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Aspergillus sydowii Strain SCAU066 and *Aspergillus versicolor* Isolate BAB-6580: Potential Source of Xylanolytic, Cellulolytic and Amylolytic Enzymes

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ABSTRACT

Fungi is known to produce a wide range of biologically active metabolites and enzymes. Enzymes produced by fungi are utilized in food and pharmaceutical industries because of their rich enzymatic profile. Filamentous fungi are particularly interesting due to their high production of extracellular enzymes which has a large industrial potential. The aim of this study is to isolate potential soil fungi species that are able to produce functional enzymes for industries. Five *Aspergillus* species were successfully isolated from antibiotic overexposed soil (GPS coordinate of N3.093219 E101.40269) by standard microbiological method. The isolated fungi were identified via morphological observations and molecular tools; polymerase chain reactions, ITS 1 (5'- TCC GTA GGT GAA CCT GCG G3') forward primer and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') reverse primer. The isolated fungi were identified as *Aspergillus sydowii* strain SCAU066, *Aspergillus tamarii* isolate TN-7, *Aspergillus candidus* strain KUFA 0062, *Aspergillus versicolor* isolate BAB-6580, and *Aspergillus protuberus* strain KAS 6024. Supernatant obtained *via* submerged fermentation of the isolated fungi in potato dextrose broth (PDB) and extracted via centrifugation was loaded onto specific media to screen for the production of xylanolytic, cellulolytic and amylolytic enzymes. The present findings indicate that *Aspergillus sydowii* strain SCAU066 and *Aspergillus versicolor* isolate BAB-6580 have great potential as an alternative source of xylanolytic, cellulolytic and amylolytic enzymes.

Keywords: *Aspergillus sydowii; Aspergillus versicolor;* Xylanolytic; Cellulolytic; Amylolytic

INTRODUCTION

Filamentous fungi are the preferred source of industrial enzymes because of their excellent capacity to produce extracellular enzymes [1]. These extracellular enzymes are synthesised inside the cell and excreted to degrade macromolecules into small molecules. Cellulose can be broken down into glucose, cellooligosaccharides and cellobiose by biological and physicochemical processes i.e. cellulase. Guo et al. [2] reported that plants, animals, fungi and some invertebrate animals possess the gene that codes for the cellulase enzyme. Xylan is a hemicellulose that is the second most abundant polysaccharide. Many bacterial

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and fungal species have been reported to synthesize and externally secrete xylanases into the environment. The function of xylanase enzyme is to degrade or depolymerize xylan. Miao et al. [3] reported that *Aspergillus* species showed a high efficiency in the secretion of xylan-degrading enzymes. According to Bajpai [4], although xylanases can be produced by bacteria, yeast and other organisms, filamentous fungi remain as the major industrial source for the production of xylanase. Xylanase is widely use in the production of food and beverages, as a supplement in animal fed and in textile production.

Amylolytic enzymes play an important role in the breakdown of starch into saccharides. Gangadharan and Sivaramakrishnan [5] reported that amylolytic enzymes produced by microorganisms make up approximately 25 – 33 % of the global marketer for industrial enzymes. According to [5], microbial enzymes are preferred over plant and animal enzymes due to their stability which significantly increases the industrial uses. In spite of a large number of non-pathogenic microorganisms that can produce useful enzymes, filamentous fungi are widely used. This is due to easy cultivation and high production that led to large industrial potential, low production cost, rapid development and the enzyme produced can be modified and recovered easily [5-7]. Therefore, the aim of this study is to isolate potential soil fungi species that are able to produce functional enzymes for industries.

EXPERIMENTAL

Soil sample collection

Soil samples were collected from Ayu Farm, Meru, Klang, a soil plot where veterinary antibiotics was used extensively. The GPS coordinate is as follows; (N3.093219 E101.40269). A weight of 500 g of soil was collected from a depth of 15 cm. Soil samples were collected and preserved in sterile plastic bags before being stored in an icebox to maintain the soil's natural properties. The soil was then taken to the laboratory and stored at 4 °C for further use.

