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# Screening of Potential Tannase-producing Fungi from Local Agri-industrial By-products using a Plate Assay and Submerged Fermentation

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***Abstract*— Tannase (Tannin Acyl Hydrolase EC 3.1.1.20) is an industrial inducible enzyme capable of hydrolyzing hydrolyzable tannin ester linkage gallotannin and ellagitannin, producing gallic acid and glucose. Tannase is extensively used in the pharmaceutical, chemical, cosmetics, textile, leather, food, feed, and beverage industries. In the beverage industry, tannase is used as a clarifying agent to clarify tannin present in coffee, coffee-flavored soft drinks, tea, and fruit juices by removing phenolic compounds. In the pharmaceutical industry, tannase is used to produce gallic acid, an intermediary compound in the production of antibacterial drug, trimethoprim, while in the food industry, tannase is used to synthesize crucial antioxidant food preservative propyl gallate (3,4,5-trihydroxybenzoate). Most of the tannase production utilizes bacteria such as *Bacillus* sp. as tannase producer under submerged fermentation, SmF. Despite the immense industrial potential of tannase, it has not fully been exploited due to lack of knowledge, and fewer studies reported filamentous fungi for tannase production. This study aimed to screen potential tannase-producing fungi from various agri-industrial by-products such as rice by-products, spent tea, spent coffee ground, banana peels, mango peels, desiccated coconut residue, soybean residue, sweet potato peels, and onions. Fungal isolate, J1 (*Aspergillus niger*) was identified as the efficient tannase-producing fungus due to the hydrolytic zone's largest diameter (60.7 ± 0.6) mm. It achieved high tannase activity with (6.86 ± 0.04) U/ml in submerged fermentation, SmF. In conclusion, filamentous fungi isolated from agri-industrial by-products have huge potential as an efficient tannase producer.***

***Keywords*— Tannase; tannase-producing fungi; agri-industrial by-products; submerged fermentation.**

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## I. INTRODUCTION

Tannin known as tannase inducer can be found widely in variety of agri-industrial by-products such as spent coffee, tea and grape [1], [2]. Tannin is natural water-soluble polyphenolic secondary metabolites with various molecular weights (500-3000Da) and generally classified into two categories which is hydrosable tannin and condensed tannin based on their structure, molecular weight and chemical properties [3]–[5]. Tannin was used as a plant defense mechanism against insects and mammalian herbivores [6]. The astringent and bitterness taste of tannin unfavored to mammalian herbivores [7], [8]. However, tannin also reported present in animal feed which can give adverse effect by lowering the nutritional value of the feed since tannin can form complexes with carbohydrate, protein, starch and minerals very well and thus prevent nutrient absorption [7], [9]. The employment of tannase in animal feed can reduce the concentration of tannase thus increase its nutritional value.

Tannin acyl hydrolase (EC 3.1.1.20) or tannase can break down hydrolyze ester linkage in hydrolyzable tannin into glucose and gallic acid [2]. The main application of tannase is to produce gallic acid. Gallic acid (3, 4, 5-tri hydroxyl

benzoic acid) is an intermediate to produce the antifolic antibacterial drug, trimethoprim and has the potential to treat breast and cervical cancer [10]–[13]. Another industrial tannase application is a clarifying agent to clarify grape juice, tea, coffee, coffee-flavored soft drink, and detannification of effluents from the leather industry [14], [15]. Recent studies showed that bacterial and fungal strain capable of synthesizing tannase mostly from the genus *Aspergillus* spp., *Penicillium,*spp. *Fusarium* spp., *Trichoderma* spp., *Klebsiella* spp. and *Bacillus* spp. [1], [13]–[15], [18]

Submerged fermentation (SmF) is a fermentation process where microorganisms grow in liquid broth with excess water, while solid-state fermentation (SSF) is a fermentation process with a very low amount of free water present in the substrate, just sufficient to support microorganism growth [17], [19]. SmF was performed in this study due to several advantages over SSF regarding ease to sterile, fully utilizing substrate media, shorter incubation time, and ease for extracellular enzyme recovery [9], [13].

