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Isolation and characterization of indigenous amylolytic enzyme-producing *Aspergillus* sp. from sweet-flavored tapai

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Abstract. *Anastasia L, Kodrat EA, Victor H, Sanjaya A, Pinontoan R. 2022. Isolation and characterization of indigenous amylolytic enzyme-producing* Aspergillus *sp. from sweet-flavored tapai. Biodiversitas 23: 5559-5565. Aspergillus* sp. is able to produce abundant amylolytic enzymes that may have a crucial role in producing the sweet flavor of tapai. This study was aimed to evaluate the amylolytic activity of *Aspergillus* sp. isolated from a widely known sweet tapai from Jember, East Java, Indonesia. The fungi isolated from sweet tapai were identified under notable morphological parameters, and its amylolytic activities were evaluated by utilizing various starch-based media, such as potato peel, potato dextrose broth, cassava, and maize. Optimum pH and temperature of partially purified amylolytic enzymes were evaluated using DNS assay. The result of this study suggested that TM3 fungal isolate isolated from sweet tapai belonged to *Aspergillus* sp. section Nigri. *Aspergillus* sp. TM3 grown in the potato peel medium displayed the highest amylolytic activity, followed by those grown in maize, potato dextrose broth, and cassava media. Further characterization of partially purified amylolytic enzymes from the isolate showed that optimal pH for the amylolytic activity was at pH 4.59 while the optimal temperature was around 54°C. Furthermore, analysis by thin-layer chromatography revealed that the amylolytic enzymes starch hydrolysis yielded dextrose as the main product and oligosaccharides as the side product, which indicated that *Aspergillus* sp. TM3 may have both alpha-amylase and glucoamylase involved in its amylolytic activity. This study explored the role of *Aspergillus* sp. TM3 in producing the sweet flavor of tapai.

Keywords: Amylolytic activity, *Aspergillus*, characterization, sweet tapai singkong

INTRODUCTION

Tapai is well known as one of the traditional fermented foods in Indonesia and available in two varieties, depending on what raw fermentation material is used. Tapai ketan utilizes glutinous rice (*Oryza sativa var. glutinosa*) whereas tapai singkong utilizes steamed white - or yellow-flesh cassava tubers (*Manihot esculenta*). White-flesh tapai singkon*g* is normally produced and available in Jakarta, Bogor and West Java regions, while yellow-flesh tapai singkong is more prevalent in Central Java and East Java regions. Yellow-flesh tapai singkong is more desirable and appealing among Indonesian consumers than the white-flesh tapai singkong. The appeal is attributed to its mild alcoholic aroma and strong sweet taste combined with its honey-like color appearance (Ayetigbo et al. 2018). Henceforth in this study, tapai singkong will be referred to as simply tapai.

In general, fermentation is a biological and enzymatic process to preserve food and to produce a desirable taste, aroma, or flavor in said food. In tapai, the fermentation agent involved is a diverse microbial consortium (consists of yeast, mold, and bacteria) utilized to produce a distinct flavor. These microbes have been identified as *Aspergillus* sp., *Rhizopus* sp., *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae*, *Candida* sp., *Hansenula* sp., *Streptococcus* sp., and lactic acid bacteria (Tamang et al. 2016; Barus et al. 2017; Sumerta and Kanti 2017; Urbahillah et al. 2021). Due to this, it is assumed that there is a series of interactions between these microbes in the fermentation process to give tapai its distinct flavor and aroma. The mold breaks down the starch content in the cassava tuber into simple sugars for the yeast to convert into alcohols. The acetic acid and lactic acid bacteria subsequently convert the alcohols into acetic acid and lactic acid respectively (Hasanah et al. 2018).