Identification of isolated soil fungi

DNA of the isolated soil fungi was extracted using two methods. The DNA of F6 and F9 were extracted using a modified DNeasy Plant Mini Kit by QIAGEN (Germany) with additional steps prior to using the kit. The additional steps involved a Cetyltrimethyldibromide (CTAB) method. This method consists of, 2 % CTAB, 5 M Potassium acetate and 10 % SDS. Scraps of isolated fungi were taken and crushed using liquid nitrogen. Three hundred microliters (300 µL) of 2 % CTAB was added and mixed using a vortex. This was followed by the introduction of 120 µL of 10 % SDS into the tube and a second vortex process. The tube was incubated in a water bath at 60 °C, for 30 minutes. Next, 300 µL of 5M Potassium acetate was added and mixed. The tubes were then allowed to cool before proceeding with the Dneasy Plant Mini Kit protocol.

The DNA of F3, F4 and F5 were extracted using innuPREP Plant DNA kit from Analytic Jena (Germany), F3, F4 and F5 involved a lysis buffer CBV. The extractions were followed according to the kit's protocol. The DNA extracted was amplified using 0.2 µM ITS 1 (5'- TCC GTA GGT GAA CCT GCG G3') forward primer and 0.2 µM ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') reverse primer. The PCR master mix was prepared using Top Taq DNA polymerase by QIAGEN which contained 10x TopTaq PCR buffer, Coralload concentrate, Q-solution, dNTP mix and TopTaq DNA polymerase. The PCR conditions were as

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follows; initial denaturation (94 °C for 3 minutes), 30 cycles of denaturation (94 °C for 30 seconds), annealing (61.4 °C, 30 seconds), extension (72 °C, 1 minute) and final extension (72 °C, 5 minutes)

Submerged fermentation

Fermentation was performed according to published procedure with some modifications using Potato Dextrose Broth (PDB) [8]. Under aseptic conditions, each culture flask that contained 100 mL PDB inoculated with a scrap of fungi. All culture flasks were incubated at 120 rpm and 25 °C for 7 days. The experiments were carried out in triplicates

Extraction of the crude enzyme from fermentation media

Extraction of the enzyme was done by a simple filtration of media using Whatman filter paper No.1 for separation of fungal mycelia. Then, the filtrate was centrifuged at 10,000 rpm for 20 minutes. The clear supernatant was used as crude enzyme.

Screening for enzyme production using a plate assay method

Amylolytic production

Preliminary screening of amylolytic activity was carried out on starch nutrient agar plates. The plate was incubated at 30 ºC for 72 hours. The clearance zone resulting from starch hydrolysis was determined by flooding the plates with Gram's iodine stain for 15 minutes and washing with distilled water to remove the excess colour. The potential amylase producers were selected based on the diameter of the clearance [9].

Asparaginolytic production

Czapek's agar addition with phenol red to produce a yellowish colour at pH 5.6 was used to screen the production of asparaginolytic. Plates were loaded with crude enzymes and incubated at 37 °C for 24 and 72 hours, respectively. A pink and purple zone was observed as positive result [10].

Proteolytic production

Proteolytic activity of the fungi was screened with gelatine nutrient agar and incubated at 28 ℃ ± 1 ℃ for 72 hours. A clear zone was detected on the plates by flooding with a saturated solution of mercuric chloride reagent or ammonium sulphate solution. A white precipitate was produced as mercuric chloride solution reacted with gelatine which made the clearing zone visible. Zone of clearance was observed for the presence of proteolytic activity [11].

Xylanolytic production

In order to detect xylanase production, the crude enzyme was loaded into the well at the centre of the xylan rich agar. The plate was incubated at 28 °C ± 2 for 72 hours. Then, the xylan rich agar was stained with iodine solution for 15 minutes and the plates were destained using warmed sterile distilled water. Zone of clearance was observed for the presence of xylanase activity [12].