To date, previous investigations were focused on the production and application of tannase for the production of gallic acid using bacteria under submerged fermentation. However, few studies reported higher tannase production by using filamentous fungi than yeast and bacteria [9], [16]. The number of studies that report utilizing fungi isolated from local agri-industrial by-products is still low, even though previous studies reported that filamentous fungi had huge potential as a tannase producer. Therefore, the present work aims to isolate and screen fungal strains from various natural agri-industrial by-products for tannase production using plate assay and submerged fermentation and later further on molecular identification of tannase-producing fungi.

## II. Materials and Method

### A. Microorganism and Maintenance

Fungal strains were isolated from various fermented agri-industrial by-products such as cocoa pod's husk, banana peels, sweet potato peels, tapioca peels, onion peels, desiccated coconut shreds, rice bran, brewer's rice, spent coffee ground, spent tea, and mango peels. The microorganism was kept in PDA slants at 4 ˚C and 16% glycerol solution at -70 ˚C for long-term storage.

### B. Preparation of Tannin Agar

For the preparation of 1% tannic acid agar, 30 g bacto agar, 0.5 g $MgSO_4.7H_20$, 1 g $KH_2PO_4$, 3 g $KNO_3$, and 0.5 g KCl were added into schoot bottle containing 850 ml distilled water. The tannic acid solution was prepared in a different schoot bottle. Ten grams of tannic acid was added into sterilized 250 ml schoot bottle containing 150 ml distilled water. The tannic acid solution was then sterilized using a cellulose nitrate filter membrane with a pore size of 0.20 µm. The bacto agar solution was homogenized first before sterilized using autoclave at 121 ˚C for 15 minutes. Both sterilized bacto agar solution and tannic acid solution were mixed well before pouring onto agar disc.

### C. Preparation of Tannin Broth

For the preparation of 1% tannic acid broth, salt solution containing 0.5 g $MgSO_4.7H_20$, 1 g $KH_2PO_4$, 3 g $KNO_3$ and 0.5 g KCl were added and mixed well into schoot bottle containing 850 ml distilled water. The tannic acid solution was prepared in a different schoot bottle. Ten grams of tannic acid was added into the sterilized 250 ml schoot bottle containing 150 ml distilled water. The tannic acid solution was then sterilized using a cellulose nitrate filter membrane with a pore size of 0.20 µm. The salt solution was homogenized first before sterilized using autoclave at 121 ˚C for 15 minutes. Both salt solution and tannic acid solution were mixed well before transferred into a sterilized 100 ml conical flask in aseptic condition.

### D. Preparation of Spore Suspension

The $1x10^{\wedge 8}$ spore suspension was prepared by using a hemocytometer using 6 days old fungal strain [20]

### E. Natural Fermentation

One hundred gram of each agri-industrial by-products such as cocoa pod, cocoa pod's husk, banana peels, sweet potato peels, tapioca peels, onion peels, desiccated coconut residue, rice bran, brewer's rice, spent coffee ground, spent tea, and mango peels were added into 250 beakers. Three milliliters of 2% molasses were added into each beaker as an enhancer for fungal growth. The substrates were kept for 7 to 14 days until fungal growth was observed.

### F. Isolation of Fungal Strain

Two grams of the fermented substrate containing fungal strain was added into the sterilized universal bottle containing 20 ml ringer solution before diluted until five times dilution. A volume of 0.1 ml of the diluted solution was pipet out onto 1% tannic acid agar plate and incubated for 3 days at 32 ˚C and then was further isolated until obtaining pure culture. The pure culture was stored in PDA slant agar at 4 ˚C .

### G. Primary Screening: Plate Assay

The fungal strain was streaked onto PDA plate and incubate for 2 days at 30˚C. Young fungus colony (before sporulation process occurs) was transferred using the 6-millimeter diameter of sterilized pipet onto 1% tannic acid plate agar. The fungus colony's diameter and the hydrolytic zone were measured on the third day [21].

### H. Secondary Screening: Submerged Fermentation (SmF)

A volume of 1ml of $1x10^{\wedge 8}$ spore suspension was added into each sterilized 250 ml conical flask containing 49 ml of 1% tannic acid solution. The media was incubated in a rotary shaker at 32 ˚C, 100 rpm for 3 days, and harvested sample every day by filtrate using No. 1 Whatman paper and then stored in a freezer -20 ˚C [21].