Meanwhile, the strong sweet flavor of tapai may be attributed to the fungal activity in the fermentation process. Previous studies have discovered the existence of fungi involved in tapai fermentation such as species of *Aspergillus*, *Mucor*, and *Rhizopus* (Surono 2016). These fungi produce amylolytic enzymes that might play a crucial role in hydrolyzing the starch content in cassava tubers into simple sugars, which significantly contribute to the sweetness of tapai. Fungi also serve as suitable organisms for producing amylolytic enzymes at an industrial scale, because of the ease of enzyme extraction and the enzyme's stability at broader range of pH and temperature. In addition, it is much preferable to commercially produce enzymes from Generally Recognized as Safe (GRAS) amylolytic microorganisms isolated from fermented foods due to their broader applications including in food and feed industries (Angelia et al. 2019; Sewalt et al. 2016). Among those fungi, *Aspergillus* sp. is known for producing large quantities of thermostable amylolytic enzymes, making it

an ideal microorganism for commercial enzyme production (Guo et al. 2021).

Amylolytic enzymes account for 30% of the global enzyme market and will continue to grow due to the increasing demand for a vast range of industrial conditions and applications, including biofuels, detergents, pharmaceuticals, paper and textile industries (Gómez-Villegas et al. 2021). The cost to produce industrial-grade enzymes is generally high. To be considered affordable, the use of abundant and low-cost carbon sources as well as optimization of external factors including pH, substrate, and temperature are essential for the microorganisms to produce commercially viable amounts of amylolytic enzymes (Balakrishnan et al. 2021). Despite the importance of amylolytic enzyme-producing fungi associated with tapai fermentation process, the research on such matter in Indonesia still remained limited. Therefore, this study aimed to evaluate the activity of amylolytic enzymes produced by *Aspergillus* sp. isolated from tapai with the use of various starch-based media and fermentation conditions.

MATERIALS AND METHODS

Sweet tapai used in this study was obtained from Jember, one of the regencies located in East Java Province, Indonesia. The Jember is well-known for its tapai as it has an alcoholic aroma and sweet flavor with honey-like appearance, making it preferable than the common tapai. Due to its popularity, tourists coming to this area often to buy the so-called tapai manis ("sweet tapai") as a souvenir.

Procedures

Isolation and morphological identification of fungi from sweet tapai

For isolation, one gram of sweet tapai was mixed with sterilized 0.85% NaCl and diluted to 10^{-3}. Then, 70 µL of the mixture was inoculated on an agar medium by spread plate method and incubated for three days at 25°C. Afterward, the fungal colony was purified by using streak plate method on fresh culture medium and incubated for another 72 hours at 25°C. Cell morphology observation of the fungal isolate was carried out using LPCB (HiMedia, India). A drop of LPCB was placed into a microscope slide, mixed with a loop of fungal isolate, and covered by a coverslip. It was left for 5 minutes at room temperature, then observed using a light microscope.

Inoculation of fungal isolates in various starch-based media and enzyme extraction

Potato peel (PP), cassava (Cs), and maize (Mz) media were prepared in jars by adding water (1:1) (w/v), while potato dextrose broth (PDB) (HiMedia, India) was prepared by suspending 2.4 g of PDB in 100 mL distilled water. All of them were autoclaved at 121°C for 21 minutes. Fungal isolate was inoculated into each medium and incubated at 25°C for 72 hours.

Qualitative screening of amylolytic activity of the fungal isolate on various starch-based media

Crude extracts of the enzyme were collected by centrifugation at 8,000 x g for 15 minutes. They were used as samples for amylolytic activity screening by well-diffusion technique. As much as 20 µL of the samples, 20 µL of commercial glucoamylase (Novozymes, Denmark) as positive control, and 20 µL of sterilized water as negative control were inoculated into each well in the starch agar and incubated at 37°C for 24 hours. The appearance of the clear zone after the starch agar was flooded with Lugol solution (Merck, Germany) indicated amylolytic activity of the crude extract.

Quantitative screening of amylolytic activity of the fungal isolate on various starch-based media

Protein concentration of crude extracts was determined through Biuret assay by measuring the absorbance at 540 nm as compared to a standard curve prepared using bovine serum albumin (Sigma-Aldrich, Germany) protein (Layne 1957). Crude extracts of the isolate were also used as the samples for amylolytic activity assay using the Miller method (Miller 1959). Briefly, 20 µL of crude extract of the enzyme was added to 480 µL acetate buffer (pH 4.5) containing soluble starch 1% (w/v), and then incubated at 60°C for 15 minutes. Afterwards, the reaction was terminated and 500 µL of DNS solution was added to estimate the reducing sugars formed. The mixture was heated in boiling water for 5 minutes, followed by adding the Rochelle salt solution and cooling to room temperature. Absorbance was measured at 540 nm by a spectrophotometer. One unit of amylolytic activity was defined as the amount of enzyme required to release 1 µg of glucose per mL per minute under the assay conditions.

