020). Hydrogen bond will be affected and conformation changes will cause inability of substrate to bond with the active site (Martgrita et al., 2023). Elements of carboxylic acids

in an enzyme would also be altered by changes in pH, therefore affecting its function (Jebor et al., 2014). Maalej et al. (2021) explained that under extreme pH values, electrostatic repulsion resulting from high net charge within the enzyme molecules will occur and cause activity loss. Partial purified amylase of strain BB3 and KBA2 had similar optimum pH in both their production and activity indicated that the fungi secreted amylase corresponds to the pH which the enzyme works best. This observation was supported by the fact that fungi secrete enzymes at pH that they could function (Peñalva et al., 2008).

Similar to characterisation of temperature, optimum pH of partial purified amylases from strain BB3 was a combination of characteristic of all amylases present. Isoforms, if present in partial purified amylase from strain BB3, might be having different optimum pH, as seen in previous studies. Sahnoun et al. (2012) found that AmyA and AmyB preferred pH 5 and 6 respectively, while both amylases averagely worked best at pH 5.6. On the other hand, Jebor et al. (2014) reported a wider difference in optimum pH values between two amylase isoforms in their study. An isoform favoured pH 6.5 while another exhibited highest activity under pH 8. However, there was also reports of amylase isoforms exhibiting similar pattern under different pH values. Ubonbal et al. (2017) reported that amylase Isoform I and Isoform II both favoured pH 4, and activities of both isoforms declined with increasing pH values. Different types of starch hydrolytic enzymes with different optimum pH might also be present. Therefore, possibility of enzymes produced by strain BB3 having various optimum pH flanking pH 7 could not be rejected unless performing further studies on isolating and characterising every enzyme present.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study successfully isolated and identified 41 indigenous fungal isolates from several waste sources. Study of amylolytic activity of the isolates revealed several species with high amylolytic potential, which are *Penicillium* sp. BB3, *P. citrinum* KBA4, *A. europaeus* BNN1 and also *A. niger* KBA2. This highlighted the importance of local wastes as sources of potential fungi. Optimising enzyme production revealed that different conditions were required for amylase production from the four selected species. Characterisation of amylase revealed that *A. niger* KBA2 produced a single isoform of amylase, while *Penicillium* sp. BB3 synthesised multiple isoforms or types of amylases with distinctive sizes. Additionally, partially purified amylases from these species exhibited variation in the requirements of enzyme production, partial purified amylase produced by the selected species favoured different conditions for their activity. Overall, the methods implemented in this work were effective to achieve the objectives of this work aligning with findings from previous studies. Results obtained in this research also laid foundation for future work in investigating practical applications of the fungal isolates and serve as reference for similar studies on amylolytic fungi. Therefore, this study contributed to the knowledge profile in indigenous fungi on local wastes.

6.2 Recommendation

The diversity of isoforms and types of amylases in *Penicillium* sp. BB3 suggested the adaptability of this species to different substrates. Therefore, future studies can focus on investigating the potential of this fungus in waste management and reutilisation, especially food waste where the fungus was isolated. Apart from that, high amylolytic

activity and ease of enzyme purification of a single type of amylase secreted by *A. niger* KBA2 suggested this species has a potential for amylase production. However, further work is needed for a more comprehensive understanding on the amylase produced by this species. Amylase production and activity should be thoroughly optimised with other applicable physicochemical parameters such as substrate concentration and effect of metal ions. Additionally, sequence and structure analysis of amylase, as well as genes coding for the protein and studies at transcriptional level are recommended.

Limitations were also discovered in this work and were discussed, which include limitation in solely investigating extracellular amylase production and usage of a single type of media (Chapter 5.3). Monitoring of pH throughout the fermentation process was also absent (Chapter 5.4.2). Identity and characteristics of purified amylases remains unknown, particularly the amylases produced by strain BB3 (Chapter 5.5; Chapter 5.6.1; Chapter 5.6.2). It should also be noted that waste samples were limited due constraints in time and therefore results in this study did not represent the entire fungal population and diversity in the local context. These limitations should be addressed to enhance future research efforts in this area.

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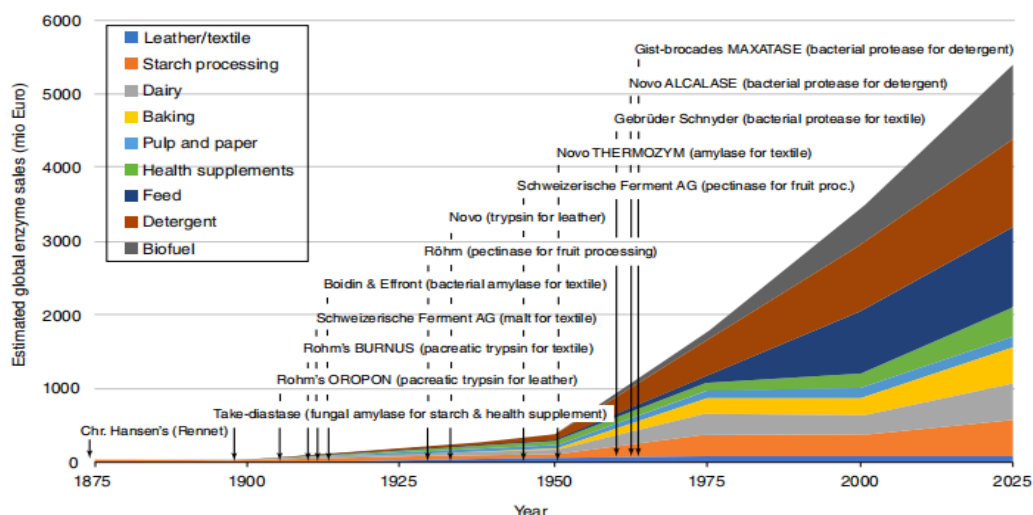
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APPENDICES

Appendix A: Development of enzyme market and estimated sales growth of enzymes by the year 2025 (Adapted from May, 2019)



Appendix B: PCR mixture preparation (Adapted from Takam et al., 2019)

Components	Required Volume (µl)
Dream Taq PCR Master Mix (2X)	10.0
ITS1 Forward Primer	1.0
ITS4 Reverse Primer	1.0
Nuclease-Free Water	6.0
DNA Template	2.0
Total Volume	20.0

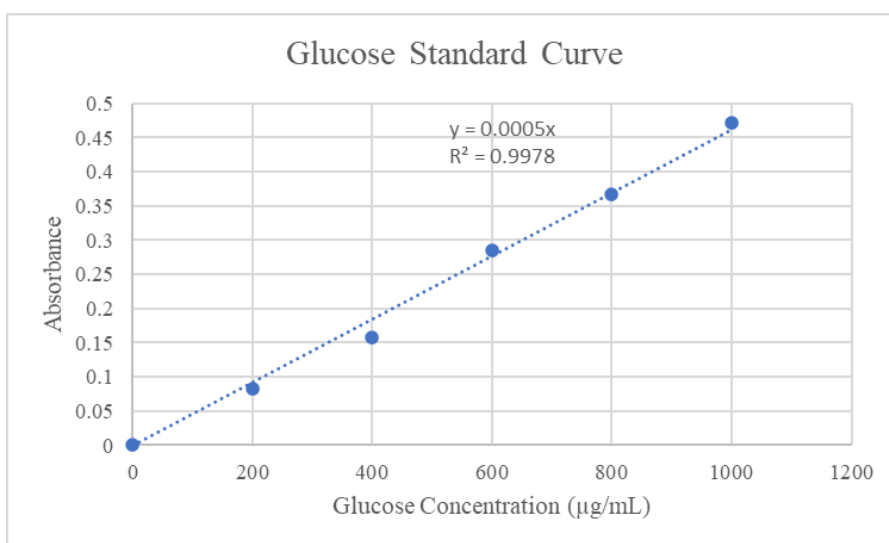
Appendix C: Criteria for PCR (Adapted and modified from Scibetta et al., 2020)