### I. Tannase Assay

Tannic acid (0.1 ml, 0.5%) solution was added into 0.7 ml of crude enzyme and incubated in a water bath at 60 ˚C for 10 minutes. Three milliliters of 1 mg/ml Bovine Serum Albumin (BSA) solution were added into the reaction mixture to stop the enzymatic reaction and then centrifuged

at 10000 rpm, 5 ˚C for 7 minutes. Three milliliters of 1% of Sodium dodecyl (SDS) was added into the filtrate after the suspension was discarded. Next, 1 ml of 0.01 M $FeCl_3$ was added into the sample and kept for 15 minutes. The absorbance of the sample was read at 530 nm using a spectrophotometer. The heated crude enzyme solution was used as a control for this assay [5]. In definition, one unit of the enzyme tannase is the amount of enzyme able to hydrolyze 1µ mole of the ester bond of tannic acid per minute at specific conditions.

### *J. Identification of Potential Fungal Strain*

The morphology of selected potential tannase-producing fungi was observed under the light microscope. Molecular identification was conducted using the polymerase chain reaction (PCR) method and DNA sequencing. Primer ITS 1 (Forward Primer) and ITS 4 (Reverse Primer) were used in this study [5].

Figure 1 shows an experimental framework for this study. Started with isolation of fungal strains from agri-industrial by-products, continued with primary and secondary screening—lastly, identification of the competent tannase-producing fungal strain.

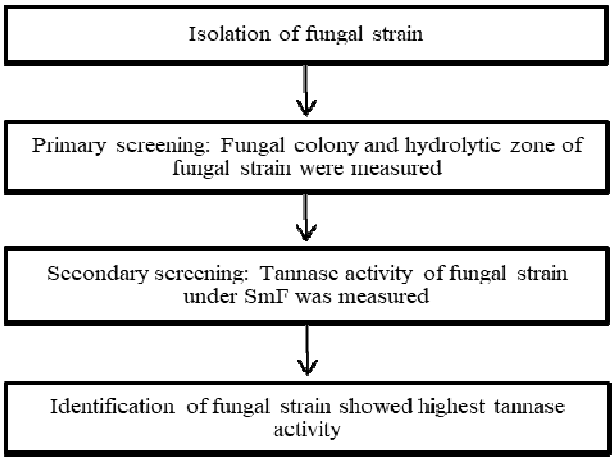


Fig. 1 Flowchart of experimental framework

## III. Result and Discussion

### *A. Isolation and Primary Screening of Tannase Producing Fungi*

A total 56 fungal strains were tested in primary screening, 51 strains isolated from 16 different local agri-industrial by-products such as spent coffee ground, spent tea, rice bran, brewer's rice, desiccated coconut residue, cocoa pod and cocoa husk, mango peels, tomato, and banana peel while other five strains from *Aspergillus* sp. family obtained from MARDI Serdang.

Primary screening was conducted by measuring the hydrolytic zone's diameter produced by fungal strain on plate assay containing tannic acid as a sole carbon source. Positive tannase-producing fungi secrete tannase to utilize the tannic acid as a carbon source, thus creating a hydrolytic zone around the colony. The fungal strains were ranked based on the diameter of the hydrolytic zone. Table 1 shows the result of the hydrolytic zone and colony's diameter of all fungal strains tested.