Characterization of amylolytic enzymes

To determine the optimum pH of amylolytic activity, the partially purified enzymes of TM3 PP were used as the sample for the amylolytic assay using the Miller method (Miller 1959). The assay was carried out at different pH using 0.1M glycine-HCl buffer (pH 3), 0.1M acetate buffer (pH 4 and pH 5), 0.1M citrate phosphate buffer (pH 6), and 0.1M phosphate buffer (pH 7). To determine the optimal temperature of amylolytic activity, the assay was carried out at various temperatures (30°C, 45°C, 60°C, 75°C) for 15 minutes in 0.1M acetate buffer (pH 5). The amount of reducing sugars formed was determined by comparing the absorbance to a standard glucose curve.

Identification of amylolytic enzyme and its products by TLC

A total of 2% (w/v) starch solution was mixed with the crude extract of TM3, partially-purified enzyme of TM3, and commercial glucoamylase (Novozymes, Denmark). The mixtures were incubated at 60°C for 1 hour. Each mixture was spotted into the Si60 plate (Merck, Germany) along with dextrose, maltose, sucrose, and fructose as the standard sugars. It was air-dried in the oven. Then, using n-butanol, 1-propanol, acetic acid, and distilled water (3:1:1:1) as the eluent, the chromatography was carried out in a sealed chamber. Visualization was done using a

reagent mixture consisting of α-naphthol, sulfuric acid, and absolute ethanol. The plate was heated in the oven to visualize sugar spots and the products of hydrolysis were identified through comparison of Rf values between the samples and standard sugars.

Data analysis

Statistical analysis of the amylolytic activity was carried out using one-way ANOVA with the Tukey comparison test in Minitab 18 software (Minitab Inc., USA) to determine the statistical differences of samples.

RESULTS AND DISCUSSION

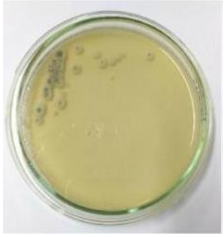
Morphological identification of fungal isolate from sweet tapai

Four isolates were successfully isolated from sweet tapai. They were named TM1, TM2, TM3, and TM4. The morphological identification of the four isolates revealed that all of them were fungi, particularly molds. The morphological description of the four isolates were presented on Table 1.

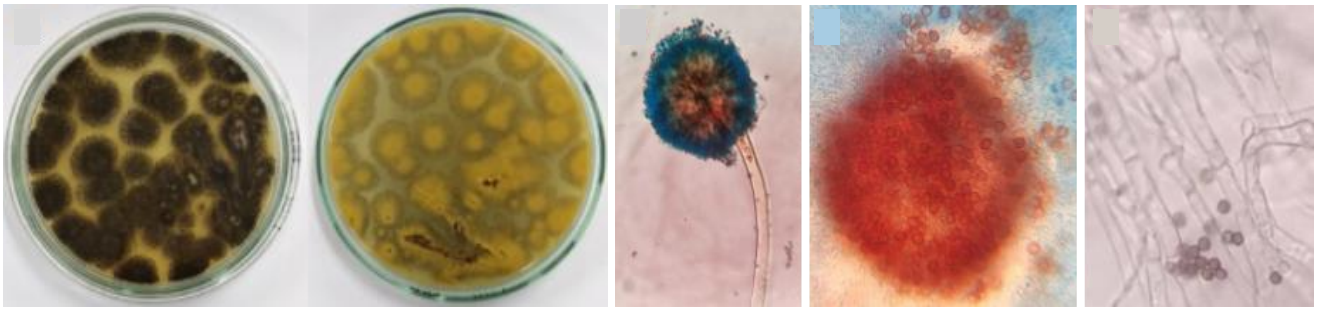
The colony of TM1 isolate had a dull green surface pigmentation with dense and velutinous textures. The cellular morphology of TM1 showed that it had branches as its distinctive feature between metulae and stipes with biverticillate branching pattern or two-staged branched. These descriptions agreed with the morphological features of *Penicillium* sp. (Ellis 2021a; Naeimi et al. 2021). While TM2 isolate was not clearly identified, it had comparable characteristics on the colony and cellular level to TM1. The characteristics are in line with features of *Penicillium* sp. (Ellis 2021a).

