Induction of Hydrolytic Enzyme Production and Antibiosis *via* a Culture of Dual Fungal Species Isolated from Soil Rich with the Residues of Woody Plants in Saudi Arabia

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Inducing hydrolytic enzymes production and antibiosis is an attractive process for industrial applications. The approach can be used to repress pathogenic microorganisms. Using a dual culture of *Aspergillus fumigatus* and *Aspergillus flavus*, the activities of cellulase, polygalacturonase, chitinase, β-1,3-glucanases, protease, xylanase, and β-glucosidase were 1.78, 3.87, 2.98, 2.79, 6.91, 2.89 U, and 1.43 U·mg^{-1} of protein. Meanwhile, the activities were 0.87, 2.78, 0.58, 1.69, 4.45, 2.06, and 0.89 U.mg^{-1} of protein for *A. flavus* alone and 0.98, 2.98, 0.87, 1.89, 4.98, 2.58, and 0.91 U.mg^{-1} of protein for *A. fumigatus* alone. The cellulase, polygalacturonase, and chitinase activities were studied at different temperatures; 40 °C and 50 °C, which were better temperatures than 20 °C in terms of enzymes activity for *A. fumigatus* and the dual culture compared to *A. flavus*. The highest antimicromial activity was observed using the dual fungal culture, where the inhibition zones were 3.13, 3.47, 2.27, 1.77, 1.03, and 2 mm compared with *A. fumigatus* alone or *A. flavus* alone, against *Staphylococcus aureus, Escherichia coli*, *Bacillus subtilis*, *Aspergillus fumigatus*, *Mucor circinelloides*, and *Fusarium moniliforme*, respectively.

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INTRODUCTION

The interactions or competition among microbial species in the environment for space and food are unique for each species. Such phenomena are considered complex mechanisms and therefore require better understanding. Numerous scientists have exploited this phenomenon for the production of enzymes and antimicrobial agents. Dullah *et al*. (2021) observed an increase in chitinase, cellulase, and xylanase *via* fungal-fungal interaction *in vitro*, and advised the application of these interactions for the stimulation of enzyme production.

Previously, dual cultures among different fungi were evaluated to study the dynamic changes of extracellular enzymes production, *e.g.*, 1,3 glucan synthase and superoxide dismutase (Chi *et al*. 2007). Biocontrol of pathogenic fungi as well as bacteria depends not only on the antifungal/antibiotic compounds but may also depend on hydrolytic enzymes to cleave the bonds in cell wall components, *e.g.*, glucans, chitin,

glycoproteins, and mannans (Bowman and Free 2006; Shaikh and Sayyed 2015; Abdel-Ghany and Bakri 2019). Therefore, the vital role of hydrolytic enzymes was documented particularly against phytopathogens. Mohammadi *et al*. (2011) proposed that numerous enzymatic activities, *e.g.*, from proteases, phosphatases, arylsulfatase, dehydrogenase, and ß– glucosidase, are considered as a primary monitor of the activities of microbial communities. In addition to the critical role that fungal extracellular enzymes play in the controlling of phytopathogens, they are also an essential tool for developing several manufactured products (Makky and Abdel-Ghany 2009; Abdel-Ghany *et al*. 2018, 2019; Nofal *et al.* 2021a).

In addition, some released microbial secondary metabolites play a vital role for metabolic pathways of other microbes (Thomashow *et al*. 1997). In nature, they are released dependent on the situation in minor amounts and are considered as the greatest effective mode of microorganism activity against competitors, permitting more prevalent bacteria and fungi producing antimicrobial agents in environments with limited nutrients (Raaijmakers and Mazzola 2012). Fungus-fungus interactions stimulate the productivity and diversity of secondary metabolites in growth containing medium. As an interesting observation in this regard, the synthesis of some compounds from a fungus that grows alone is inhibited in the case of dual cultures, and new compounds are synthesized instead (Chatterjee *et al*. 2016). For example, *Aspergillus niger* suppressed the production of compounds by other fungi that were present in dual cultures.

Various microbial metabolic pathways play a major role in the transformation of lignins and organic matter and therefore support the occurrence of microorganisms in nature. In this context, according to several reports, the application of fungal co-cultures is one potential resource for creating enzymes for the biotechnological industrial and ecological processes (Wiberth *et al*. 2019; Nofal *et al.* 2021b,c). The induction of active metabolites and enzyme production *via* the dual culture of fungi are major goals of the current study to achieve safe industrial development.