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Cellulolytic production

Carboxymethylcellulose (CMC) agar plate was incubated at 37 °C for 24 - 48 hours. A clear zone was formed on the CMC agar plate after the application of Gram's iodine solution which indicated cellulose enzyme production [13].

Lipolytic production

Lipase activity of fungi was screened on tributyrin nutrient agar (TBA) plates. The plates were incubated for 48 hours at 37 °C. Halo zones were produced which indicate the hydrolysis of lipase [14].

RESULTS AND DISCUSSION

Identification of the isolated fungi

Five isolated fungi were labeled as F3, F4, F5, F6 and F9 (Figure 1). The isolated fungi were identified by morphological and molecular identifications. As shown in Figure 1(a), F3 has a two-toned green coloured colony. The growth of F3 on the plate shows a green coloured colony with a light green lining and an outer white lining. The texture is moist as extrolites formed after six days of incubation. No colour change on the reverse plate was observed after 14 days of incubation. After 14 days of incubation, the colony had a diameter of 4.0 cm. Microscopic observations showed long conidiophores, with conidiospores and no hyphae as shown in Figure 1(b).

F4 showed yellow to a green coloured colony (Figure 1(c)). It was observed to be in circular and powdery form. The diameter of the colonies was 8.4 cm after four days of incubation. No colour change was observed after 14 days of incubation. Microscopic observations showed long conidiophores with vesicle attached to phialides and at the end of it was conidiospores (Figure 1(d)). Figure 1(e) shows a flat white yellowish colony of F5. After 14 days of incubation, the colony had a diameter of 3.5 cm. No colour change was observed after 14 days of incubation. Microscopic observations showed conidiophores, vesicle and conidiospores (Figure 1(f)).

Figure 1(g) showed F6 colony with a greenish-brown coloured colony. There were wrinkles in the middle of the colony. After five days of incubation, it started to show brown droplets of extrolites in the middle of the plate. The reverse plate started to change colour to dark purple after five days of incubation. The colony diameter was 3.9 cm after 14 days of incubation. Microscopic characterization showed conidiophores with no hyphae observed. Phialides attached with conidiospores at the end of it were also observed (Figure 1(h)). F9 is yellow and green coloured with a white lining on the outer area of the colony as shown in Figure 1(i). It is a flat colony that is circular in shape. The diameter of the colony was 4.1 cm after 14 days of incubation. Microscopic observation showed long conidiophores, conidiospores and vesicle with no hyphae present (Figure 1(j)).