TM3 isolate, on the other side, showed a black surface pigmentation and filamentous shape with entire margin, flat elevation, as well as rough and granular textures (Figure 1A). The utilization of LPCB for cellular morphological identification of fungi was recommended by previous studies (Zhou and Li 2015; Ellis 2021d). The cellular morphology revealed that TM3 isolate had a conidiophore which terminated in a vesicle covered with phialide and metulae (biseriate species). Its conidial head was arranged in divergent chains (radiate), conidial shape was globose with rough-walled ascospore, and all of them were dark brown in coloration (Figure 1B, 1C, 1D). These descriptions are in line with the morphological characteristics of *Aspergillus* section Nigri (Vyzantiadis 2012; Houbraken et al. 2020; Ellis 2021c). Since most of the species in section Nigri have a very similar morphology, it is unlikely to identify TM3 isolate to the species level solely through the morphological identification. It is necessary to identify the *Aspergillus* sp. TM3 further through molecular identification approaches. TM3 isolate in this study was denoted temporarily as *Aspergillus* sp. TM3.

Table 1. The colony and cellular morphology characteristics of isolates from sweet tapai

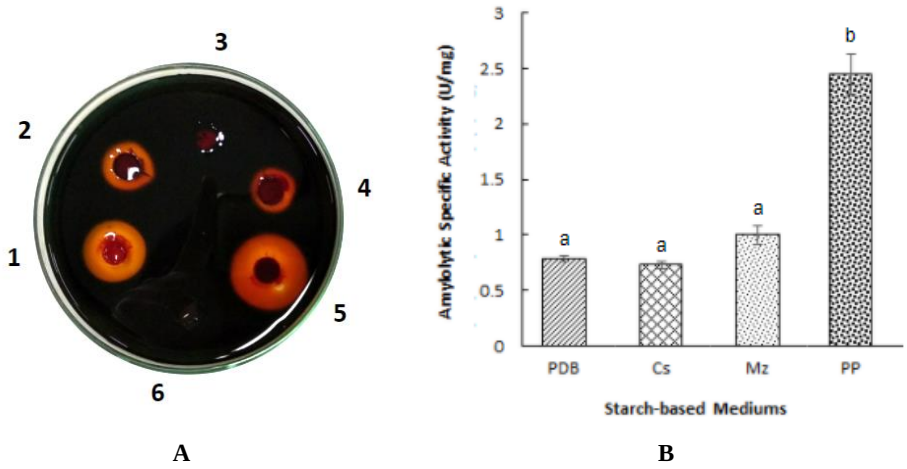
Characteristics	Isolate name			
	TM1	**TM2**	**TM3**	**TM4**
Colony morphology				
Surface pigmentation	Dull green	White *)	White **)	Dull green to grey
Reverse pigmentation	Greyish	Greyish	Yellowish **)	Greyish
Shape	Filamentous	Filamentous	Filamentous	Filamentous
Texture	Dense, velutinous	Dense, rough, velutinous	Rough, granular	Dense, rough, velutinous
Margin	Entire	Entire	Entire	Filiform
Elevation	Flat	Raised	Flat	Raised
Cellular morphology				
Arrangement of conidia	Diverge	n.d.	Radiate	Disarticulate
Arrangement of conidial chain	Basipetal	n.d.	Basipetal	Acropetal
Shape of conidia	Subglobose	Globose	Globose	Ellipsoidal
Color of conidia	Hyaline	Hyaline	Dark brown	Dark brown
Shape of ascospore	n.d.	n.d.	Globose	n.d.
Texture of ascospore	n.d.	n.d.	Rough-walled	n.d.
Color of ascospore	n.d.	n.d.	Dark brown	n.d.
Vegetative hyphae	Septate	Septate	Septate	Septate
Color of vegetative hyphae	Hyaline	Hyaline	Hyaline	Dark brown
Conidiophore (aerial hyphae)	Straight	n.d.	Straight	Straight
Color of conidiophore	Hyaline	Hyaline	Hyaline	Dark brown
Distinctive features	Bi-verticillate branching pattern	n.d.	Biseriate conidial head	Ramoconidia
Identification	*Penicillium* sp.	*Penicillium* sp.	*Aspergillus* sp.	*Cladosporium* sp.