EXPERIMENTAL

Fungal Isolates and their Interaction

Soil samples, rich with woody plant residues, from the Abu Arish region, Jazan, Saudi Arabia were used a source of fungal isolates. Eight different fungi isolates were isolated using potato dextrose agar (PDA) and Czapek Dox agar media and were identified according to macro- and microscopic features previously described by Raper and Fennell (1973), Schipper (1978), and Domsch *et al*. (1980).

Fugal Isolates Growth at Different Temperatures

The fungal isolates were cultivated in sterile plates containing PDA medium and incubated at different temperatures ranging from 10 to 50 °C to select the two most potent thermotolerant isolates, depending on colony radius.

Dual Fungal Culture in Broth Medium

Two fungal cultures were cultivated separately and joined using potato dextrose broth medium and incubated at a temperature of 30 °C for 10 d. The collected mycelia from the fungal growth medium after the incubation period were dried in oven for 28 h at 60 °C and weighed several times to obtain a constant weight to determine their dry weight; the

growth medium was filtered through Whatman no. 1 filter paper; then it was used as a source of enzymes.

Enzyme Production at Different Incubation Temperatures

The production of various enzymes was evaluated at different incubation temperatures (10 to 50 °C) in terms of production amounts. The medium containing flasks were separately inoculated with the tested fungi and joined in a dual culture before being incubated for 10 d. After the incubation period, the produced enzymes were evaluated.

Chemicals Used

All chemicals used in the current experiment were obtained from Sigma-Aldrich, St Louis, MO.

Filtrate Enzymes Assays

Cellulase activity

The reaction mixture for measuring the cellulase activity contained 100 µL of the fungal culture filtrate with 400 µL of 100 mM sodium citrate buffer (pH 5.2) and 1% carboxy methyl cellulose (CMC) as the substrate. Next, the reaction mixture was incubated at a temperature of 50 °C; the released glucose was quantifiably measured *via* the dinitrosalicylic acid (DNSA) method.

A recognized amount of aliquot was placed in a test tube and filled to 1 mL with distilled water; then, 0.5 mL of DNSA reagent was added, followed by being carefully mixed. The test tube was placed in a boiling water bath for 5 min, followed by the addition of 1 mL of sodium potassium tartrate (40%). The tube content was filled up to 5 mL with distilled water. Then the absorbance was read *via* a spectrophotometer at 540 nm. The enzyme aliquot was replaced by 1.0 mL of distilled water for the blank measurement. A glucose standard at a different concentration was calibrated, and the enzyme activity was recorded (Sadasivam and Manickam 1992; Collmer *et al*. 1988).

Polygalacturonase (PG) activity

The reaction mixture for measuring the PG activity contained 100 µL of the fungal culture filtrate with 400 µL of 100 mM sodium citrate buffer (pH 5.2) and sodium polypectate (0.25%) (Collmer *et al*. 1988). After incubation the reaction mixture at a temperature of 37 °C for 1 h, the released galacturonic acid was assessed quantifiably *via* the DNSA method (Sadasivam and Manickam 1992).

Chitinase activity

The reaction mixture for measuring the chitinase activity contained 100 µL of the fungal culture filtrate with 200 µL of chitin (0.5 %) in 10 mM sodium acetate buffer adjusted to a pH of 5.2. Next, the reaction mixture was incubated at a temperature of 50 °C for 1 h; the released N-acetylglucosamine was evaluated *via* dimethylamino benzaldehyde (DMAB) reagent. In a test tube, a recognized volume (0.5 mL) of the reaction mixture, with 0.5 mL of 120 mM potassium borate buffer (at a pH of 8.9), was heated in a boiling water bath for 3 min, followed by cooling and the addition of 3 mL of DMAB reagent, followed by incubation for 20 min at a temperature of 38 °C. Subsequently after cooling at a temperature of 20 °C, the absorbance was read *via* a spectrophotometer at 544 nm. A standard curve of N-acetylglucosamine in borate buffer was calibrated to detect the released N-acetylglucosamine (Reissig *et al*. 1955).