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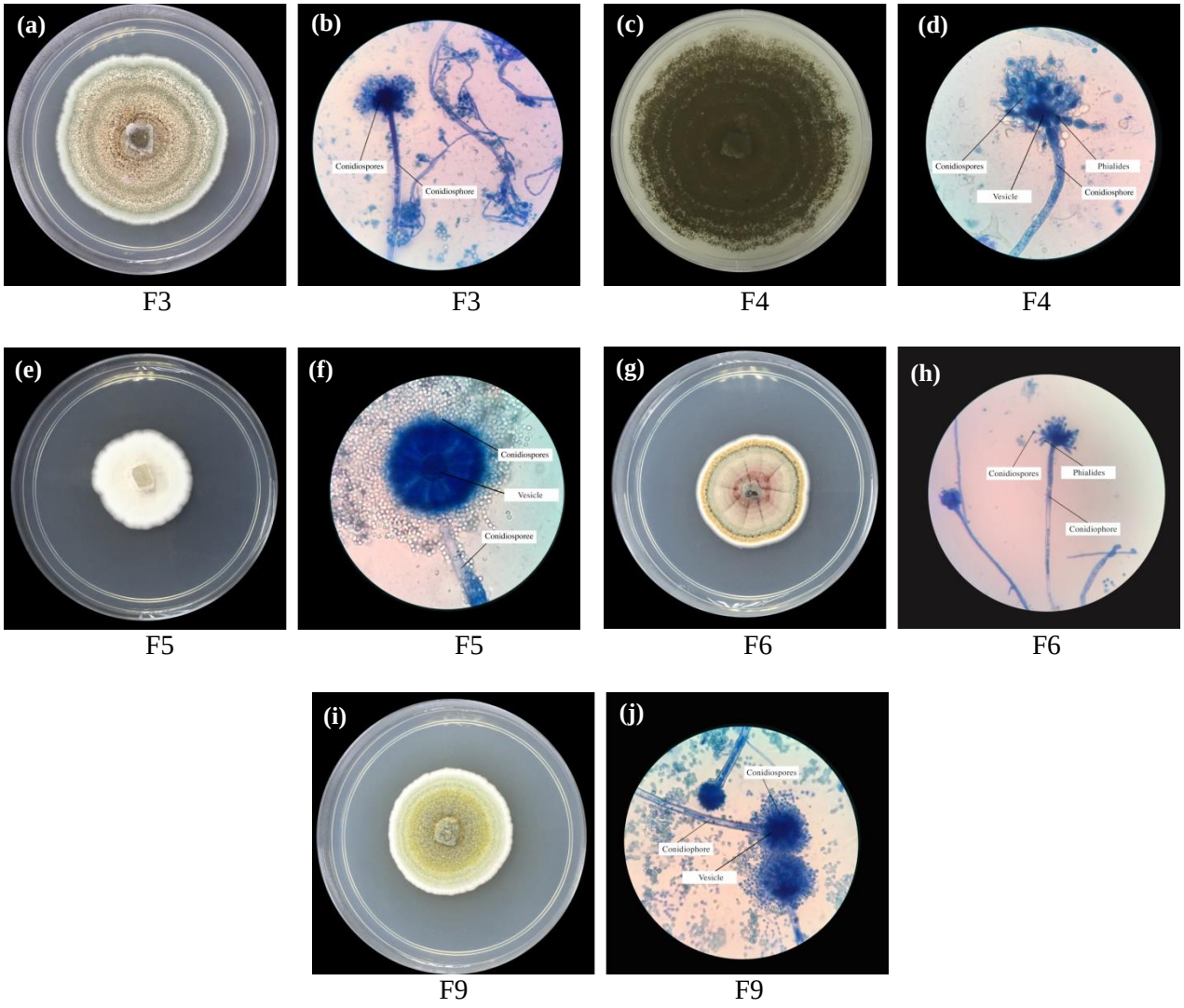

Figure 1: Macroscopic and microscopic observation with 1000X total magnification of isolated soil fungi.

The isolated fungi were further identified with molecular tools. Genomic DNA of the isolated fungi were amplified with fungal universal primer, Internal Transcribed Spacer (ITS) region primer and identified as *Aspergillus versicolor* isolate BAB-6580 (F3), *Aspergillus protuberus* strain KAS 6024 (F4), *Aspergillus sydowii* strain SCAU066 (F5), *Aspergillus tamarii* isolate TN-7 (F6) and *Aspergillus candidus* strain KUFA 0062 (F9). Figure 2 shows the PCR products separated on 1% agarose gel containing 1x GelRed stain.