Note: Hyaline means no pigmentation; *) incubated in 3 days and turned dark green to grey in 7 days; **) incubated in 3 days and turned black in 7 days; n.d. not determined.



A.1 **A.2** **B** **C** **D**

Figure 1. Colony and cellular morphology of TM3 isolate. A: Obverse and reverse view of TM3 isolate grown on a medium; B: Overview of conidiophore and conidial head observed at 400x total magnification; C: Conidia observed at 1000x total magnification; D: Vegetative hyphae and ascospore observed at 1000x total magnification



A **B**

Figure 2. (A) Qualitative screening of amylolytic activity of *Aspergillus* sp. TM3 grown in various starch-based mediums using well-diffusion technique. 1: *Aspergillus* sp. TM3 PP crude extract; 2: *Aspergillus* sp. TM3 Cs crude extract; 3: *Aspergillus* sp. TM3 PDB crude extract; 4: *Aspergillus* sp. TM3 Mz crude extract; 5: 1% commercial amylase; 6: Sterilized water. The plate was incubated at 37°C for 24h. (B) Quantitative screening of amylolytic activity of *Aspergillus* sp. TM3 grown in various starch-based mediums using DNS assay. The data provided were the mean of four independent experiments (n=4) showing consistent results. Means that do not share a letter are significantly different

The colony morphology of TM4 isolate had a dull green to grey surface pigmentation with dense, rough, and velutinous textures. TM4 isolate cellular morphology had acropetal conidial chains, where the youngest conidia appeared at the tip. It also had a distinctive feature, ramoconidia, resulting from the ramification of conidia. The disarticulated conidial arrangement also found to be the distinctive feature of TM4 isolate. These descriptions are all in line with the morphological characteristics of *Cladosporium* sp. (El-Dawy et al. 2021; Ellis 2021b).

After morphological identification, all of the four isolates were grown on a medium and their crude extracts were screened by the well-diffusion method to evaluate its ability to hydrolyze starch. TM3 isolate, which showed the most promising potential in amylolytic activity (data not shown), was further characterized in this study.

Amylolytic activity of *Aspergillus* sp. grown in various starch-based media

The crude extracts of *Aspergillus* sp. TM3 grown in various starch-based media PP, PDB, Cs, and Mz respectively were screened qualitatively for the amylolytic activity in starch agar. After the agar was flooded with Lugol solution, the clear zones appeared. As shown in Figure 2A, the clear zone from the crude extract of *Aspergillus* sp. TM3 grown in PP was relatively larger than others, while the crude extract of *Aspergillus* sp. TM3 grown in PDB medium did not produce any clear zone. The appearance of a clear zone was associated with the amylolytic activity of the crude extract, hence in this qualitative screening, *Aspergillus* sp. TM3 isolated from sweet tapai has proven to have amylolytic activity and it might produce higher yield of amylolytic enzymes when grown in PP media. This result is also complementary with the previous study, where *Aspergillus niger* aggregate from fermented cassava gatot was able to grow well in potato-based medium and produced extracellular amylase (Angelia et al. 2019). In addition, Biuret assay results demonstrated that the crude extracts of *Aspergillus* sp. TM3 grown in various starch-based media had different quantities of protein produced.

Aspergillus sp. TM3 grown in Cs media had the most abundant protein content, followed by the *Aspergillus* sp. TM3 grown in PP media (data not shown). The four crude

extracts of *Aspergillus* sp. TM3 harvested from various starch-based media were also assayed quantitatively for the amylolytic activity using DNS assay. The assay was performed to detect the reducing sugars released from various starch-based substrates hydrolyzed by *Aspergillus* sp. TM3 amylolytic enzyme. The specific activity of amylolytic enzyme was found to be highest when using PP medium as the substrate, which was three times higher than the amylolytic activity from extracts obtained from PDB and Cs media, respectively (Figure 2B). Thus, the crude extract of *Aspergillus* sp. TM3 grown in PP media was partially purified using acetone for further characterization.