β-1,3-glucanase activity

The reaction mixture for measuring the *β*-1,3 glucanase activity contained 100 µL of the fungal culture filtrate with 100 µL of laminarin (4%) in 50 mM sodium acetate buffer adjusted to a pH of 5.2. Next, the reaction mixture was incubated at a temperature of 37 °C for 10 min. Then the released glucose was measured *via* the DNSA method (Kauffman *et al.* 1987; Sadasivam and Manickam 1992). Generally, the activity of the enzymes, including cellulase, PG, chitinase, and β-1,3 glucanase, was measured in Unit·mg^{-1} of protein. However, the quantity of enzyme required to create 1 mM of corresponding reducing sugar per min per mL of the supernatants was defined as unit activity.

Protease activity

The reaction mixture for measuring the protease activity contained 500 mL of the fungal culture filtrate containing enzyme with 500 mL of casein (0.36 %) in 2 mL of 100 mM acetate buffer adjusted at a pH of 3.6. Next, the mixture was incubated at a temperature of 50 °C for 1 h. Then 3 mL of trichloroacetic acid (5%) was added to stop the reaction (Malik and Singh 1980). The reaction mixture was centrifuged for 10 min at 5000 rpm, and then 500 µL of the supernatant was used to assessment the released free amino acids *via* the ninhydrin protocol. The quantity of protein required to release the amino acids per min per mL of supernatant containing enzyme was expressed as Unit·mg^{-1} of protein, and one unit of enzyme activity was defined as the quantity of protein necessary to release the amino acids per min per mL of culture supernatant (Malik and Singh 1980).

Xylanase and β-glucosidase activity

Activity of the xylanase was assessed *via* the previous assay using oat spelt xylan as the substrate. The DNS method was used to evaluate the released sugar (Bailey *et al*. 1992). The sugar quantity was measured *via* the standard curve of xylose (Miller *et al*. 1960). The *β*-glucosidase activity was assessed *via* the previous assay using an addition of 1.0 mL of p-nitrophenyl-β-D-glucoside (2 mM) in 0.05 M sodium acetate buffer adjusted at a pH of 5.0 as the substrate to 100 µL of the fungal culture filtrate containing enzyme (Herr *et al*. 1978). The reaction mixture was incubated at 30 °C for 10 min, and 2.0 mL of a 1 M Na_2CO_3 solution was added to stop the reaction mixture. According to the absorbency guide of 18.5 cm^2/µmol at 405 nm for p-nitrophenol, one unit of *β*-glucosidase was defined as the quantity of enzyme releasing 1/µmol of *p*-nitrophenol per min.

Antimicrobial Activity of the Fungal Extract

The well agar diffusion assay was applied testing the antimicrobial activity of the fungal extract against three bacteria and three fungi, including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Mucor circinelloides* (as filamentous black fungus), *Fusarium moniliforme*, and *Aspergillus fumigatus*. A sterile cork borer (6 mm) was used to making a hole of agar, streaking with the test organisms. Then, 100 µL of the extract was dissolved in dimethyl sulfoxide (DMSO) for each fungal culture alone, as well as for the dual culture, were potted in the well. The plates were preserved for 30 min in a refrigerator for suitable diffusion of the extract. Then they were incubated at a temperature of 37 °C for bacteria and 28 °C for fungi for 1 and 3 d, respectively. Ketoconazole and gentamycin were used as the antifungal and antibiotic agent as the positive control; at the same time the well was loaded with DMSO as the extracted solvent. The inhibition of microbial growth, in the form of the inhibition zone around wells, were measured (mm) (Abdel-Ghany *et al*. 2021).

Statistical Analysis

The experimental results were performed with three replicates and calculated as the mean ± the standard deviation (SD).

RESULTS AND DISCUSSION

Eight fungal isolates were isolated from three rhizospheric soil samples rich with woody plant residues (at a depth of 5 to 20 cm) (Fig. 1) located in the Abu Arish governorate, Jazan Region, Saudi Arabia (Fig. 2).

Fig. 1. Soil rich with woody plant residues as a source of fungal isolates

Fig. 2. Map of Abu Arish, Jazan Region, Saudi Arabia (location of the collected soil samples, blue location on map)

Based on the identification, 5 fungal isolates belonged to *Aspergillus fumigatus*, three to *A. flavus*, two belonged to *Mucor* spp., and one to *Alternaria alternata*. All isolates were cultivated at different temperatures ranged from 20 to 60 °C. The two fungal species (*A. fumigatus* followed by *A. flavus*) most resistant to temperature were selected for the dual culture for enzyme production and antibiosis. *Aspergillus fumigatus* was the dominant fungal isolate and was more resistant to temperature. Jazan is a sub-tropical region, characterized by a warm climate that favors the proliferation of thermophilic/ thermotolerant fungi (Tirado *et al*. 2010).