Stage	Temperature (° C)	Duration	Remark
Initial denaturation	95	2 minutes	-
Denaturation	95	30 seconds	30 Cycles
Annealing	55	30 seconds	
Initial extension	72	1 minute	
Final extension	72	5 minutes	-

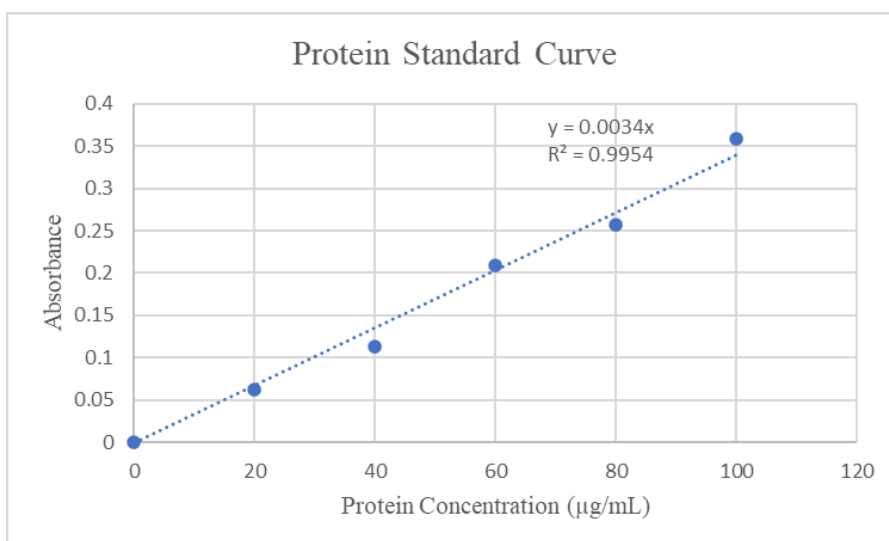
Appendix D: Preparation of DNS solution (Adapted from Lam et al., 2021)

Components	Volume of Solution (ml)
1g DNS powder	20.0 ml NaOH
30g Rochelle salt	50.0 ml distilled water
Distilled water	30.0
Total volume	100.0

Appendix E: Glucose standard curve for amylase assay



Appendix F: Protein standard curve for Bradford assay



Appendix G: Composition of 5% stacking gel (Adapted from Laemmli, 1970)

Components	Required Volume
30% acrylamide/bisacrylamide	0.425 ml
0.5M Tris-HCl (pH 6.8)	0.625 ml
10% SDS	25 µl
10% Ammonium persulphate	12.5 µl
TEMED	2.5 µl
Distilled water	1.425 ml

Appendix H: Composition of 12% resolving gel (Adapted from Laemmli, 1970)

Components	Required Volume
30% acrylamide/bisacrylamide	2.0 ml
1.5M Tris-HCl (pH 8.8)	1.25 ml
10% SDS	50 µl
10% Ammonium persulphate	25 µl
TEMED	2.5 µl
Distilled water	1.70 ml

Appendix I: Composition of 1X SDS-PAGE running buffer (Adapted from Gallagher, 2012)

Components	Required Volume/Amount
Tris Base	3.03 g
Glycine	14.4 g
SDS	1.0 g
Distilled water	1 l

Appendix J: Composition of 1X SDS-PAGE sample buffer, also known as Laemmli buffer (Adapted from Laemmli, 1970)

Components	Concentration
Tris-HCl buffer (pH 6.8)	31.5 mM
Glycerol	10%
SDS	1%
Bromophenol blue	0.005%

Appendix K: Composition of Coomassie staining solution (Adapted from Laemmli, 1970)

Components	Required Volume/Amount
Ethanol	150 ml
Glacial acetic acid	50 ml
Distilled water	300 ml
Coomassie brilliant blue R-250	1 g

Appendix L: Composition of 1X SDS-PAGE running buffer (Adapted from Gallagher, 2012)

Components	Required Volume
Tris Base	3.03 g
Glycine	14.4 g
Distilled water	1 l

Appendix M: Composition of buffers for amylase assay under different pH (Adapted from Karim et al., 2018)

Sodium citrate buffer (0.1 M, pH 3)	
Components	Required Volume/Amount
Sodium citrate	1.01 g
Citric acid	0.88 g
Starch	0.8 g
Distilled water	80 ml
Sodium acetate buffer (0.1 M, pH 4)	
Components	Required Volume/Amount
Sodium acetate	0.10 g
Acetic acid	0.39 ml
Starch	0.8 g
Distilled water	100 ml

Appendix M continued

Sodium acetate buffer (0.1 M, pH 5)	
Components	Required Volume/Amount
Sodium acetate	0.42 g
Acetic acid	0.15 ml
Starch	0.8 g
Distilled water	100 ml
Potassium phosphate buffer (0.1 M, pH 6)	
Components	Required Volume/Amount
Potassium phosphate monobasic	1.02 g
Potassium phosphate dibasic	0.08 g
Starch	0.8 g
Distilled water	80 ml

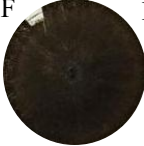
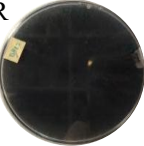
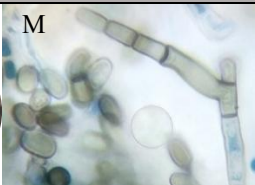
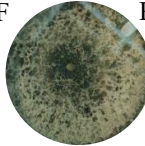
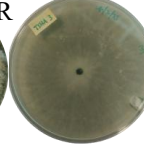
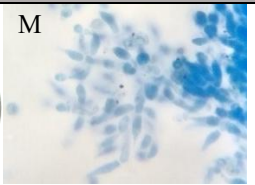
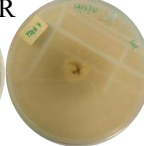
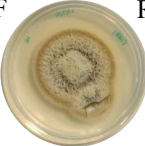
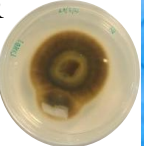
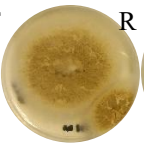
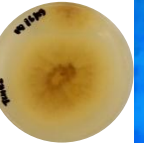
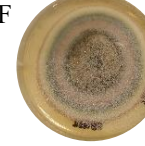
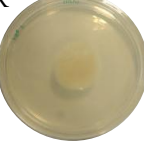
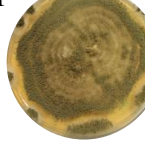
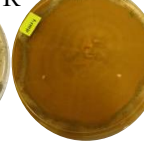
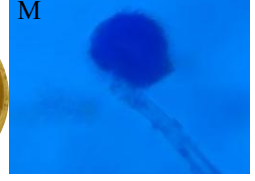
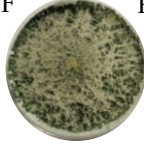
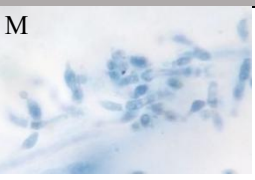
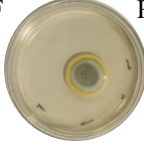
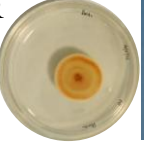
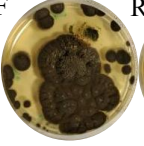
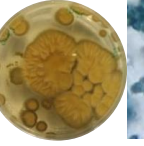
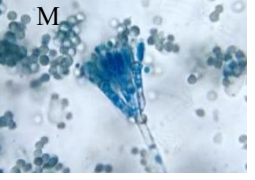
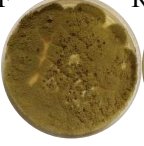
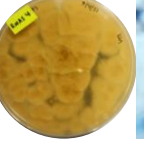
Appendix M continued

