Assiut University Journal of Multidisciplinary Scientific Research (AUNJMSR)
Faculty of Science, Assiut University, Assiut, Egypt.
Printed ISSN 2812-5029
Online ISSN 2812-5037
Vol. 52(1): 48- 66 (2023)
https://aunj.journals.ekb.eg/

Production of cocktail enzymes and partial purified pectinase utilizing lignocellulosic wastes by specific *Didymella* species

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ARTICLE INFO

Article History:
Received:
Accepted:
Online:

Keywords:
Cocktail enzymes; *Didymella*; Fermentation; ITS; Pectinase

ABSTRACT

Three possible novel *Didymella* species, isolated from Mango and Guava juices in Assiut, Egypt, were identified based on morphology and sequencing of the internal transcribed spacers region (ITS). The three strains could ferment five lignocellulosic residues, namely bean straw, corn cobs, rice husk, wheat bran, and wheat straw in solid-state fermentation (SSF) and produce cocktail enzymes (endoglucanase, exoglucanase, laccase, pectinase, and xylanase). *Didymella* sp. AUMC 15636 and AUMC 15637 produced the most endoglucanase, exoglucanase and xylanase activity (20 and 23, 10 and 17, 18 and 18 U/gds, respectively) on bean straw, and laccase (0.7 and 2.6 U/gds) on corn cobs and wheat bran, in addition to pectinase (37 and 26 U/gds) on rice husk and corn cobs, respectively. *Didymella* sp. AUMC 15640 released the maximum endoglucanase, exoglucanase, laccase, pectinase, and xylanase enzymes on the bean straw residues (24, 24, 10, 31 and 27 U/gds), respectively. *Didymella* sp. AUMC 15636 yielded 0.9 g crude pectinase per liter fermentation medium in submerged fermentation (SmF). At pH 6.0, a peak in pectinase activity (80 U/gds) was detected, which rose to 100 U/gds at 45 ºC. Presence of Mn^{2+} ions activated the pectinase by 30% over the control. Zn^{2+} and EDTA had no effect on pectinase activity, while sodium dodecyl sulfate and other ions lowered pectinase activity by 10 to 30%, while K^{+} and Ca^{2+} having the highest impact. The target of this study, which is advocated here by utilizing such strains, is to

bioconvert lignocellulosic residues into value-added product.

INTRODUCTION

Five billion metric tonnes of lignocellulosic biomass are generated annually worldwide. Therefore, it is important to convert these wastes into useful outputs with industrial and commercial value and minimizing their detrimental impact on the environment [1]. Lignocellulose makes up 50 % of all plant matter. It is made up of cellulose (35–50%), hemicellulose (20–35%), and lignin (15–25%) [2-4].

When substantial environmental and health risks are posed by fields that have been burnt, they are still mostly unexplored in Egypt. These residues should not be considered "waste," but rather as "natural resources" for use in later industrial operations and may be employed as a suitable source of enzyme synthesis since they are typically plentiful in proteins, carbs, and minerals. Thus, more than one enzyme is used in their biodegradation by microorganisms. These leftovers may be used by fungi to produce beneficial minerals and compounds [4, 5].

Cellulases are utilized in several industries including biofuel, textile, pulp and paper, detergent, animal feed, and food sectors [6-8]. Xylanases are used to improve the clarity of fruit juices, de-icing of paper waste, biobleaching, and the consistency of milk, feed, and fiber, as well as the saccharification of hemicelluloses to xylose sugars [9]. Ligninases are utilized commonly in the biofuel generation. They are also used in the textile, food, paper, cosmetic, and pharmaceutical sectors, as well as in organic synthesis, wastewater treatment, and bioremediation [6, 10]. Laccase is now the top priority because to its several uses, which include dye decolourization, wastewater detoxification, and biological remediation. Laccase can oxidize a wide range of substrates, including ortho and paradiphenols, methoxy-substituted phenols, aromatics, phenolic acids, and many other molecules [11, 12].

Pectinases are enzymes that specifically target pectin and depolymerize it by the processes of hydrolysis, transelimination, and deesterification, which hydrolyze the ester bond between the carboxyl and methyl groups of pectin. These enzymes break down pectin, a complex polymer hold the cellulose network together. Pectinases make almost tenth of all commercial enzymes produced worldwide, and their market is expanding daily [13-16]. In order to produce cocktail enzymes by three *Didymella* species in SSF and partially purify pectinase enzyme in SmF, the current research aims to employ some remaining lignocellulosic wastes as an alternative carbon source.

MATERIALS AND METHODS

Strains isolation and maintenance

Three strains are involved in this investigation. The strains were isolated using the pour plate method [17] from samples of Mango and Guava juice obtained from a neighborhood market in Assiut Governorate, using oat agar medium (OA; [18] at 25 °C. The isolated fungi were purified and preserved as pure cultures [19], in Assiut University Mycological Centre as AUMC 15636, AUMC 15637, and AUMC 15640.

Morphological identification of the strains

Didymella strains were cultured on malt extract agar (MEA) (malt extract powder, 20; peptone, 1.0; glucose, 20; and agar 20), potato dextrose agar (PDA) (potato infusion from 200 g; glucose, 20; agar, 20) and oat agar (OA) (oat extract, 30; agar, 20) [18]. Microscopic features on OA were examined after 7 days of incubation at 25 °C, and the strains in this study was identified according to their macroscopic and microscopic characteristics [20].

Molecular identification of the strain

DNA extraction, PCR and sequencing

For DNA isolation, the method described in Moubasher *et al.* [21] was employed. PCR reaction was carried out [22], using ITS1 and ITS4 primer pairs [23] for ITS region amplification.

Phylogenetic analysis

Contiguous sequences of the Didymella species used in this study were obtained by DNASTAR (version 5.0). An outgroup sequence for Macroventuria wentii CBS 526.71 (MH860250), three sequences for Didymella spp. in this work and 16 sequences from the genus Didymella retrieved from GenBank made up the 20 sequences in the total ITS dataset included for phylogenetic analysis. MAFFT (version 6.861b) with the default options [24] was used for alignment of ITS sequences in this study. BMGE [25] optimized alignment gaps and sparse uninformative characters. PhyML 3.0 [26] was used to conduct maximum-likelihood (ML) and maximum-parsimony (MP) phylogenetic analyses, and the robustness of the most parsimonious trees was evaluated by 1000 replications [27]. Utilizing Modeltest 3.7's Akaike Information Criterion (AIC), the optimum nucleotide substitution model for ML analysis was identified [28]. The phylogenetic tree was drawn and visualized using MEGA X 10.2.6 [29]. The resulting tree was edited and saved as TIF format [30].