TABLE I
PRIMARY SCREENING OF FUNGI ISOLATES

| No. | Source of Fungal strain | Isolates | Diameter of colony(mm) | Diameter of hydrolytic zone (mm) |
|---|---|---|---|---|
| 1 | | A1 | 24.7 ± 0.6 | 29.7 ± 0.6 |
| 2 | Brewer's | A2 | 43.8 ± 1.2 | 55.7 ± 0.6 |
| 3 | rice | A3 | 27.3 ± 0.6 | 31.7 ± 0.6 |
| 4 | | A4 | 22.0 ± 0.0 | 32.7 ± 0.6 |
| 5 | | B1 | 28.7 ± 1.2 | 34.0 ± 1.0 |
| 6 | Rice bran | B2 | 42.3 ± 0.6 | 53.7 ± 0.6 |
| 7 | | C1 | 23.7 ± 0.6 | 38.3 ± 0.6 |
| 8 | Spent | C2 | 41.0 ± 0.0 | 56.7 ± 1.2 |
| 9 | coffee | C3 | 31.7 ± 1.2 | 43.3 ± 1.5 |
| 10 | ground | C4 | 38.3 ± 0.6 | 54.0 ± 0.0 |
| 11 | | D1 | 18.7 ± 0.6 | 23.0 ± 0.0 |
| 12 | Spent tea | D2 | 33.0 ± 0.0 | 40.7 ± 1.5 |
| 13 | | D3 | 29.3 ± 1.6 | 39.7 ± 1.5 |
| 14 | | D4 | 27.3 ± 0.6 | 35.0 ± 1.0 |
| 15 | | E1 | 33.0 ± 1.7 | 43.3 ± 4.2 |
| 16 | Mango | E2 | 43.0 ± 1.0 | 54.3 ± 0.6 |
| 17 | peels | E3 | 35.7 ± 1.5 | 46.0 ± 1.0 |
| 18 | | E4 | 37.0 ± 2.6 | 47.7 ± 4.0 |
| 19 | | G1 | 44.0 ± 1.0 | 56.7 ± 0.6 |
| 20 | Cocoa | G2 | 30.3 ± 1.5 | 38.3 ± 2.3 |
| 21 | peels | G3 | 23.0 ± 1.7 | 32.3 ± 1.2 |
| 22 | | G4 | 24.0 ± 2.6 | 33.3 ± 1.5 |
| 23 | | H1 | 32.0 ± 2.0 | 43.7 ± 1.5 |
| 24 | | H2 | 31.0 ± 1.0 | 43.3 ± 1.2 |
| 25 | Cocoa pod | H3 | 24.7 ± 0.6 | 34.3 ± 0.6 |
| 26 | and seed | H4 | 31.0 ± 0.0 | 43.0 ± 0.0 |
| 27 | | H5 | 39.0 ± 0.0 | 51.7 ± 1.2 |
| 28 | | H6 | 28.7 ± 1.2 | 39.7 ± 1.5 |
| 29 | | H7 | 23.7 ± 1.2 | 35.3 ± 3.1 |
| 30 | | I1 | 45.3 ± 0.6 | 59.3 ± 0.6 |
| 31 | Tapioca | I2 | 42.7 ± 3.1 | 53.3 ± 2.5 |
| 32 | peels | I3 | 26.0 ± 1.7 | 35.7 ± 1.6 |
| 33 | | I4 | 34.7 ± 0.6 | 46.7 ± 0.6 |
| 34 | Banana | J1 | 45.3 ± 0.6 | 60.7 ± 0.6 |
| 35 | peels | J2 | 20.0 ± 1.0 | 27.3 ± 0.6 |
| 36 | (*Nangka*) | J3 | 29.3 ± 0.6 | 38.0 ± 1.0 |
| 37 | Banana | K1 | 23.0 ± 2.0 | 28.7 ± 2.9 |
| 38 | peels (*Abu*) | K2 | 15.0 ± 2.0 | 18.3 ± 1.5 |
| 39 | | L1 | 21.3 ± 0.6 | 28.3 ± 0.6 |
| 40 | | L2 | 20.3 ± 0.6 | 28.0 ± 0.0 |
| 41 | Banana | L3 | 21.3 ± 0.6 | 28.7 ± 0.6 |
| 42 | peels | L4 | 23.3 ± 0.6 | 31.3 ± 0.6 |
| 43 | (*Tanduk*) | L5 | 22.0 ± 1.0 | 29.3 ± 1.2 |
| 44 | | L6 | 35.0 ± 4.0 | 45.0 ± 5.0 |
| 45 | | L7 | 14.0 ± 4.0 | 21.0 ± 1.7 |
| 46 | | L8 | 20.3 ± 0.6 | 29.7 ± 0.6 |
| 47 | Desiccated coconut shred | M1 | 41.3 ± 0.6 | 56.0 ± 1.0 |
| 48 | Soya bean residue | N1 | 25.3 ± 0.6 | 30.0 ± 0.0 |
| 49 | Red onion | O1 | 39.3 ± 0.6 | 55.7 ± 0.6 |
| 50 | Sweet potato peels | P1 | 20.3 ± 1.5 | 30.3 ± 0.6 |
| 51 | Tomato | Q1 | 20.3 ± 0.6 | 26.0 ± 0.0 |
| 52 | *Aspergillus oryzae* | F0011 | 24.7 ± 0.6 | 31.0 ± 1.0 |
| 53 | *Aspergillus flavus* | F0070 | 20.0 ± 0.0 | 27.0 ± 0.0 |
| 54 | *Aspergillus flavus var. columnaris* | F0009 | 27.3 ± 1.5 | 32.3 ± 1.5 |
| 55 | *Aspergillus sojae* | F0023 | 30.0 ± 0.0 | 33.3 ± 0.6 |
| 56 | *Aspergillus niger* | F0018 | 36.7 ± 0.6 | 51.3 ± 1.2 |