Potassium phosphate buffer (0.1 M, pH 7)	
Components	Required Volume/Amount
Potassium phosphate monobasic	0.66 g
Potassium phosphate dibasic	0.54 g
Starch	0.8 g
Distilled water	80 ml
Tris-HCl buffer (0.1 M, pH 8)	
Components	Required Volume/Amount
Tris Base	0.43 g
HCl	0.13 ml
Starch	0.8 g
Distilled water	80 ml

Appendix M continued

Tris-HCl buffer (0.1 M, pH 9)	
Components	Required Volume/Amount
Tris Base	0.86 g
HCl	0.027 ml
Starch	0.8 g
Distilled water	80 ml

Appendix N: Images of macroscopic and microscopic characteristics of fungal isolates

Sago Pith Waste		
F	R	M
		
TSHA1		
F	R	M
		
TSHA2		
F	R	M
		
TSHA3		
F	R	M
		
TSHB1		
F	R	M
		
TSHB2		
F	R	M
		
TSHB3		
F	R	M
		
MSHB2		
F	R	M
		
MSHB3		
Washed Rice Water		
F	R	M
		
RWA1		
F	R	M
		
RWA2		
F	R	M
		
RWA3		
F	R	M
		
RWA4		

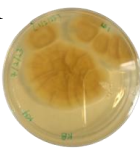
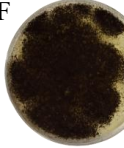
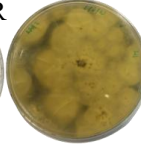
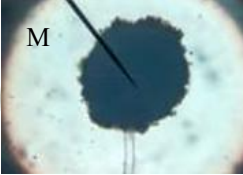
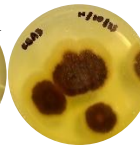
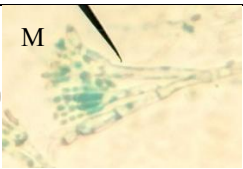
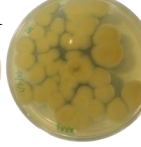
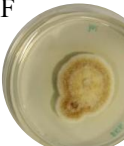
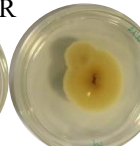
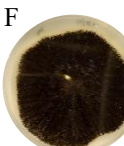
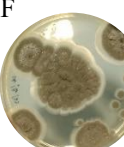
Appendix N continued

F R M	RWA5
F R M	RWB1
F R M	RWB2
F R M	RWB4
F R M	RWB5
F R M	RWB7
F R M	RWB8
Potato	
F R M	PA1
F R M	PA2
F R M	PA3
F R M	PB1

Appendix N continued

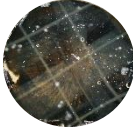
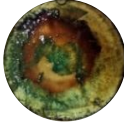
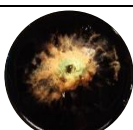
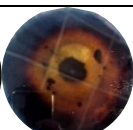
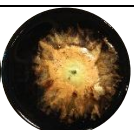
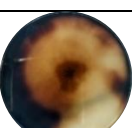
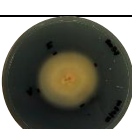
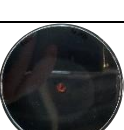
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PB2	PB3
F R M	-
PB4	
Bread	
F R M	F R M
BA1	BA2
F R M	F R M
BA3	BA4
F R M	F R M
BA5	BB1
F R M	F R M
BB2	BB3

Appendix N continued

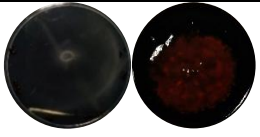
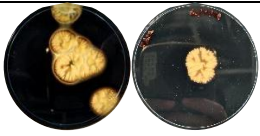
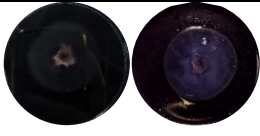
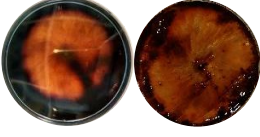
Glutinous Rice Cake		
 F  R  M	 F  R  M	
KBA1		KBA2
 F  R  M	 F  R  M	
KBA3		KBA4
Banana Cake		
 F  R  M	 F  R  M	
BNN1		BNN2
 F  R  M		
BNN3		

Note: F=Front view of isolates, R=Reverse view of isolates, M=Microscopic view of isolates

Appendix O: Results of amylase screening in fungal isolated from starch waste

Starch Waste	Code	Iodine Staining		Code	Iodine Staining	
Sago Pith Waste	TSHA1			TSHB2		
	TSHA2			TSHB3		
	TSHA3			MSHB2		
	TSHB1			MSHB3		
Washed Rice Water	RWA1			RWB2		
	RWA2			RWB4		
	RWA3			RWB5		
	RWA4			RWB7		
	RWA5			RWB8		
	RWB1			-	-	

Appendix O continued

Potato	PA1		PB2	
	PA2		PB3	
	PA3		PB4	
	PB1		-	-
Bread	BA1		BA5	
	BA2		BB1	
	BA3		BB2	
	BA4		BB3	
Glutinous Rice Cake	KBA1		KBA3	
	KBA2		KBA4	
Banana Cake	BNN1		BNN3	

Appendix O continued

Banana Cake	BNN2		-	-
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Appendix P: Tabular results, One-Way ANOVA and homogenous subsets of EAI of amylolytic isolates

Source	Species	Code	EAI
Sago Pith Waste	<i>Trichoderma asperellum</i>	TSHA2	0.82±0.05
	<i>Paecilomyces maximus</i>	TSHA3	0.94±0.01
	<i>Pleurostoma richardsiae</i>	TSHB1	0.71±0.09
	<i>Paecilomyces maximus</i>	TSHB2	0.91±0.01
	<i>Talaromyces siamensis</i>	TSHB3	0.85±0.06
	<i>Ophiostoma valdivianum</i>	MSHB2	0.85±0.05
	<i>Aspergillus nomiae</i>	MSHB3	0.94±0.08
Washed Rice Water	<i>Trichoderma asperellum</i>	RWA1	1.00±0.00
	<i>Talaromyces siamensis</i>	RWA2	0.91±0.02
	<i>Penicillium citrinum</i>	RWA3	1.22±0.08
	<i>Aspergillus flavus</i>	RWA4	1.15±0.03
	<i>Aspergillus fumigatus</i>	RWA5	0.72±0.05
	<i>Trichoderma asperellum</i>	RWB2	0.79±0.01
	<i>Aspergillus oryzae</i>	RWB4	0.93±0.01
	<i>Aspergillus fumigatus</i>	RWB5	0.72±0.08
	<i>Talaromyces siamensis</i>	RWB7	1.00±0.01
	<i>Aspergillus tubingensis</i>	RWB8	0.96±0.02

Appendix P continued

Potato	<i>Aspergillus niger</i>	PA1	1.07±0.08
	<i>Penicillium citrinum</i>	PA2	1.25±0.12
	<i>Agroathelia delphinii</i>	PB1	0.74±0.06
	<i>Aspergillus niger</i>	PB3	0.91±0.06
	<i>Penicillium citrinum</i>	PB4	1.20±0.03
Bread	<i>Aspergillus</i> sp.	BA2	1.14±0.27
	<i>Aspergillus fumigatus</i>	BA3	0.73±0.05
	<i>Aspergillus niger</i>	BA4	1.02±0.09
	<i>Penicillium citrinum</i>	BA5	1.12±0.02
	<i>Penicillium</i> sp.	BB3	1.45±0.10
Glutinous Rice Cake	<i>Aspergillus flocculosus</i>	KBA1	1.07±0.04
	<i>Aspergillus niger</i>	KBA2	1.16±0.02
	<i>Penicilium expansum</i>	KBA3	0.99±0.05
	<i>Penicillium citrinum</i>	KBA4	1.36±0.02
Banana Cake	<i>Aspergillus europaeus</i>	BNN1	1.19±0.04
	<i>Aspergillus niger</i>	BNN2	0.92±0.02
	<i>Penicillium citrinum</i>	BNN3	1.13±0.05