Production of cocktail enzymes from lignocellulosic biomass in solid state fermentation (SSF)

Biomass residue preparation

Bean straw (BS), corn cobs (CC), rice husk (RH), wheat bran (WB), and wheat straw (WS) are the five different agro-industrial residues that were chosen for enzyme processing in submerged (SmF) and solid state fermentation (SSF). These materials were all gathered from marketplaces in Assiut Governorate, Egypt. They underwent purified water cleaning, oven drying at 50 °C to a constant weight, and grinding to fine particles.

Production of cocktail enzymes in SSF

For enzyme production in SSF, separate Erlenmeyer flasks (500 mL) were loaded with ten grams of each of the analyzed agro-industrial residues. The biomass was moistened by adding 10 mL of 0.1% sucrose Czapek's broth to each flask. The surface was then moistened to an additional 80% moisture content using distilled water. A 1.0 mL of spore suspension from the 7-day-old culture containing 1×10^8 spore/mL inoculated each flask, and the flasks underwent 15 days of stagnant incubation at 30 °C.

Extraction and assay of cocktail enzymes

Each flask's contents was taken out separately and filtered through two layers of cheesecloth in 100 mL of a 50 mM sodium citrate buffer (pH 5.0). The cell-free supernatants were obtained by centrifugation (10,000 rpm for 10 minutes at 4 ºC), and used to undertake enzyme tests.

Enzymes assay and protein determination

Assays for endoglucanase, exoglucanase, laccase, pectinase, and xylanase were performed in accordance with Al-Kolaibe *et al*. [31]. Total protein content was measured by the method suggested by Lowry *et al.* [32]. All experiments were conducted in triplicates.

Pectinase production by *Didymella* sp. AUMC 15636 in submerged fermentation (SmF)

For pectinase production, the fungus was cultured in Erlenmeyer flasks (500 ml) at 30 ^{o}C using sucrose-free Czapek's broth ($NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4$; 0.5; KCl, 0.5; $ZnSO_4$, 0.01; $CuSO_4$, 0.005; initial pH 7.0) supplemented with 1.0% pectin (as a sole carbon source), using 1.0 mL containing 1.5×10^8 spore/ml spore suspension from 7–day–old culture. The incubation period lasted 7 days at 150 rpm. The cell-free supernatant was then obtained after centrifugation (10,000 rpm, 10 min at 4 ºC).

Partial purification and dialysis of pectinase

Total protein from 1.0 L fermentation medium was isolated from the solution at 4 ºC using triple volume cold ethanol (96.0 %), and dried by freeze dryer (VirTis, model # 6 KBTES-55, NY, USA). A 0.9 g enzyme powder was dissolved in 5 mL 100 mM citrate buffer (pH 5.0) and dialyzed according to Moharram et al. [16], and utilized as partly pure fungal pectinase in characterization tests.

Characterization of the partially-purified pectinase

A 0.01 g enzyme powder and 0.01 g citrus peel pectin (each dissolved in 1.0 ml of 50 mmol citrate buffer) were included in this test in a water bath. The effect of pH (3.0–10.0) and temperature (30–60 ºC) on pectinase activity were investigated. After the reaction time (20 min), the reaction was terminated by introducing 2.0 ml of 3,5–dinitrosalicylic acid (DNS) according to Miller [33] and Jayani *et al*. [34], and the pectinase activity was determined as the amount of the enzyme that releases 1 µmol galacturonic acid. min^{-1} under standard assay conditions. Citrate buffer (pH 3.0–6.0), phosphate buffer (pH 7.0–8.0), and borate buffer (pH 9.0–10.0) were the buffers employed. Additionally, a few ions (Na^+, K^+, Ca^{+2}, Co^{+2}, Ni^{+2}, Cu^{+2}, Fe^{+2}, Mg^{+2}, Mn^{+2}, and Zn^{+2}) were tested by being added at concentrations of 5.0 mmol/ml as NaCl, KCl, $CaCl_2$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, and $ZnSO_4$. An enzyme inhibitors

were tested using ethylenediamine tetra acetic acid (EDTA) and sodium dodecyl sulfate (SDS) at a concentration of 5.0 mmol/mL. Three repeats of each experiment were carried out.

RESULTS

Description of *Didymella* spp.

***Didymella* sp. AUMC 15636:** Colonies on MEA and OA attaining 50–55 and 40–45 mm in diameter after 7 days at 25 ºC, respectively. On OA, olivaceous green to olivaceous grey. Pycnidia scattered, arranged at margin or partly submerged in medium, subglobose, often non-ostiolate, 50–270 µm, solitary or confluent. Conidia oblong to ellipsoidal, with several small polar guttules 4–7 × 2–3 (–3.5) µm. Chlamydospores intercalary or terminal, unicellular, pale olivaceous, 5–8 µm (Figure 1).