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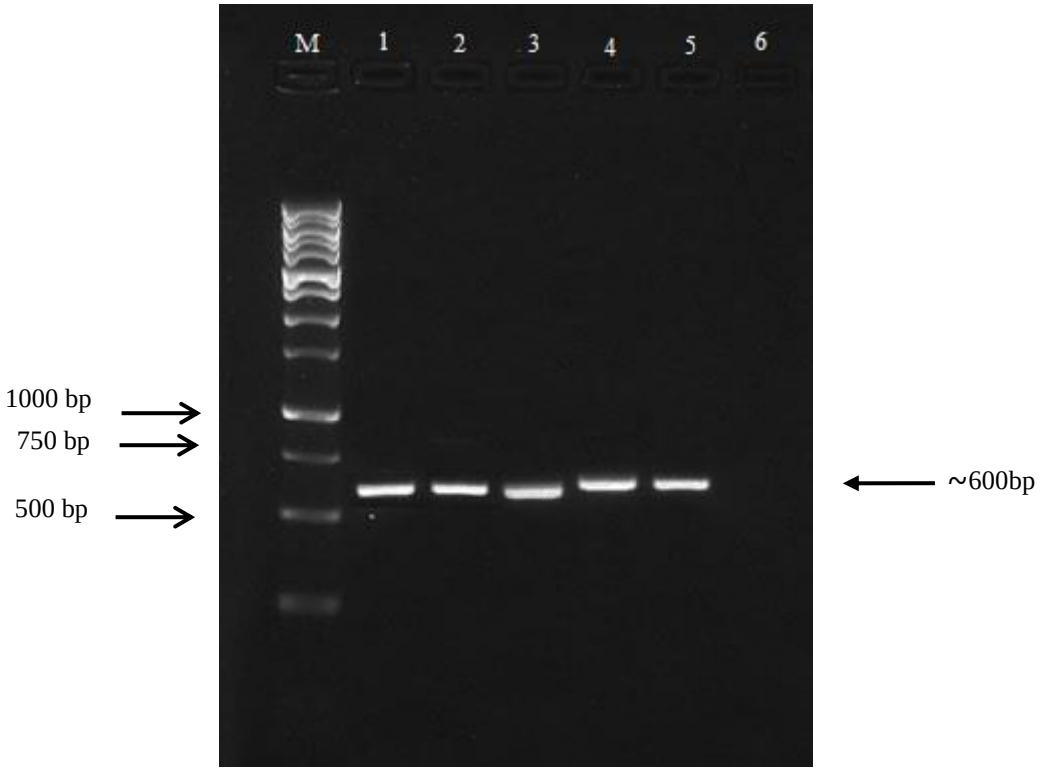

Figure 2: Agarose gel electrophoresis (1%) of PCR products amplified with ITS1 and ITS4 primers. Lane M: 1kb DNA ladder marker (Solis Biodyne) 1. *Aspergillus versicolor* isolate BAB-6580; 2. *Aspergillus protuberus* strain KAS 6024 ; 3. *Aspergillus sydowii* strain SCAU066; 4. *Aspergillus tamarii* isolate TN-7; 5. *Aspergillus candidus* strain KUFA 0062; 6. Negative control.

Lipolytic, proteolytic, xylanolytic, cellulolytic, amylolytic and asparaginolytic activities of soil fungi

Aspergillus sydowii strain SCAU066, *Aspergillus tamarii* isolate TN-7, *Aspergillus candidus* strain KUFA 0062, *Aspergillus versicolor* isolate BAB-6580 and *Aspergillus protuberus* strain KAS 6024 that were isolated from soil showed great potential for the production of xylanolytic (Figure 3) and cellulolytic enzymes. The halo zone diameter of the enzymatic production is tabulated in Table 1. The halo zone showed on CMC agar plates in Figure 4 indicated that the isolated *Aspergillus* species were able to degrade cellulose through the production of cellulose enzymes. CMC agar was used for cellulolytic production which contained 1% CMC (carboxymethylcellulose). Gram's Iodine solution was flooded onto the CMC agar plates which formed a brown-black complex with cellulose. The fungi with the capability to produce cellulase formed a distinct clearance zone while the other non-producing cellulase fungi remained brown-black. Other microorganisms such as *Fibrobacter*, *Streptococcus*, *Escherichia* and *Aspergillus oryzae* also demonstrated the capability to break down cellulose. According to Khatiwada et al. [13], a lot of studies give more attention to fungi as they can produce cellulase.

Figure 5 showed only *Aspergillus sydowii* strain SCAU066 and *Aspergillus versicolor* isolate BAB-6580 with amylolytic activity. In this assay, starch agar consisting of soluble starch that acts as a carbohydrate

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source was used. Starch agar plate that was flooded with Gram's iodine produced blue coloured which formed a complex with starch that existed on the agar. An amylolytic activity was indicated by the formation of halo zone around the fungi that were grown on the starch agar.