ANOVA

EAI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.520	33	.107	21.268	<.001
Within Groups	.341	68	.005		
Total	3.861	101			

EAI

Tukey HSD^a

		Subset for alpha = 0.05											
Fungl_Species	N	1	2	3	4	5	6	7	8	9	10	11	12
TSHB1	3	.7133											
RWA5	3	.7167											
RWB5	3	.7200											
BA3	3	.7300	.7300										
PB1	3	.7400	.7400										
RWB2	3	.7933	.7933	.7933									
TSHA2	3	.8167	.8167	.8167									
MSHB2	3	.8467	.8467	.8467	.8467								
TSHB3	3	.8533	.8533	.8533	.8533								
TSHB2	3	.9100	.9100	.9100	.9100	.9100							
RWA2	3	.9133	.9133	.9133	.9133	.9133							
PB3	3	.9133	.9133	.9133	.9133	.9133							
BNN2	3	.9167	.9167	.9167	.9167	.9167							
RWB4	3	.9300	.9300	.9300	.9300	.9300	.9300						
MSHB3	3	.9367	.9367	.9367	.9367	.9367	.9367	.9367					
TSHA3	3	.9433	.9433	.9433	.9433	.9433	.9433	.9433					
RWB8	3		.9600	.9600	.9600	.9600	.9600	.9600	.9600				
KBA3	3			.9933	.9933	.9933	.9933	.9933	.9933	.9933			
RWB7	3			.9967	.9967	.9967	.9967	.9967	.9967	.9967			
RWA1	3			1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000			
BA4	3			1.0200	1.0200	1.0200	1.0200	1.0200	1.0200	1.0200	1.0200		
PA1	3				1.0733	1.0733	1.0733	1.0733	1.0733	1.0733	1.0733		
KBA1	3				1.0733	1.0733	1.0733	1.0733	1.0733	1.0733	1.0733		
BA5	3					1.1233	1.1233	1.1233	1.1233	1.1233	1.1233		
BNN3	3					1.1267	1.1267	1.1267	1.1267	1.1267	1.1267		
BA2	3					1.1400	1.1400	1.1400	1.1400	1.1400	1.1400	1.1400	
RWA4	3						1.1533	1.1533	1.1533	1.1533	1.1533	1.1533	
KBA2	3							1.1633	1.1633	1.1633	1.1633	1.1633	
BNN1	3								1.1867	1.1867	1.1867	1.1867	
PB4	3									1.2000	1.2000	1.2000	
RWA3	3									1.2200	1.2200	1.2200	1.2200
PA2	3										1.2500	1.2500	1.2500
KBA4	3											1.3600	1.3600
BB3	3												1.4467
Sig.		.051	.051	.060	.060	.051	.070	.060	.060	.060	.051	.082	.060

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix Q: Post Hoc test in General Linear Model (Multivariate) analysis of optimum pH for amylase production in four selected species

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Amylase_Activity_A. europaeus	3	4	-.4767 [*]	.04959	<.001	-.6432	-.3101
		5	-.1367	.04959	.134	-.3032	.0299
		6	-.1633	.04959	.056	-.3299	.0032
		7	-.3100 [*]	.04959	<.001	-.4766	-.1434
		8	-.4833 [*]	.04959	<.001	-.6499	-.3168
	4	3	.4767 [*]	.04959	<.001	.3101	.6432
		5	.3400 [*]	.04959	<.001	.1734	.5066
		6	.3133 [*]	.04959	<.001	.1468	.4799
		7	.1667 [*]	.04959	.050	.0001	.3332
		8	-.0067	.04959	1.000	-.1732	.1599
	5	3	.1367	.04959	.134	-.0299	.3032
		4	-.3400 [*]	.04959	<.001	-.5066	-.1734
		6	-.0267	.04959	.993	-.1932	.1399
		7	-.1733 [*]	.04959	.040	-.3399	-.0068
		8	-.3467 [*]	.04959	<.001	-.5132	-.1801
	6	3	.1633	.04959	.056	-.0032	.3299
		4	-.3133 [*]	.04959	<.001	-.4799	-.1468
		5	.0267	.04959	.993	-.1399	.1932
		7	-.1467	.04959	.097	-.3132	.0199
		8	-.3200 [*]	.04959	<.001	-.4866	-.1534
	7	3	.3100 [*]	.04959	<.001	.1434	.4766
		4	-.1667 [*]	.04959	.050	-.3332	-.0001
		5	.1733 [*]	.04959	.040	.0068	.3399
		6	.1467	.04959	.097	-.0199	.3132
		8	-.1733 [*]	.04959	.040	-.3399	-.0068
	8	3	.4833 [*]	.04959	<.001	.3168	.6499
		4	.0067	.04959	1.000	-.1599	.1732
		5	.3467 [*]	.04959	<.001	.1801	.5132
		6	.3200 [*]	.04959	<.001	.1534	.4866
		7	.1733 [*]	.04959	.040	.0068	.3399
Fungal_Biomass_A. europaeus	3	4	-.0900	.05796	.641	-.2847	.1047
		5	.0267	.05796	.997	-.1680	.2213
		6	-.1867	.05796	.063	-.3813	.0080
		7	-.0633	.05796	.875	-.2580	.1313
		8	-.0667	.05796	.851	-.2613	.1280
	4	3	.0900	.05796	.641	-.1047	.2847
		5	.1167	.05796	.389	-.0780	.3113
		6	-.0967	.05796	.574	-.2913	.0980
		7	.0267	.05796	.997	-.1680	.2213
		8	.0233	.05796	.998	-.1713	.2180
	5	3	-.0267	.05796	.997	-.2213	.1680
		4	-.1167	.05796	.389	-.3113	.0780
		6	-.2133 [*]	.05796	.029	-.4080	-.0187
		7	-.0900	.05796	.641	-.2847	.1047
		8	-.0933	.05796	.607	-.2880	.1013
	6	3	.1867	.05796	.063	-.0080	.3813
		4	.0967	.05796	.574	-.0980	.2913
		5	.2133 [*]	.05796	.029	.0187	.4080
		7	.1233	.05796	.336	-.0713	.3180
		8	.1200	.05796	.362	-.0747	.3147
	7	3	.0633	.05796	.875	-.1313	.2580
		4	-.0267	.05796	.997	-.2213	.1680
		5	.0900	.05796	.641	-.1047	.2847
		6	-.1233	.05796	.336	-.3180	.0713
		8	-.0033	.05796	1.000	-.1980	.1913
	8	3	.0667	.05796	.851	-.1280	.2613
		4	-.0233	.05796	.998	-.2180	.1713
		5	.0933	.05796	.607	-.1013	.2880
		6	-.1200	.05796	.362	-.3147	.0747
		7	.0033	.05796	1.000	-.1913	.1980

Based on observed means.

The error term is Mean Square(Error) = .005.