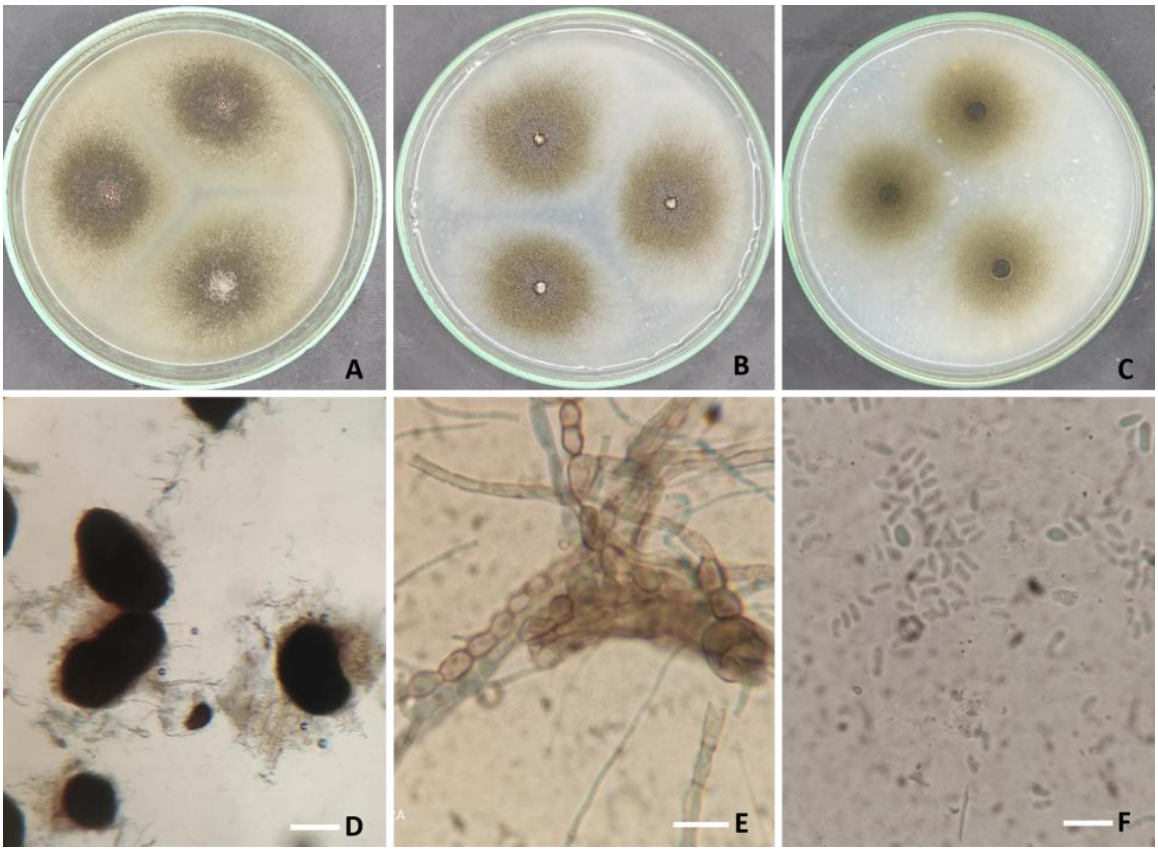

Figure 1: *Didymella* sp. AUMC 15636. A–C, 7–day-old colonies on MEA, PDA, and OA at 25 ºC. D, Pycnidia. E, chlamydospores. F, conidia. (Scale bars: D = 100 µm; E–F = 10 µm).

***Didymella* sp. AUMC 15637:** Colonies on MEA and OA attaining 50–55and 35–40 mm in diameter after 7 days at 25 ºC, respectively. On OA, dull green to olivaceous black.

Pycnidia subglobose-conical or papillate, indistinctly uniostiolate, 50–200 µm, glabrous, solitary or sometimes confluent. Chlamydospores barrel shaped or subglobose to ellipsoidal, single, in chains, intercalary, terminal, 13–32 µm. Conidia ellipsoidal, with 2 large polar guttules, (3.5–) 4–6 (–7) × (1.5–) 2–2.5(–3) µm (Figure 2).

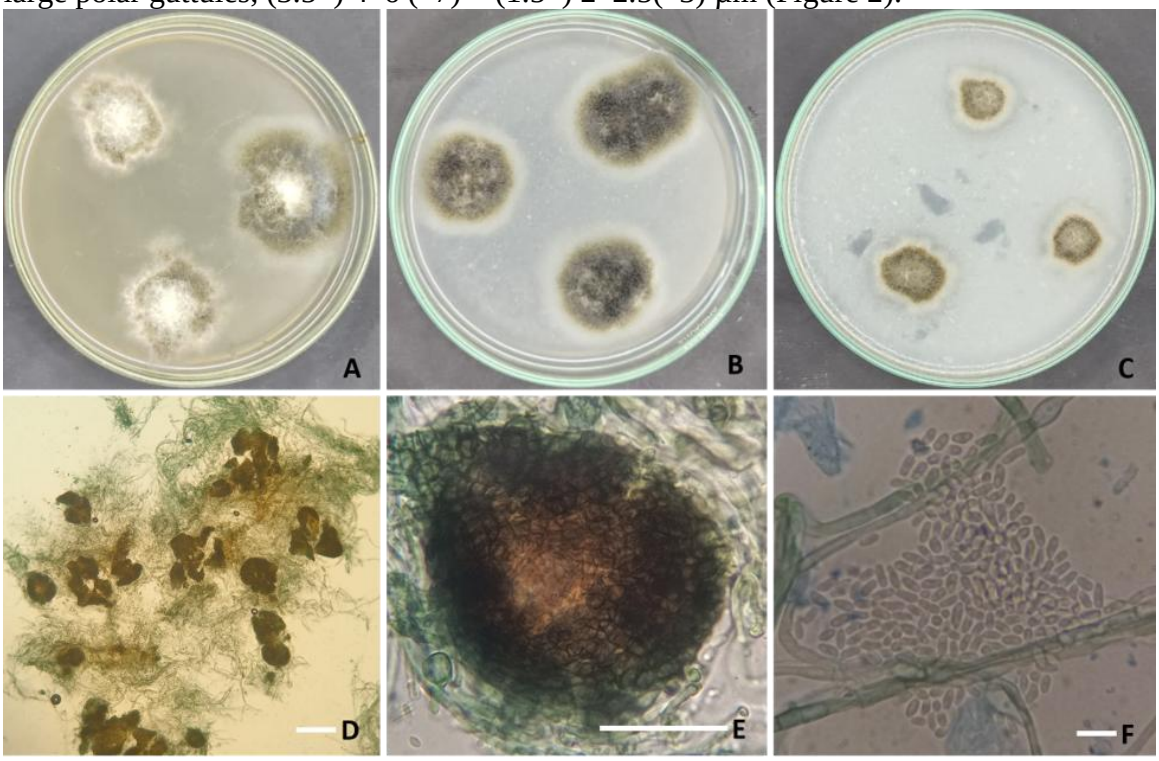

Figure 2: *Didymella* sp. AUMC 15637. A–C, 7–day-old colonies on MEA, PDA, and OA at 25 ºC. D–E, Pycnidia. F, conidia. (Scale bars: D = 200 µm; E = 100 µm; F = 10 µm).