The percentage of positive tannase producers obtained from each agri-industrial by-product was shown in Figure 2.

Most of the positive tannase-producing fungi were isolated from banana peels with 13 fungal isolates, followed by cocoa residue and rice by-products with 11 fungal isolates and 6 fungal isolates, respectively. The least number of positive tannase producers was found from soybean, desiccated coconut, tomato, and onion, with only 1 fungal isolate obtained. The addition of molasses in the initial stage before the natural fermentation process promotes the growth of fungi. Tannase producer fungi can be found in high tannin-rich agri-industrial by-products such as Jamun (*Syzgium cumini*), amaltash (*Cassia fistula*), tamarind (*Tamarindus indica*), mulberry (*Morus macroura*), keeker (*Acacia nilotica*), pomegranate (*Punica granatum*) and mango (*Magnifera indica*) [22]. Fungal isolates were also obtained from different environmental sources such as various tea dump sites, agri-industrial waste sites, and site nearby tannery industries [5].

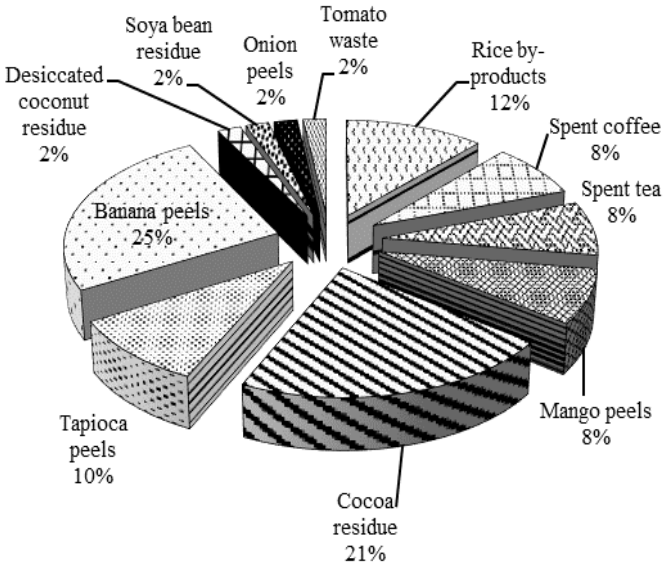


Fig. 2 Percentage of positive tannase-producing fungi isolated from various agri-industrial by-products.

From 56 isolates tested, only 13 isolates which are JI, O1, M1, F0018, I1, I2, H5, G1, E2, A2, B2, C2, and C4 were selected as potential tannase producer due to their large diameter of hydrolytic zone ranging from 51 mm to 61 mm after incubation at 30˚C for 72 hours and these fungal strains were subjected to submerged fermentation for secondary screening.

### *B. Secondary Screening under Submerged Fermentation*

The results showed that fungal strain J1 isolated from banana peels showed the highest tannase activity with (6.86 ± 0.04) U/ml at 72 hours followed by fungal strain I1 and G1 with tannase activity of (6.13 ± 0.08) U/ml and (4.39 ± 0.02) U/ml respectively (Figure 3). Isolate H5 showed the least tannase activity. In general, the highest tannase activity for all fungal strains was observed after 72 hours. The low tannase activity before 48 hours because less mycelium is produced in the early stage; thus, less extracellular tannase is synthesized to break down the medium's tannin. J1 isolate was chosen as the best tannase-producing fungus due to its highest tannase activity.