*, The mean difference is significant at the .05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Amylase_Activity_A.niger	3	4	.3433	.16792	.374	-.2207	.9074
		5	-.6267*	.16792	.027	-1.1907	-.0626
		6	-.5967*	.16792	.036	-1.1607	-.0326
		7	-.5900*	.16792	.039	-1.1540	-.0260
		8	.1067	.16792	.986	-.4574	.6707
	4	3	-.3433	.16792	.374	-.9074	.2207
		5	-.9700*	.16792	<.001	-1.5340	-.4060
		6	-.9400*	.16792	.001	-1.5040	-.3760
		7	-.9333*	.16792	.001	-1.4974	-.3693
		8	-.2367	.16792	.722	-.8007	.3274
	5	3	.6267*	.16792	.027	.0626	1.1907
		4	.9700*	.16792	<.001	.4060	1.5340
		6	.0300	.16792	1.000	-.5340	.5940
		7	.0367	.16792	1.000	-.5274	.6007
		8	.7333*	.16792	.009	.1693	1.2974
	6	3	.5967*	.16792	.036	.0326	1.1607
		4	.9400*	.16792	.001	.3760	1.5040
		5	-.0300	.16792	1.000	-.5940	.5340
		7	.0067	.16792	1.000	-.5574	.5707
		8	.7033*	.16792	.012	.1393	1.2674
	7	3	.5900*	.16792	.039	.0260	1.1540
		4	.9333*	.16792	.001	.3693	1.4974
		5	-.0367	.16792	1.000	-.6007	.5274
		6	-.0067	.16792	1.000	-.5707	.5574
		8	.6967*	.16792	.013	.1326	1.2607
	8	3	-.1067	.16792	.986	-.6707	.4574
		4	.2367	.16792	.722	-.3274	.8007
		5	-.7333*	.16792	.009	-1.2974	-.1693
		6	-.7033*	.16792	.012	-1.2674	-.1393
		7	-.6967*	.16792	.013	-1.2607	-.1326
Fungal_Biomass_A.niger	3	4	-.2800	.10921	.180	-.6468	.0868
		5	.0033	.10921	1.000	-.3635	.3701
		6	-.0033	.10921	1.000	-.3701	.3635
		7	.0067	.10921	1.000	-.3601	.3735
		8	-.0467	.10921	.998	-.4135	.3201
	4	3	.2800	.10921	.180	-.0868	.6468
		5	.2833	.10921	.172	-.0835	.6501
		6	.2767	.10921	.189	-.0901	.6435
		7	.2867	.10921	.164	-.0801	.6535
		8	.2333	.10921	.332	-.1335	.6001
	5	3	-.0033	.10921	1.000	-.3701	.3635
		4	-.2833	.10921	.172	-.6501	.0835
		6	-.0067	.10921	1.000	-.3735	.3601
		7	.0033	.10921	1.000	-.3635	.3701
		8	-.0500	.10921	.997	-.4168	.3168
	6	3	.0033	.10921	1.000	-.3635	.3701
		4	-.2767	.10921	.189	-.6435	.0901
		5	.0067	.10921	1.000	-.3601	.3735
		7	.0100	.10921	1.000	-.3568	.3768
		8	-.0433	.10921	.998	-.4101	.3235
	7	3	-.0067	.10921	1.000	-.3735	.3601
		4	-.2867	.10921	.164	-.6535	.0801
		5	-.0033	.10921	1.000	-.3701	.3635
		6	-.0100	.10921	1.000	-.3768	.3568
		8	-.0533	.10921	.996	-.4201	.3135
	8	3	.0467	.10921	.998	-.3201	.4135
		4	-.2333	.10921	.332	-.6001	.1335
		5	.0500	.10921	.997	-.3168	.4168
		6	.0433	.10921	.998	-.3235	.4101
		7	.0533	.10921	.996	-.3135	.4201

Based on observed means.

The error term is Mean Square(Error) = .018.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Amylase_Activity_P. citrinum	3	4	-.2700*	.03590	<.001	-.3906	-.1494
		5	-.4833*	.03590	<.001	-.6039	-.3627
		6	-.0500	.03590	.731	-.1706	.0706
		7	-.2133*	.03590	<.001	-.3339	-.0927
	4	3	.2700*	.03590	<.001	.1494	.3906
		5	-.2133*	.03590	<.001	-.3339	-.0927
		6	.2200*	.03590	<.001	.0994	.3406
		7	.0567	.03590	.626	-.0639	.1773
	5	3	.1900*	.03590	.002	.0694	.3106
		4	.4833*	.03590	<.001	.3627	.6039
		6	.2133*	.03590	<.001	.0927	.3339
		7	.4333*	.03590	<.001	.3127	.5539
	6	3	.2700*	.03590	<.001	.1494	.3906
		4	.4033*	.03590	<.001	.2827	.5239
		5	.0500	.03590	.731	-.0706	.1706
		7	-.2200*	.03590	<.001	-.3406	-.0994
	7	3	-.4333*	.03590	<.001	-.5539	-.3127
		4	-.1633*	.03590	.007	-.2839	-.0427
		5	-.0300	.03590	.955	-.1506	.0906
		8	.2133*	.03590	<.001	.0927	.3339
	8	3	-.0567	.03590	.626	-.1773	.0639
		4	-.2700*	.03590	<.001	-.3906	-.1494
		5	.1633*	.03590	.007	.0427	.2839
		6	.1333*	.03590	.027	.0127	.2539
Fungal_Biomass_P. citrinum	3	4	.0800	.03590	.293	-.0406	.2006
		5	-.1900*	.03590	.002	-.3106	-.0694
		6	-.4033*	.03590	<.001	-.5239	-.2827
		7	.0300	.03590	.955	-.0906	.1506
	4	3	-.1333*	.03590	.027	-.2539	-.0127
		5	-.3933*	.04753	<.001	-.5530	-.2337
		6	-.4367*	.04753	<.001	-.5963	-.2770
		7	-.3567*	.04753	<.001	-.5163	-.1970
	5	3	-.3400*	.04753	<.001	-.4997	-.1803
		4	-.1033	.04753	.316	-.2630	.0563
		6	.3933*	.04753	<.001	.2337	.5530
		7	-.0433	.04753	.936	-.2030	.1163
	6	3	.0367	.04753	.967	-.1230	.1963
		4	.0533	.04753	.863	-.1063	.2130
		5	.2900*	.04753	<.001	.1303	.4497
		7	.4367*	.04753	<.001	.2770	.5963
	7	3	.0433	.04753	.936	-.1163	.2030
		4	.0800	.04753	.566	-.0797	.2397
		5	.0967	.04753	.379	-.0630	.2563
		6	.3333*	.04753	<.001	.1737	.4930
	8	3	.3567*	.04753	<.001	.1970	.5163
		4	-.0367	.04753	.967	-.1963	.1230
		5	-.0800	.04753	.566	-.2397	.0797
		6	.0167	.04753	.999	-.1430	.1763
	3	4	.2533*	.04753	.002	.0937	.4130
		5	.3400*	.04753	<.001	.1803	.4997
		6	-.0533	.04753	.863	-.2130	.1063
		7	-.0967	.04753	.379	-.2563	.0630
	4	3	-.0167	.04753	.999	-.1763	.1430
		5	.2367*	.04753	.003	.0770	.3963
		6	.1033	.04753	.316	-.0563	.2630
		7	-.2900*	.04753	<.001	-.4497	-.1303
	5	3	-.3333*	.04753	<.001	-.4930	-.1737
		4	-.2533*	.04753	.002	-.4130	-.0937
		6	-.2367*	.04753	.003	-.3963	-.0770
		7	-.2367*	.04753	.003	-.3963	-.0770

Based on observed means.