***Didymella* sp. AUMC 15640:** Colonies on MEA and OA reaching45–50 and 30–35 mm in diameter after 7 days at 25 ºC, respectively. On OA, olivaceous grey at the center, greenish towards margin. Pycnidia abundant, mostly in greyish concentric zones, subglobose with 1–2 ostioles, often with distinct necks, glabrous, solitary or confluent, 100–250 µm. Micropycnidia usually submerged, 45–80 µm. Conidia oblong to ellipsoidal, mostly 3.5–6.5 × 1.5–2 µm (Figure 3).

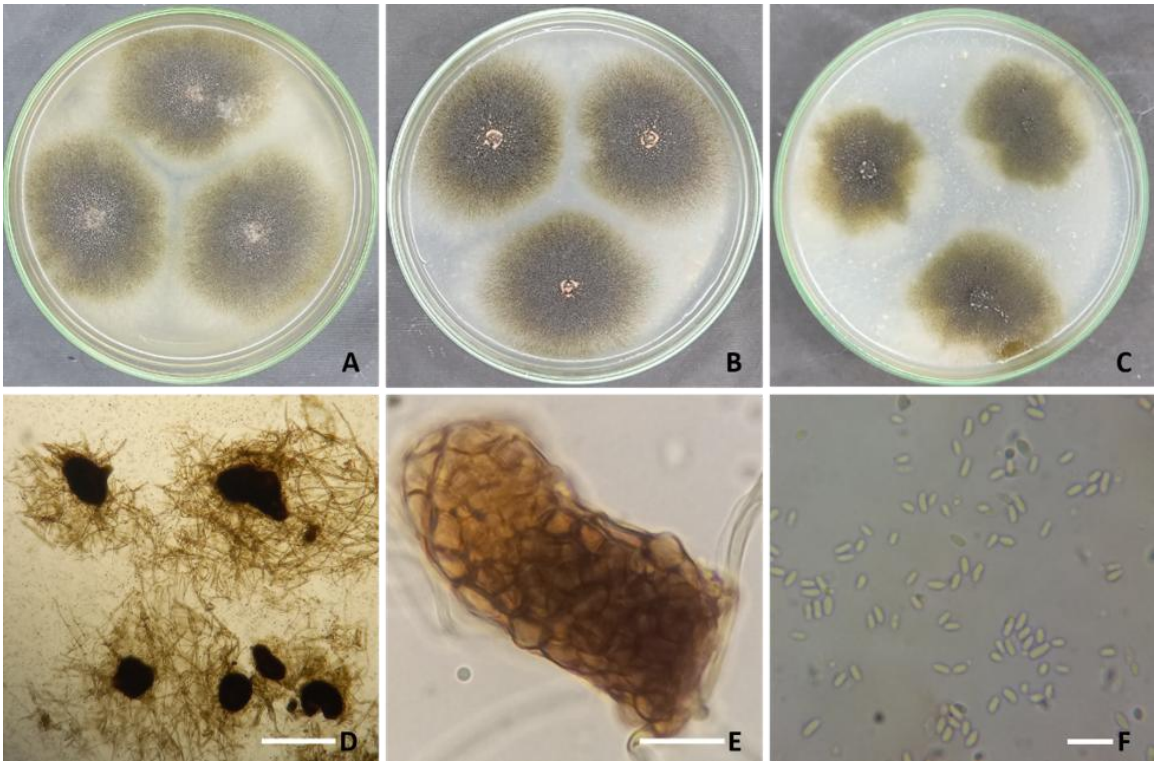

Figure 3: *Didymella* sp. AUMC 15640. A–C, 7–day-old colonies on MEA, PDA, and OA at 25 ºC. D–E, Pycnidia. F, conidia. (Scale bars: D = 200 µm; E = 100 µm; F = 10 µm).

Phylogenetic analysis

Didymella spp. AUMC 15636, AUMC 15637, and AUMC 15640 were compared to other *Didymella* species using a phylogenetic analysis based on ITS sequencing data. 20 sequences totaling 540 characters were created from the ITS data set, of which 495 could be successfully aligned (with no gaps or N), 23 characters (4.6% of complete) were recorded as variable, and 10 characters (2.0% of complete) were rated as informative. Two trees were generated by the Maximum Parsimony approach, which was used to infer the evolutionary history. Figure 1 depicts the phylogenetic tree with the maximum log likelihood (-1027.69), consistency index (0.545455), retention index (0.687500), and composite index of (0.375000). The three strains in this investigation were placed at a single clade, according to the ITS tree (Figure 4), indicating their possible originality. To correctly identify these strains, beta-tubulin (TUB) and large subunit (LSU) sequences must be obtained. ITS sequences of the strains were uploaded to GenBank database (https://www.ncbi.nlm.nih.gov/genbank) as OP605526, OP605525, and OP605537, respectively.