However, all the fungal isolate showed no halo zone for asparaginolytic, proteolytic and lipolytic enzyme activities. Thus, these fungi did not produce any asparaginase, protease or lipase. Since asparaginase was not produced by the fungi, the asparagine that existed in the agar cannot be hydrolyzed which resulted in no halo zone forming for proteolysis. For screening of proteolytic production, no halo zone formed on the gelatin agar because all the selected soil fungi did not produce any protease to break down gelatin in the agar. Moreover, the fungi also could not hydrolyze triglycerides in TBA agar since they did not produce lipase; therefore no halo zone was formed.

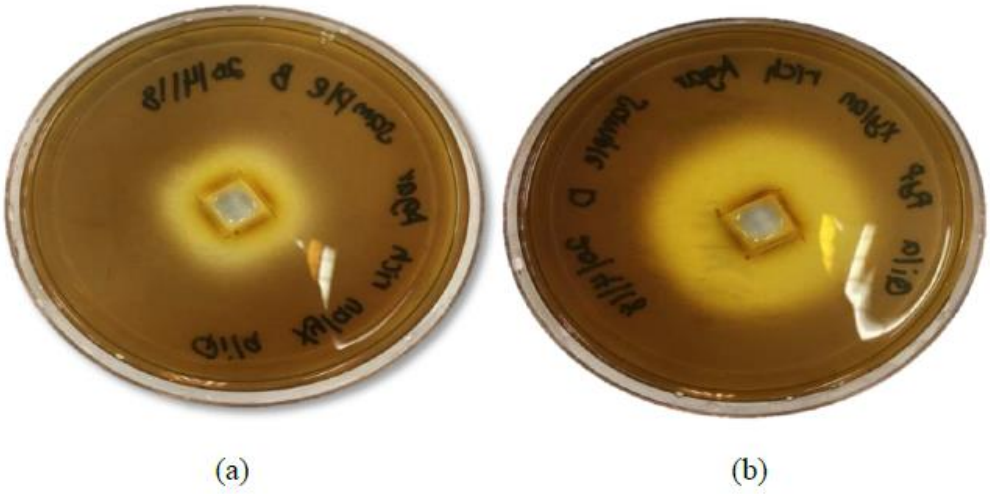

Figure 3: Halo zone of xylanolytic production by (a) *Aspergillus sydowii* strain SCAU066 and (b) *Aspergillus versicolor* isolate BAB-6580.

Table 1: The mean diameter of the halo zone (cm) ± standard deviation of the isolated fungi

Isolate	Amylolytic (cm)	Asparaginolytic (cm)	Proteolytic (cm)	Xylanolytic (cm)	Cellulolytic (cm)	Lipolytic (cm)
F3	1.40 ± 0.17	-	-	2.70 ± 0.20	2.00 ± 0.42	-
F4	-	-	-	2.60 ± 0.21	2.30 ± 0.42	-
F5	-	-	-	2.50 ± 0.11	2.10 ± 0.33	-
F6	2.30 ± 0.26	-	-	4.30 ± 0.15	3.40 ± 0.66	-
F9	-	-	-	2.90 ± 0.14	2.30 ± 0.46	-

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(a) (b)

Figure 4: Halo zone of cellulolytic production by (a) *Aspergillus sydowii* strain SCAU066 and (b) *Aspergillus versicolor* isolate BAB-6580.

(a) (b)

Figure 5: Halo zone of amylolytic production by (a) *Aspergillus sydowii* strain SCAU066 and (b) *Aspergillus versicolor* isolate BAB-6580.

CONCLUSIONS

The *Aspergillus* species, specifically *Aspergillus sydowii* strain SCAU066 and *Aspergillus versicolor* isolate BAB-6580 showed potentiality as amylolytic, xylanolytic and cellulolytic enzymes producers. This demonstrates that soil fungi possess the capability to produce amylase, xylanase and cellulase. These enzymes provide a lot of benefits to biotechnological and pharmaceutical industries.

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