The error term is Mean Square(Error) = .003.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Amylase_Activity_Penicillium_sp	3	4	-.5567*	.08602	<.001	-.8456	-.2677
		5	-.1300	.08602	.664	-.4189	.1589
		6	-.3967*	.08602	.006	-.6856	-.1077
		7	-.7800*	.08602	<.001	-1.0689	-.4911
		8	-.3767*	.08602	.009	-.6656	-.0877
	4	3	.5567*	.08602	<.001	.2677	.8456
		5	.4267*	.08602	.003	.1377	.7156
		6	.1600	.08602	.468	-.1289	.4489
		7	-.2233	.08602	.172	-.5123	.0656
	5	3	.1300	.08602	.664	-.1589	.4189
		4	-.4267*	.08602	.003	-.7156	-.1377
		6	-.2667	.08602	.077	-.5556	.0223
		7	-.6500*	.08602	<.001	-.9389	-.3611
	6	3	-.2467	.08602	.112	-.5356	.0423
		4	.3967*	.08602	.006	.1077	.6856
		5	.2667	.08602	.077	-.0223	.5556
		7	-.3833*	.08602	.008	-.6723	-.0944
	7	3	.0200	.08602	1.000	-.2689	.3089
		4	.7800*	.08602	<.001	.4911	1.0689
		5	.2233	.08602	.172	-.0656	.5123
		6	.6500*	.08602	<.001	.3611	.9389
	8	3	.3833*	.08602	.008	.0944	.6723
		4	.4033*	.08602	.005	.1144	.6923
		5	.3767*	.08602	.009	.0877	.6656
		6	-.1800	.08602	.352	-.4689	.1089
Fungal_Biomass_Penicillium_sp	3	4	-.1100	.07118	.645	-.3491	.1291
		5	-.1300	.07118	.486	-.3691	.1091
		6	-.1500	.07118	.345	-.3891	.0891
		7	-.2333	.07118	.057	-.4724	.0058
		8	-.3400*	.07118	.005	-.5791	-.1009
	4	3	.1100	.07118	.645	-.1291	.3491
		5	-.0200	.07118	1.000	-.2591	.2191
		6	-.0400	.07118	.992	-.2791	.1991
	5	3	-.1233	.07118	.538	-.3624	.1158
		4	-.2300	.07118	.062	-.4691	.0091
	6	3	.2300	.07118	.062	-.4691	.0091
		4	.1300	.07118	.486	-.1091	.3691
		5	.0200	.07118	1.000	-.2591	.2191
		7	-.1033	.07118	.698	-.3424	.1358
	7	3	-.2100	.07118	.098	-.4491	.0291
		4	.1500	.07118	.345	-.0891	.3891
		5	.0400	.07118	.992	-.1991	.2791
		6	.0200	.07118	1.000	-.2591	.2191
	8	3	-.0833	.07118	.842	-.3224	.1558
		4	-.1900	.07118	.153	-.4291	.0491
		5	.2333	.07118	.057	-.0058	.4724
		6	.1233	.07118	.538	-.1158	.3624
	9	3	.1033	.07118	.698	-.1358	.3424
		4	.0833	.07118	.842	-.1558	.3224
		5	-.1067	.07118	.672	-.3458	.1324
		6	-.3400*	.07118	.005	-.5791	-.1009
	10	3	.2300	.07118	.062	-.4691	.0091
		4	.1300	.07118	.486	-.1091	.3691
		5	.0200	.07118	1.000	-.2591	.2191
		6	.1900	.07118	.153	-.0491	.4291
	11	3	.1067	.07118	.672	-.1324	.3458
		4	.1067	.07118	.672	-.1324	.3458
		5	.1067	.07118	.672	-.1324	.3458
		6	.1067	.07118	.672	-.1324	.3458

Based on observed means.

The error term is Mean Square(Error) = .008.

*. The mean difference is significant at the .05 level.

Appendix R: Post Hoc test in General Linear Model (Multivariate) analysis of optimum incubation period for amylase production in four selected species

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Incubation_Period	(J) Incubation_Period	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
Amylase_Activity_A. europaeus	3	5	.4000*	.04552	<.001	.2542	.5458
		7	.0700	.04552	.461	-.0758	.2158
		9	.4367*	.04552	<.001	.2909	.5824
	5	3	-.4000*	.04552	<.001	-.5458	-.2542
		7	-.3300*	.04552	<.001	-.4758	-.1842
		9	.0367	.04552	.850	-.1091	.1824
	7	3	-.0700	.04552	.461	-.2158	.0758
		5	.3300*	.04552	<.001	.1842	.4758
		9	.3667*	.04552	<.001	.2209	.5124
	9	3	-.4367*	.04552	<.001	-.5824	-.2909
		5	-.0367	.04552	.850	-.1824	.1091
		7	-.3667*	.04552	<.001	-.5124	-.2209
Fungal_Biomass_A. europaeus	3	5	-.1967*	.03464	.002	-.3076	-.0857
		7	-.4267*	.03464	<.001	-.5376	-.3157
		9	-.7133*	.03464	<.001	-.8243	-.6024
	5	3	.1967*	.03464	.002	.0857	.3076
		7	-.2300*	.03464	<.001	-.3409	-.1191
		9	-.5167*	.03464	<.001	-.6276	-.4057
	7	3	.4267*	.03464	<.001	.3157	.5376
		5	.2300*	.03464	<.001	.1191	.3409
		9	-.2867*	.03464	<.001	-.3976	-.1757
	9	3	.7133*	.03464	<.001	.6024	.8243
		5	.5167*	.03464	<.001	.4057	.6276
		7	.2867*	.03464	<.001	.1757	.3976

Based on observed means.

The error term is Mean Square(Error) = .002.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Incubation_Period	(J) Incubation_Period	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Amylase_Activity_A.niger	3	5	-.2500*	.06280	.017	-.4511	-.0489
		7	-.0133	.06280	.996	-.2145	.1878
		9	.0567	.06280	.804	-.1445	.2578
	5	3	.2500*	.06280	.017	.0489	.4511
		7	.2367*	.06280	.023	.0355	.4378
		9	.3067*	.06280	.005	.1055	.5078
	7	3	-.0133	.06280	.996	-.1878	.2145
		5	-.2367*	.06280	.023	-.4378	-.0355
		9	.0700	.06280	.691	-.1311	.2711
	9	3	-.0567	.06280	.804	-.2578	.1445
		5	-.3067*	.06280	.005	-.5078	-.1055
		7	-.0700	.06280	.691	-.2711	.1311
Fungal_Biomass_A.niger	3	5	-.1200	.06536	.325	-.3293	.0893
		7	-.3500*	.06536	.003	-.5593	-.1407
		9	-.5467*	.06536	<.001	-.7560	-.3374
	5	3	.1200	.06536	.325	-.0893	.3293
		7	-.2300*	.06536	.032	-.4393	-.0207
		9	-.4267*	.06536	<.001	-.6360	-.2174
	7	3	.3500*	.06536	.003	.1407	.5593
		5	.2300*	.06536	.032	.0207	.4393
		9	-.1967	.06536	.066	-.4060	.0126
	9	3	.5467*	.06536	<.001	.3374	.7560
		5	.4267*	.06536	<.001	.2174	.6360
		7	.1967	.06536	.066	-.0126	.4060

Based on observed means.