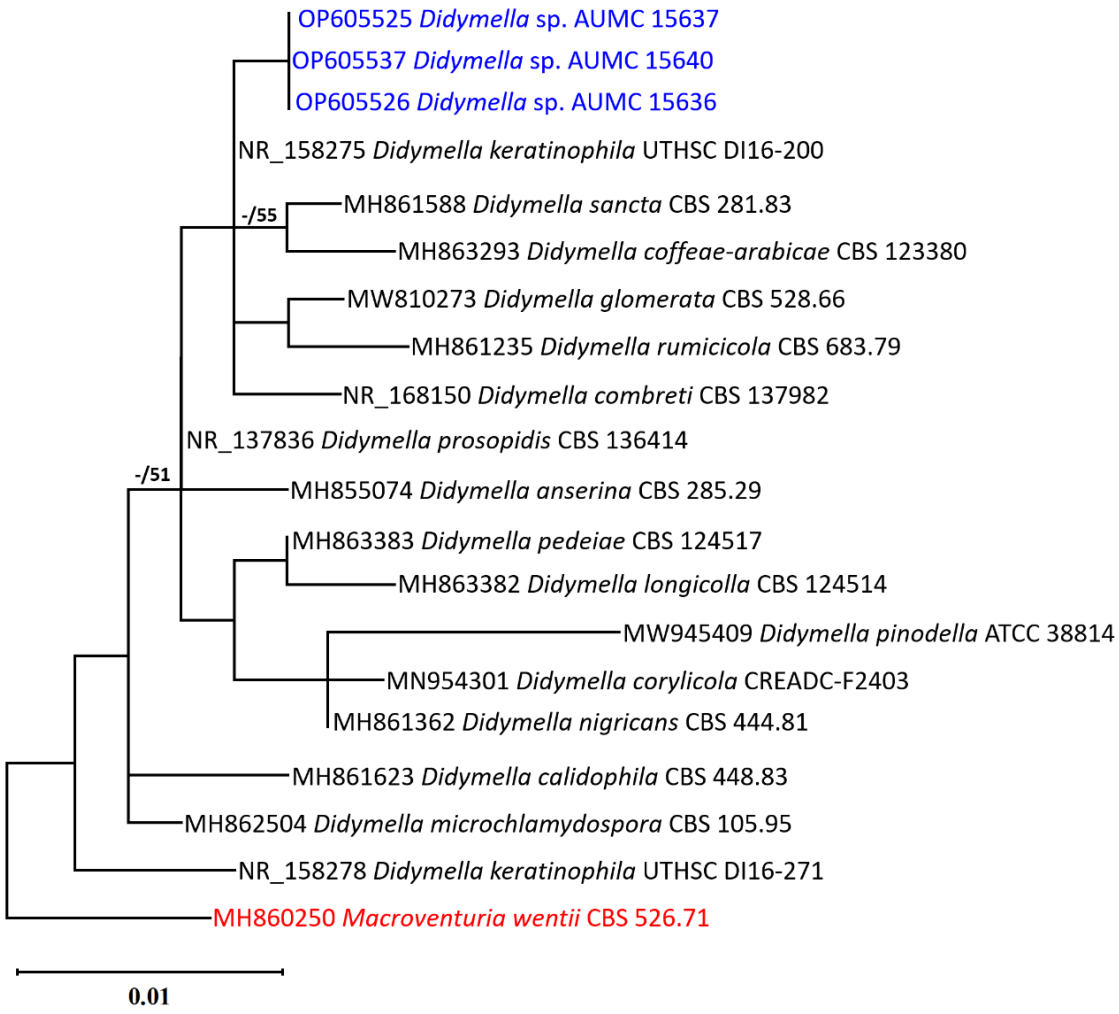

Figure 4: Evolutionary tree based on ML/MP analysis using ITS sequences of *Didymella* spp. AUMC 15636, AUMC 15637, and AUMC 15640 in comparison to GenBank's closely related matches (in blue color). The relevant nodes are marked above/below by the bootstrap support values for ML/MP equal to or greater than 50%. The *Macroventuria wentii* CBS 526.71 outgroup serves as the tree's root (in red color).

Production of cocktail enzymes in SSF

Didymella sp. AUMC 15636 and AUMC 15637 could produce the most endoglucanase, exoglucanase and xylanase activity (20±1.4 and 23±2.2, 10±0.84 and 17±1.3, 18±1.1 and 18±1.8 U/gds, respectively) on bean straw, laccase (0.7±0.1 and 2.6±0.12 U/gds) on corn cobs and wheat bran, and pectinase (37±3.2 and 26±3.4 U/gds) on rice husk and corn cobs, respectively. *Didymella* sp. AUMC 15640 released the maximum endoglucanase, exoglucanase, laccase, pectinase, and xylanase enzymes on the bean straw residues (24±2, 24±1.8, 10±1.3, 31±3.5 and 27±2.7 U/gds), respectively (Figures 5–7).

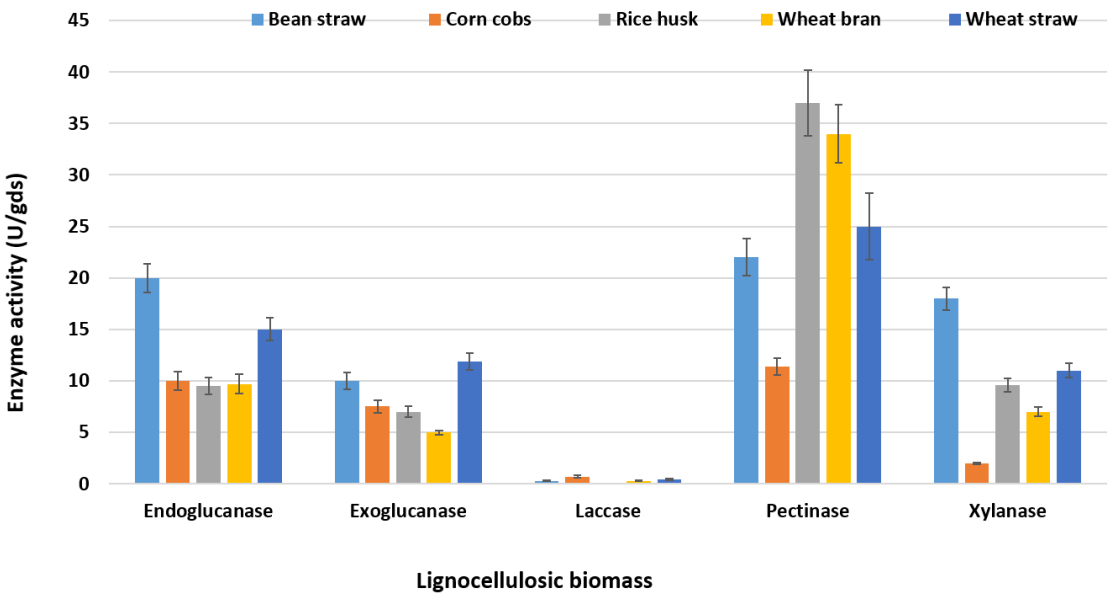

Figure 5: Extracellular enzymes produced by *Didymella* sp. AUMC 15636 in SSF at 30 ºC.