The tannase activities show a high correlation with r= 0.9204 between the hydrolytic zone diameter and tannase activity in all 13 fungal isolates. These results are in line with previous studies [10], [23], [24]. Their studies showed a correlation between tannase activity and the diameter of hydrolytic zone produced by isolates on plate assay.

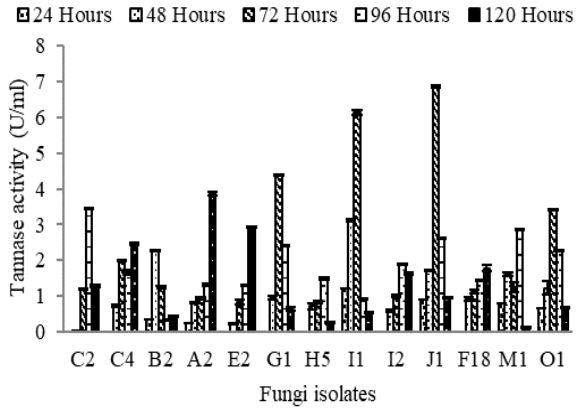


Fig. 3 Tannase activity of selected fungal strains under SmF

### *C. Identification of Fungal Strain*

Fungal strain J1 showed dark green conidia on PDA plate, while black conidia were observed on TAA plate. Besides, white mycelium was observed at the end of the colony on both PDA and TAA, and there was no pigmentation on the reverse side of both plates. A hydrolytic zone can be seen clearly on TAA plate, as shown in Figure 4. Under the light microscope, it was observed that J1 isolate has septate hyphae, biserrate phialides radiate around the conidiophore, long and globose conidiophore, and round-shaped conidia, as shown in Figure 5.

For molecular identification, purified DNA was amplified using polymerase chain reaction (PCR). Two types of primers were used in this study: ITS 1 (Forward Primer) and ITS 4 (Reverse Primer). The band produced was sent to First BASE Laboratories Sdn Bhd for the DNA sequencing. The result from the sequencing analysis was analyzed by using the software; NCBI BLAST Website. It was found that PN1 isolate is *Aspergillus niger*.

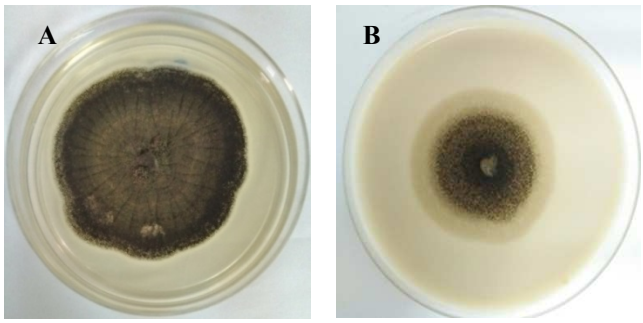


Fig. 4 Growth of J1 Isolate (A) on PDA (B) on TAA

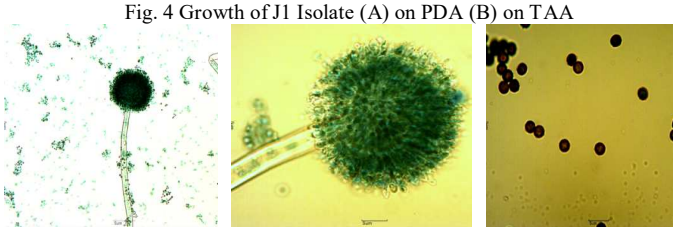

Fig. 5 Light microscopy of J1 isolate

## IV. Conclusion

This report showed that tannase-producing fungal strain could be isolated from local agri-industrial by-products such as banana peels, rice bran, brewer's rice, red onion, cocoa pod and husk, mango peels, and desiccated coconut residue. Besides, fungal strain J1 from banana peels was identified as the most potential tannase-producing fungi due to the largest hydrolytic zone diameter of (60.7 ± 0.6) mm and the highest tannase activity (6.86 ± 0.04) U/ml. The study reveals the low-cost agri-industrial by-products' potential as the best source to isolate potential tannase producer fungi.

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