The error term is Mean Square(Error) = .006.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Incubation_Period	(J) Incubation_Period	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Amylase_Activity_P. citrinum	3	5	.1067	.03697	.078	-.0117	.2251
		7	-.0567	.03697	.463	-.1751	.0617
		9	-.0867	.03697	.167	-.2051	.0317
	5	3	-.1067	.03697	.078	-.2251	.0117
		7	-.1633*	.03697	.010	-.2817	-.0449
		9	-.1933*	.03697	.004	-.3117	-.0749
	7	3	.0567	.03697	.463	-.0617	.1751
		5	.1633*	.03697	.010	.0449	.2817
		9	-.0300	.03697	.847	-.1484	.0884
	9	3	.0867	.03697	.167	-.0317	.2051
		5	.1933*	.03697	.004	.0749	.3117
		7	.0300	.03697	.847	-.0884	.1484
Fungal_Biomass_P. citrinum	3	5	-.3267*	.03749	<.001	-.4467	-.2066
		7	-.2400*	.03749	<.001	-.3601	-.1199
		9	-.3667*	.03749	<.001	-.4867	-.2466
	5	3	.3267*	.03749	<.001	.2066	.4467
		7	.0867	.03749	.174	-.0334	.2067
		9	-.0400	.03749	.718	-.1601	.0801
	7	3	.2400*	.03749	<.001	.1199	.3601
		5	-.0867	.03749	.174	-.2067	.0334
		9	-.1267*	.03749	.039	-.2467	-.0066
	9	3	.3667*	.03749	<.001	.2466	.4867
		5	.0400	.03749	.718	-.0801	.1601
		7	.1267*	.03749	.039	.0066	.2467

Based on observed means.

The error term is Mean Square(Error) = .002.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Incubation_Period	(J) Incubation_Period	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Amylase_Activity_Penicillium_sp	3	5	.0167	.04062	.975	-.1134	.1467
		7	-.1800*	.04062	.009	-.3101	-.0499
		9	.1033	.04062	.126	-.0267	.2334
	5	3	-.0167	.04062	.975	-.1467	.1134
		7	-.1967*	.04062	.006	-.3267	-.0666
		9	.0867	.04062	.222	-.0434	.2167
	7	3	.1800*	.04062	.009	.0499	.3101
		5	.1967*	.04062	.006	.0666	.3267
		9	.2833*	.04062	<.001	.1533	.4134
	9	3	-.1033	.04062	.126	-.2334	.0267
		5	-.0867	.04062	.222	-.2167	.0434
		7	-.2833*	.04062	<.001	-.4134	-.1533
Fungal_Biomass_Penicillium_sp	3	5	-.3000*	.06960	.011	-.5229	-.0771
		7	-.3267*	.06960	.007	-.5496	-.1038
		9	-.5367*	.06960	<.001	-.7596	-.3138
	5	3	.3000*	.06960	.011	.0771	.5229
		7	-.0267	.06960	.980	-.2496	.1962
		9	-.2367*	.06960	.038	-.4596	-.0138
	7	3	.3267*	.06960	.007	.1038	.5496
		5	.0267	.06960	.980	-.1962	.2496
		9	-.2100	.06960	.065	-.4329	.0129
	9	3	.5367*	.06960	<.001	.3138	.7596
		5	.2367*	.06960	.038	.0138	.4596
		7	.2100	.06960	.065	-.0129	.4329

Based on observed means.

The error term is Mean Square(Error) = .007.

*. The mean difference is significant at the .05 level.

Appendix S: Statistical analysis of optimum temperature for activity of partial purified amylase with One-Way ANOVA and Tukey post hoc test

ANOVA

Amylase_Activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.616	6	.436	34.356	<.001
Within Groups	.178	14	.013		
Total	2.794	20			

A. niger KBA2

ANOVA

Amylase_Activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21.641	6	3.607	271.385	<.001
Within Groups	.186	14	.013		
Total	21.827	20			

Penicillium sp. BB3

Multiple Comparisons

Dependent Variable: Amylase_Activity

Tukey HSD

(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
30	40	-.07333	.09198	.981	-.3874	.2407
	50	-.86000*	.09198	<.001	-1.1741	-.5459
	60	-.30000	.09198	.066	-.6141	.0141
	70	-.18667	.09198	.439	-.5007	.1274
	80	-.18333	.09198	.459	-.4974	.1307
	90	.41333*	.09198	.007	.0993	.7274
40	30	.07333	.09198	.981	-.2407	.3874
	50	-.78667*	.09198	<.001	-1.1007	-.4726
	60	-.22667	.09198	.243	-.5407	.0874
	70	-.11333	.09198	.870	-.4274	.2007
	80	-.11000	.09198	.885	-.4241	.2041
	90	.48667*	.09198	.002	.1726	.8007
50	30	.86000*	.09198	<.001	.5459	1.1741
	40	.78667*	.09198	<.001	.4726	1.1007
	60	.56000*	.09198	<.001	.2459	.8741
	70	.67333*	.09198	<.001	.3593	.9874
	80	.67667*	.09198	<.001	.3626	.9907
	90	1.27333*	.09198	<.001	.9593	1.5874
60	30	.30000	.09198	.066	-.0141	.6141
	40	.22667	.09198	.243	-.0874	.5407
	50	-.56000*	.09198	<.001	-.8741	-.2459
	70	.11333	.09198	.870	-.2007	.4274
	80	.11667	.09198	.855	-.1974	.4307
	90	.71333*	.09198	<.001	.3993	1.0274
70	30	.18667	.09198	.439	-.1274	.5007
	40	.11333	.09198	.870	-.2007	.4274
	50	-.67333*	.09198	<.001	-.9874	-.3593
	60	-.11333	.09198	.870	-.4274	.2007
	80	.00333	.09198	1.000	-.3107	.3174
	90	.60000*	.09198	<.001	.2859	.9141
80	30	.18333	.09198	.459	-.1307	.4974
	40	.11000	.09198	.885	-.2041	.4241
	50	-.67667*	.09198	<.001	-.9907	-.3626
	60	-.11667	.09198	.855	-.4307	.1974
	70	-.00333	.09198	1.000	-.3174	.3107
	90	.59667*	.09198	<.001	.2826	.9107
90	30	-.41333*	.09198	.007	-.7274	-.0993
	40	-.48667*	.09198	.002	-.8007	-.1726
	50	-1.27333*	.09198	<.001	-1.5874	-.9593
	60	-.71333*	.09198	<.001	-1.0274	-.3993
	70	-.60000*	.09198	<.001	-.9141	-.2859
	80	-.59667*	.09198	<.001	-.9107	-.2826

*. The mean difference is significant at the 0.05 level.

A. niger KBA2

Multiple Comparisons

Dependent Variable: Amylase_Activity

Tukey HSD

(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
30	40	-2.85667*	.09413	<.001	-3.1781	-2.5353
	50	-.44000*	.09413	.005	-.7614	-.1186
	60	.02000	.09413	1.000	-.3014	.3414
	70	-.11667	.09413	.867	-.4381	.2047
	80	.07667	.09413	.979	-.2447	.3981
	90	.33333*	.09413	.040	.0119	.6547
40	30	2.85667*	.09413	<.001	2.5353	3.1781
	50	2.41667*	.09413	<.001	2.0953	2.7381
	60	2.87667*	.09413	<.001	2.5553	3.1981
	70	2.74000*	.09413	<.001	2.4186	3.0614
	80	2.93333*	.09413	<.001	2.6119	3.2547
	90	3.19000*	.09413	<.001	2.8686	3.5114
50	30	.44000*	.09413	.005	.1186	.7614
	40	-2.41667*	.09413	<.001	-2.7381	-2.0953
	60	.46000*	.09413	.003	.1386	.7814
	70	.32333*	.09413	.048	.0019	.6447
	80	.51667*	.09413	.001	.1953	.8381
	90	.77333*	.09413	<.001	.4519	1.0947
60	30	-.02000	.09413	1.000	-.3414	.3014
	40	-2.87667*	.09413	<.001	-3.1981	-2.5553
	50	-.46000*	.09413	.003	-.7814	-.1386
	70	-.13667	.09413	.767	-.4581	.1847
	80	.05667	.09413	.996	-.2647	.3781
	90	.31333	.09413	.058	-.0081	.6347
70	30	.11667	.09413	.867	-.2047	.4381
	40	-2.74000*	.09413	<.001	-3.0614	-2.4186
	50	-.32333*	.09413	.048	-.6447	-.0019
	60	.13667	.09413	.767	-.1847	.4581
	80	.19333	.09413	.426	-.1281	.5147
	90	.45000*	.09413	.004	.1286	.7714
80	30	-.07667	.09413	.979	-.3981	.2447
	40	-2.93333*	.09413	<.001	-3.2547	-2.6119
	50	-.51667*	.09413	.001	-.8381	-.1953
	60	-.05667	.09413	.996	-.3781	.2647
	70	-.19333	.09413	.426	-.5147	.1281
	90	.25667	.09413	.162	-.0647	.5781
90	30	-.33333*	.09413	.040	-.6547	-.0119
	40	-3.19000*	.09413	<.001	-3.5114	-2.8686
	50	-.77333*	.09413	<.001	-1.0947	-.4519
	60	-.31333	.09413	.058	-.6347	.0081
	70	-.45000*	.09413	.004	-.7714	-.1286
	80	-.25667	.09413	.162	-.5781	.0647