Characterization of amylolytic activity from *Aspergillus* sp. grown in PP medium

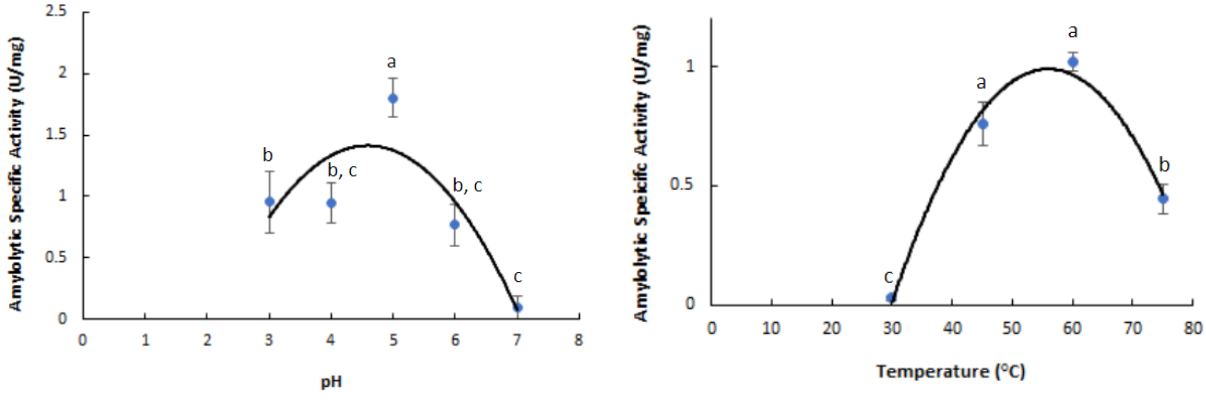
The ability of *Aspergillus* sp. TM3 to grow in PP medium and produce amylolytic enzymes with a significantly higher amylolytic activity might be affected by several factors. Since the PP was arranged into one layer and sterilized, the treatment might increase the surface area of the substrate and make it easier for fungi to grow aerobically. Moreover, a study claimed that *Aspergillus* sp. are able to utilize the substrate in potato peel better than in other substrates probably because the potato peel contains inducer compounds (Mukherjee et al. 2019). Potato peel is also known for its high starch content which accounts for more than 50% of its dry weight (Gaudino et al. 2020). More importantly, potato peel has a porous surface with a non-rigid texture, thus providing a large surface area to hold water to keep it moist. Overall, the abundance of the starch content and large surface area of potato peel might support the growth of *Aspergillus* sp. TM3, as well as its capability to produce amylolytic enzymes optimally.

Characterization of amylolytic activity produced by *Aspergillus* sp. TM3 was done by determining its optimal pH and temperature (Figure 3). The data in Figure 3A indicated that amylolytic enzyme from *Aspergillus* sp. TM3 used in this study displayed the variance of its activity in different pH ranges. Figure 3A revealed that the optimal specific activity of the amylolytic enzyme was at pH 5 and it was found to be decreasing at higher pH values, which were pH 6 and pH 7 (p-value < 0.05). Equations generated from the graph of pH was presented below:

$$y = -0.2307x^2 + 2.1163x - 3.4407$$

From the above equation with $R^2 = 0.7443$, the optimal pH value approximated for the amylolytic activity of *Aspergillus* sp. TM3 was at pH 4.59. To date, the optimal pH for amylolytic activity of *Aspergillus* sp. is usually within the range of pH 4.0 – 6.0. Supported by other studies, generally, fungal amylolytic activity is known to be higher at relatively acidic pH values (Bagheri et al. 2014; Wang et al. 2016; Jain and Katyal 2018).