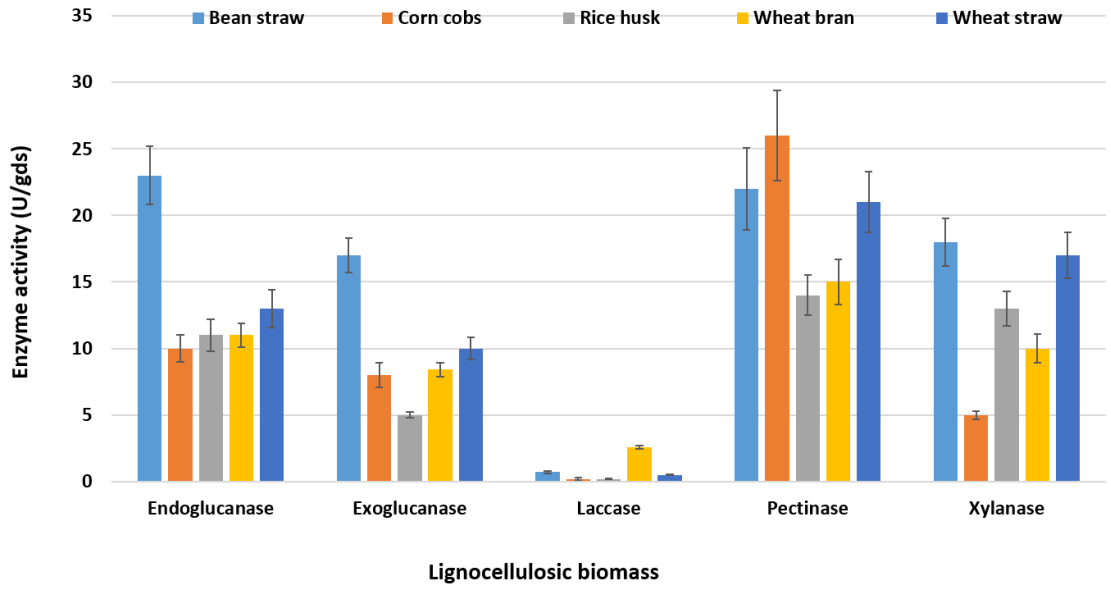

Figure 6: Extracellular enzymes produced by *Didymella* sp. AUMC 15637 in SSF at 30 ºC.

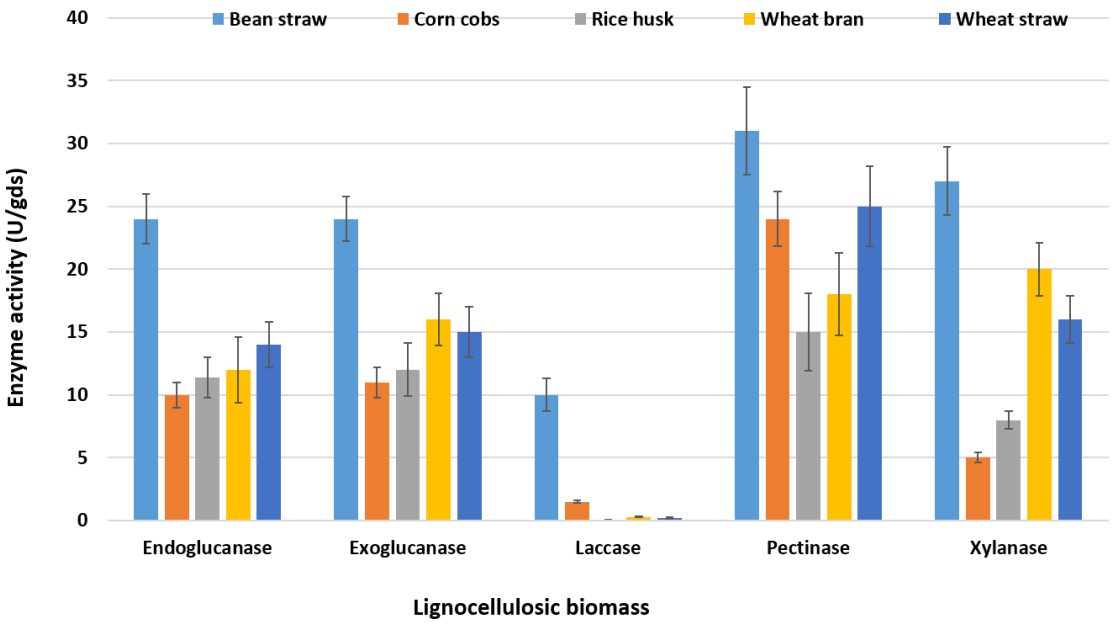

Figure 7: Extracellular enzymes produced by *Didymella* sp. AUMC 15640 in SSF at 30 ºC.

Production of pectinase enzyme by *Didymella* sp. AUMC 15636

Citrus pectin could be fermented by the strain *Didymella* sp. AUMC 15636 to produce pectinase in SmF, and produce 0.9 g of crude pectinase powder per liter fermentation medium.

Effect of pH and temperature on the activity of *Didymella* sp. AUMC 15636's pectinase

It was discovered that pH 6.0 yielded the highest activity of 80 U/gds (Figure 8), which increased to 100 U/gds at 45 ºC (Figure 9).

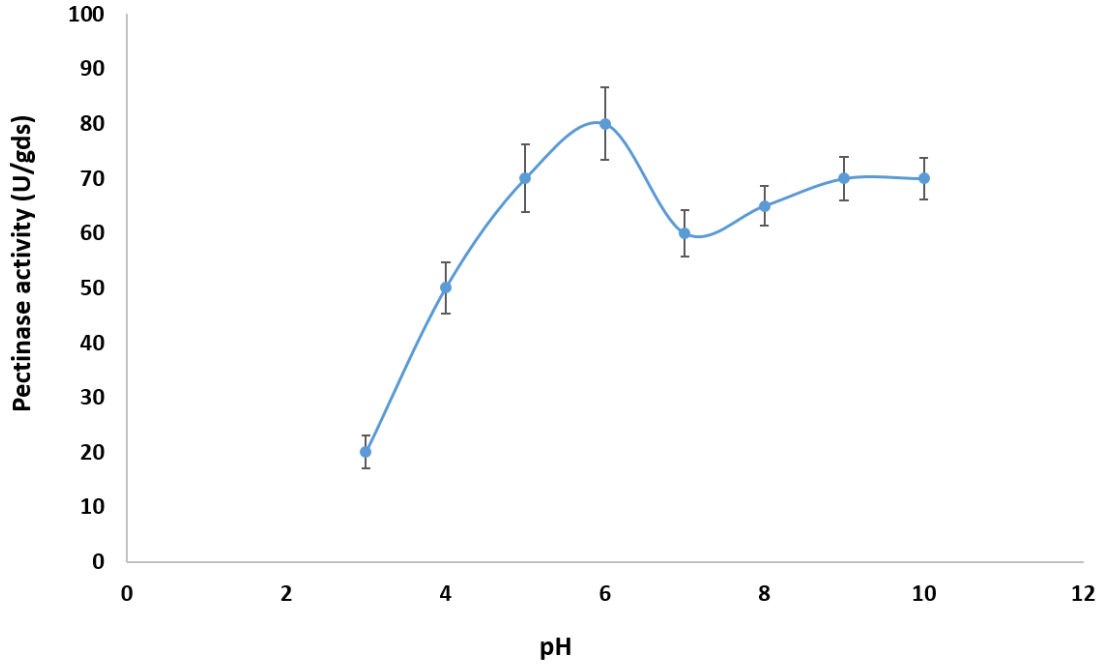

Figure 8: Effect of pH on the activity of pectinase produced by *Didymella* sp. AUMC 15636