*. The mean difference is significant at the 0.05 level.

Penicillium sp. BB3

Appendix T: Statistical analysis of optimum pH for activity of partial purified amylase with One-Way ANOVA and Tukey post hoc test

ANOVA

Amylase_Activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	254.413	6	42.402	3272.500	<.001
Within Groups	.181	14	.013		
Total	254.595	20			

A. niger KBA2

ANOVA

Amylase_Activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.636	6	.106	10.643	<.001
Within Groups	.139	14	.010		
Total	.776	20			

Penicillium sp. BB3

Multiple Comparisons

Dependent Variable: Amylase_Activity

Tukey HSD

(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
3	4	-3.85000 [*]	.09294	<.001	-4.1674	-3.5326
	5	-10.68333 [*]	.09294	<.001	-11.0007	-10.3660
	6	-1.61333 [*]	.09294	<.001	-1.9307	-1.2960
	7	-1.30667 [*]	.09294	<.001	-1.6240	-.9893
	8	-.87000 [*]	.09294	<.001	-1.1874	-.5526
	9	-.25000	.09294	.172	-.5674	.0674
4	3	3.85000 [*]	.09294	<.001	3.5326	4.1674
	5	-6.83333 [*]	.09294	<.001	-7.1507	-6.5160
	6	2.23667 [*]	.09294	<.001	1.9193	2.5540
	7	2.54333 [*]	.09294	<.001	2.2260	2.8607
	8	2.98000 [*]	.09294	<.001	2.6626	3.2974
	9	3.60000 [*]	.09294	<.001	3.2826	3.9174
5	3	10.68333 [*]	.09294	<.001	10.3660	11.0007
	4	6.83333 [*]	.09294	<.001	6.5160	7.1507
	6	9.07000 [*]	.09294	<.001	8.7526	9.3874
	7	9.37667 [*]	.09294	<.001	9.0593	9.6940
	8	9.81333 [*]	.09294	<.001	9.4960	10.1307
	9	10.43333 [*]	.09294	<.001	10.1160	10.7507
6	3	1.61333 [*]	.09294	<.001	1.2960	1.9307
	4	-2.23667 [*]	.09294	<.001	-2.5540	-1.9193
	5	-9.07000 [*]	.09294	<.001	-9.3874	-8.7526
	7	.30667	.09294	.061	-.0107	.6240
	8	.74333 [*]	.09294	<.001	.4260	1.0607
	9	1.36333 [*]	.09294	<.001	1.0460	1.6807
7	3	1.30667 [*]	.09294	<.001	.9893	1.6240
	4	-2.54333 [*]	.09294	<.001	-2.8607	-2.2260
	5	-9.37667 [*]	.09294	<.001	-9.6940	-9.0593
	6	-.30667	.09294	.061	-.6240	.0107
	8	.43667 [*]	.09294	.005	.1193	.7540
	9	1.05667 [*]	.09294	<.001	.7393	1.3740
8	3	.87000 [*]	.09294	<.001	.5526	1.1874
	4	-2.98000 [*]	.09294	<.001	-3.2974	-2.6626
	5	-9.81333 [*]	.09294	<.001	-10.1307	-9.4960
	6	-.74333 [*]	.09294	<.001	-1.0607	-.4260
	7	-.43667 [*]	.09294	.005	-.7540	-.1193
	9	.62000 [*]	.09294	<.001	.3026	.9374
9	3	.25000	.09294	.172	-.0674	.5674
	4	-3.60000 [*]	.09294	<.001	-3.9174	-3.2826
	5	-10.43333 [*]	.09294	<.001	-10.7507	-10.1160
	6	-1.36333 [*]	.09294	<.001	-1.6807	-1.0460
	7	-1.05667 [*]	.09294	<.001	-1.3740	-.7393
	8	-.62000 [*]	.09294	<.001	-.9374	-.3026

*. The mean difference is significant at the 0.05 level.

A. niger KBA2

Multiple Comparisons

Dependent Variable: Amylase_Activity

Tukey HSD

(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
3	4	-.19000	.08149	.295	-.4683	.0883
	5	-.31333*	.08149	.023	-.5916	-.0351
	6	-.37333*	.08149	.006	-.6516	-.0951
	7	-.43333*	.08149	.002	-.7116	-.1551
	8	-.12667	.08149	.711	-.4049	.1516
	9	.06333	.08149	.984	-.2149	.3416
4	3	.19000	.08149	.295	-.0883	.4683
	5	-.12333	.08149	.733	-.4016	.1549
	6	-.18333	.08149	.331	-.4616	.0949
	7	-.24333	.08149	.105	-.5216	.0349
	8	.06333	.08149	.984	-.2149	.3416
	9	.25333	.08149	.086	-.0249	.5316
5	3	.31333*	.08149	.023	.0351	.5916
	4	.12333	.08149	.733	-.1549	.4016
	6	-.06000	.08149	.988	-.3383	.2183
	7	-.12000	.08149	.756	-.3983	.1583
	8	.18667	.08149	.312	-.0916	.4649
	9	.37667*	.08149	.006	.0984	.6549
6	3	.37333*	.08149	.006	.0951	.6516
	4	.18333	.08149	.331	-.0949	.4616
	5	.06000	.08149	.988	-.2183	.3383
	7	-.06000	.08149	.988	-.3383	.2183
	8	.24667	.08149	.098	-.0316	.5249
	9	.43667*	.08149	.002	.1584	.7149
7	3	.43333*	.08149	.002	.1551	.7116
	4	.24333	.08149	.105	-.0349	.5216
	5	.12000	.08149	.756	-.1583	.3983
	6	.06000	.08149	.988	-.2183	.3383
	8	.30667*	.08149	.027	.0284	.5849
	9	.49667*	.08149	<.001	.2184	.7749
8	3	.12667	.08149	.711	-.1516	.4049
	4	-.06333	.08149	.984	-.3416	.2149
	5	-.18667	.08149	.312	-.4649	.0916
	6	-.24667	.08149	.098	-.5249	.0316
	7	-.30667*	.08149	.027	-.5849	-.0284
	9	.19000	.08149	.295	-.0883	.4683
9	3	-.06333	.08149	.984	-.3416	.2149
	4	-.25333	.08149	.086	-.5316	.0249
	5	-.37667*	.08149	.006	-.6549	-.0984
	6	-.43667*	.08149	.002	-.7149	-.1584
	7	-.49667*	.08149	<.001	-.7749	-.2184
	8	-.19000	.08149	.295	-.4683	.0883

*. The mean difference is significant at the 0.05 level.

Penicillium sp. BB3