Figure 4. Identification of amylolytic enzyme and its hydrolyzed product using TLC. 1: 1% sucrose, 2: 1% maltose, 3: 1% fructose, 4: 1% dextrose, 5: Hydrolysis of 2% starch by partially-purified enzyme of *Aspergillus* sp. TM3 PP, 6: Hydrolysis of 2% starch by crude extract of *Aspergillus* sp. TM3 PP, 7: Hydrolysis of 2% starch by commercial glucoamylase. Starch hydrolysis was done in 60 minutes



A **B**

Figure 3. Effects of pH values (A) and temperatures (B) on amylolytic activity of *Aspergillus* sp. TM3 grown in PP medium. The assay was done in 60°C for 15 min. Vertical bars presented the standard errors of the mean (SEM) obtained from four independent experiments (n=4). Means that do not share a letter are significantly different ($p < 0.05$). One unit of amylolytic activity was defined as the amount of enzyme required to release one µg of glucose per mL per minute

The effect of temperature on the specific activity of amylolytic enzyme is presented in Figure 3B. The amylolytic enzyme exhibited optimal specific activity in a temperature of 60°C, and a rapid loss of activity was observed above it (p-value < 0.05). Equations generated from the graph of temperature was presented below:

$$y = -0.0015x^2 + 0.1626x - 3.5596$$

From the above equation with R^2 = 0.9874, it was known that the optimal temperature for the amylolytic activity of *Aspergillus* sp. TM3 was around 54°C. The optimal amylolytic activity of *Aspergillus niger* has been reported at 60°C, with the maximum activity of its alpha-amylase at 45°C and the glucoamylase at around 60°C (Bagheri et al. 2014; Rizk et al. 2019). The results, at this point, were not sufficient to determine whether *Aspergillus* sp. TM3 exhibited alpha-amylase or glucoamylase activity to hydrolyze the starch content in PP. Therefore, TLC was performed to identify the enzymes by identifying their products.

Identification of amylolytic enzyme and its products by TLC

TLC analysis of *Aspergillus* sp. TM3 amylolytic enzyme starch hydrolysis showed a spot containing dextrose as the main product and a thin streak which Yi et al. (2018) suggested to be containing maltotriose or other oligosaccharides (Figure 4 Lane 5 and 6). It was suggested that these products resulted from alpha-amylase and glucoamylase activities of *Aspergillus* sp. TM3, supported by several studies on the glucoamylase activity of *Aspergillus* sp. and fungal alpha-amylase activity of a starter (Lago et al. 2021; Yi et al. 2018). Another study reported that the hydrolysis of starch by glucoamylase from *Aspergillus niger* resulted in dextrose as the product and no other reducing sugars (Gautam et al. 2011; Angelia et al. 2019).

This study showed that TM3 isolate from sweet tapai belonged to *Aspergillus* sp. section Nigri. To date, the Nigri section is still subject to debates, evaluations, assessments, and revisits due to its complexity. A number of past studies classified black fungal isolates as *Aspergillus niger* solely based on their phenotypic characteristics. However, recent studies reported that the phenotypic approach is insufficient for species-level identification of the Nigri section. Both morphological and molecular identification are necessary to clearly distinguish a species from the remaining species within the Nigri section, such as *A. niger*, *A. awamori*, *A. welwitschiae*, *A. brasiliensis*, and *A. luchuensis* (Varga et al. 2011; Hong et al. 2014). Our preliminary result indicated that TM3 might belong to *A. welwitschiae*.

Aspergillus sp. TM3 in this study displayed the highest amylolytic activity when grown in PP medium compared to the other starch-based media. The starch content and large surface area of PP might support the growth of *Aspergillus* sp. TM3. Moreover, PP as a medium has an advantage over the laboratory medium from an economical perspective. Further characterization revealed that the amylolytic enzymes of *Aspergillus* sp. TM3 isolated from sweet tapai has optimal activity at pH 4.59 and temperature around 54°C. The starch hydrolysis by the amylolytic enzymes produced dextrose as the main product and oligosaccharides as the side product, which indicated that the amylolytic activity of *Aspergillus* sp. TM3 involved both alpha-amylase and glucoamylase. However, HPLC assay and further evaluation need to be carried out to further investigate the role of *Aspergillus* sp. TM3 in producing the sugary flavor of sweet tapai from Jember.

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