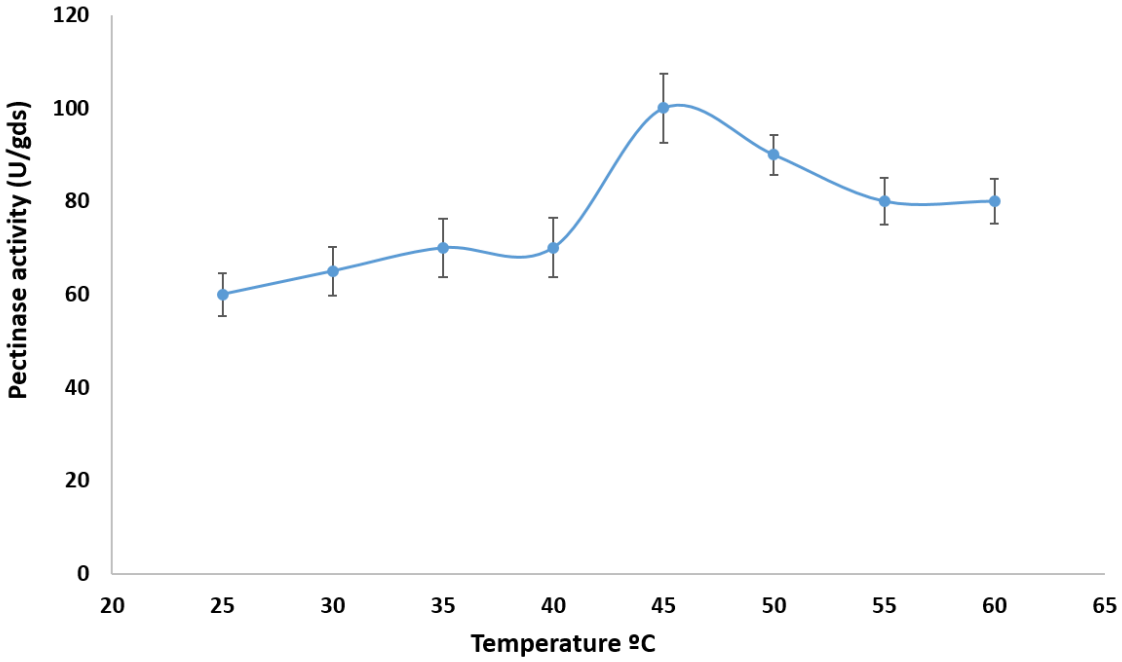

Figure 9: Effect of temperature on the activity of pectinase produced by *Didymella* sp. AUMC 15636 at pH 6.0.

Effect of some metal ions and inhibitors on pectinase activity

Mn ions had a significant activating influence on the activity of the pectinase produced when tested under ideal conditions (pH 6.0 and 45 ºC), reporting 130 U/gds with a 30% increase in activity over the control. Zn and EDTA had no influence on pectinase activity, which was the same as the control. SDS and other ions could reduce pectinase activity by 10 to 30%, with K and Ca having the greatest impact (Figure 10).

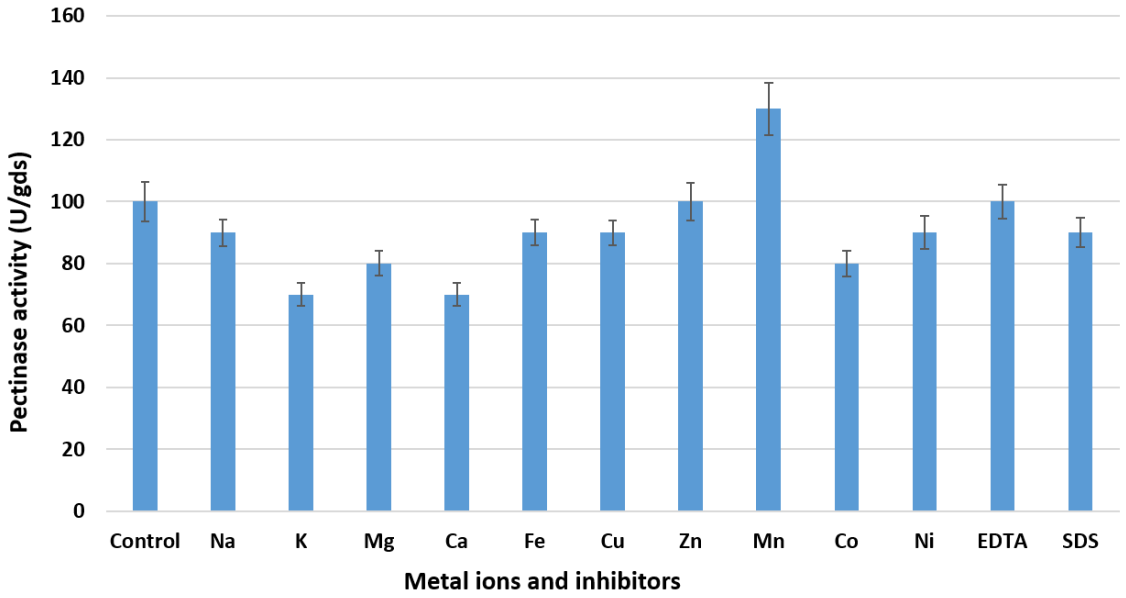

Figure 11: Effect of some metal ions and inhibitors at pH 6.0 and 45 ºC on the activity of pectinase produced by *Didymella* sp. AUMC 15636.

DISCUSSION

Three *Didymella* strains were recovered from Mango and Guava juices in the current study. To identify the relationship between our strains and other members of the *Didymella* genus, we used phylogenetic analysis based on ITS region sequencing. According to the ITS tree, the three strains in this study were positioned in a single clade, indicating their likely uniqueness. Beta-tubulin (TUB) and large subunit (LSU) sequences should be obtained to appropriately identify these strains in a future work. The three *Didymella* strains in this study shown an exceptional capacity to break down all residues while also generating significant levels of endoglucanase, exoglucanase, laccase, pectinase, and xylanase. Industrial bioprocesses for the production of industrial commodities are anticipated to be developed in the near future [35].

According to the present findings, the *Didymella* strains used the five biomass as an enzyme synthesis substrate in SSF, enabling high levels of five enzymes production. Due to the enormous amounts of agricultural residue that are produced each year on a worldwide scale, it is becoming more and more crucial to dispose of biomass in ways that are ecologically friendly. Finding a useful use for these wastes is a study area that promotes ecosystem preservation. Regarding this, several recent initiatives have been

launched in Egypt to recycle lignocellulosic wastes into useful products, and these substrates have been researched for their potential as a carbon source for the manufacture of many enzymes [31, 36, 37]. Numerous advantages have resulted from these processes, including higher productivity and cheaper production costs.

Due to large percentages of cellulose and hemicellulose in their structure (Table 1), bean straw, corn cobs, rice husk, wheat bran, and wheat straw are all appealing candidates for commercial production of important enzymes in Egypt.

Table 1: Cellulose, hemicellulose and lignin content of lignocellulosic biomass in this study.

Biomass	Composition (% dry weight basis)			References
	Cellulose	Hemicellulose	Lignin	
Bean straw	31	24	10	Montoya-Rosales, Peces [38]
Corn cob	45	35	15	Limayem and Ricke [3]
Rice husk	22	23	15	Megawati, Sediawan [39]
Wheat bran	30	50	15	Graminha, Gonçalves [40]
Wheat straw	27	21	23	Adapa, Schonenau [41]

About 30 million tonnes of dry beans are generated annually throughout the world, particularly in tropical regions of India, Brazil, Myanmar, China, the United States, and Mexico [42]. The processing of sweet corn results in the beneficial byproduct of corn cobs [43]. Because of its nutritional value, maize cob may be utilized as a substrate for the growth of microbes or transformed into molecules that have additional benefits, such as lactic acid, citric acid, sugars, ethanol, and enzymes [31, 36, 44-46]. Additionally, corn cob is the best substrate for the manufacture of xylanase since it contains the most xylan of any agricultural waste (40%).

Rice straw is one of the most common lignocellulosic waste materials on the earth. It is produced in vast quantities each year, reaching over 731 million tonnes, which are spread among Africa (20.9 million tonnes), Asia (667.6 million tonnes), Europe (3.9 million tonnes), and America (37.2 million tonnes) [47-49].

Rice husk is one of the most readily accessible agricultural byproducts (20 %) in many rice-producing regions across the world [50]. Rice husk comprises 75-90% cellulose, hemicellulose, and lignin [39, 51].

About 150 million tonnes of wheat bran produced annually and used in the feed business [52]. There has recently been an upsurge in attempts to generate enzymes by solid state fermentation utilizing wheat bran, which has been the favored choice in some research in Egypt[31, 36]. Its cell-wall polysaccharides containing 40% xylan [53]. The hydrolyzed wheat bran has a composition of 42.5 % glucose, 15.4 % xylose, 3.1 % arabinose, and 2.7 % galactose of its dry weight which are required to initiate microorganism growth and replication [54]. However, it can be expensive carbon source for large scale usage, which can represent problems for its industrial application [55].

In the current study, *Didymella* strains showed the highest pectinases production in SSF utilizing all biomass tested with sufficient productivity. The powerful strain (*Didymella* sp. AUMC 15636) was used to create pectinase enzyme in SmF. Pectinase has been used for a long time to degrade pectin and speed up various processing procedures, such as juice extraction, clarification, and liquefaction [16, 56]. Pectinases are one of the most used enzymes in food, accounting for 40% of all dietary enzymes [57, 58]. Chemical synthesis is outperformed by microbial enzymes because of their high yields, superior selectivity, non-toxicity, and biodegradability [59]. The global enzyme market, which was worth $9.9 billion in 2019, is expected to grow at a 7.1% annual rate from 2020 to 2027 [60]. The majority of mesophilic or thermophilic enzymes are already commercially accessible, with pectinase being one among them.

About 25% of all sales of food enzymes worldwide are pectinases produced by microorganisms. It is generated primarily from filamentous fungi, particularly *Aspergillus niger*[34, 61]. *Didymella* sp. AUMC 15636 produced pectinase in this study at a temperature of 30 ºC, reaching its highest activity at 45 ºC and pH 6.0. We are aware of no instances of *Didymella* species producing pectinases other than the virulence factors polygalacturonase (PG), pectate lyase (PL), and pectin lyase (PNL) that have been detected in cantaloupe fruit [62].

Even filamentous fungi that are psychrophilic or psychrotolerant have this trait. Pathogenic fungus *Sclerotinia borealis*, which thrives in extremely cold environments and cannot grow over 20 °C, produces pectinases, which are most active at 40 °C [63]. Another example of a thermotolerant fungus is *Mucor flavus*, which produced pectinases that are most active at 45 °C [64]. The pectinases produced by *Botrytis cinerea*, a phytopathogenic fungus, are most active between 34 and 37 ºC [65]. It is challenging to compare the values of enzyme activity reported by various studies due to slight variations in technique. As a result, comparisons need to be done carefully.

CONCLUSION

This publication isolates and describes three *Didymella* species that were thought to be new species. It was discovered that the *Didymella* strains produced endoglucanase, exoglucanase, laccase, pectinase, and xylanase from five lignocellulosic biomass in SSF. In SmF, citrus pectin was fermented in order to develop the most potent strain of pectinase enzyme. A portion of the generated pectinase enzyme was purified using cold ethanol, and its activity was assessed in relation to pH, temperature, metal ions, and inhibitors. At pH 6.0 and 45 ^{o}C, the partly pure pectinase's activity peaked. The pectinase was 30% more active by Mn ions than the control. SDS and other ions reduced pectinase activity by 10 to 30%, with K and Ca having the highest effects. Zn and EDTA had no effect on pectinase activity.

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