The *A. flavus* and *A. fumigatus* dual culture performed in broth medium showed proliferation of *A. flavus* (as shown in Fig. 3A) compared with *A. fumigatus* (as shown in Fig. 3B). Remarkably, the growth of the two cultures jointly (as shown in Fig. 3AB) showed low growth. The dry weight of *A. flavus*, *A. fumigatus*, and two fungal species jointly was 1.77, 1.62, and 1.60 g, respectively. El-Debaiky (2017) used the dual culture method for studying the interactions of *Aspergillus piperis* against numerous fungi, *i.e.*, *Sclerotium cepivorum, Sclerotinia sclerotiorum, Alternaria alternata, Botrytis cinerea* and *Alternaria solani*. They summarized the mechanisms of the antagonists through antibiosis *via* lysis, breaking, and the denaturation of the mycelia and spores. Other reported mechanisms have included mycoparasitism *via* coiling and the penetration of the fungal hyphae. Different enzymes were detected in the culture filtrate of the dual culture of *A. flavus* and *A. fumigatus*. Figure 4 shows that all detected enzymes by *A. flavus* were less detected in the culture filtrate of *A. fumigatus*, while the inoculated dual fungal culture induced more enzyme productivity. The activities of cellulase, polygalacturonase, chitinase, *β*-1,3-glucanases, protease, xylanase, and *β*-glucosidase were 0.87, 2.78, 0.58, 1.69, 4.45, 2.06, and 0.89 $U \cdot mg^{-1}$ of protein, respectively for *A. flavus*. Comparatively, the activity was 0.98, 2.98, 0.87, 1.89, 4.98, 2.58, and 0.91 $U \cdot mg^{-1}$ of protein, respectively, for *A. fumigatus*. The activity was 1.78, 3.87, 2.98, 2.79, 6.91, 2.89, and 1.43 $U \cdot mg^{-1}$ of protein, respectively, for the dual culture. Chitinase, lipase, and protease, as cell wall degrading enzymes, were detected during interaction among *Botrytis cinerea* and *Talaromyces pinophilus* (Abdel-Rahim and Abo-Elyousr 2018). As observed in the current study, the presence of the two joint fungal cultures encouraged the productivity of hydrolytic enzymes; these results were in agreement with Schirmböck *et al*. (1994), who found that the culture filtrate of *Trichoderma harzianum* grown in medium containing mycelia of *Botrytis cinerea* showed hydrolase activities, but when grown without cell walls did not show any hydrolysis potential. The obtained results were similar to those of Qiu *et al.* (2017), where the deactivated mycelia of *Pleurotus ostreatus* induced *Trichoderma asperellum* to produce hydrolytic enzymes. Not only filamentous fungi were induced for the production of enzymes, as unicellular fungus *Candida oleophila* was also induced by the existence of mycelia fragments of *Penicillium digitatum* as a content of the growth medium to produce protease, chitinase and exo-β-1,3-glucanase (Bar-Shimon *et al.* 2004). However, the activity of hydrolytic enzymes by fungi was induced by the presence of other fungal mycelia in the medium growth, but the activity was dependent on the type of mycelia, as mentioned previously by Elad *et al*. (1985), where the highest β-1,3-glucanase and chitinase activity were produced by *Pythium nunn* grown on medium containing mycelia of *Sclerotium rolfsii* and *Rhizoctonia solani*, while lowest activity was observed when grown on mycelia of *Fusarium oxysporum.* These may be due to the components of the fungal cell walls. Recently, Zhao *et al.* (2021) studied the co-culture of *Aspergillus flavus* and *Aspergillus penicillioides* as a potent method for hydrolytic enzyme production, and observed the maximum production of carboxy methyl cellulase, *β*-xylosidase, and

xylanase from the co-culture of these fungi compared to the production from the separately cultivated fungi.

Fig. 3. Culture of *A. flavus* (A); *A. fumigatus* (B); and the dual culture of *A. flavus* and *A. fumigatus* (AB)

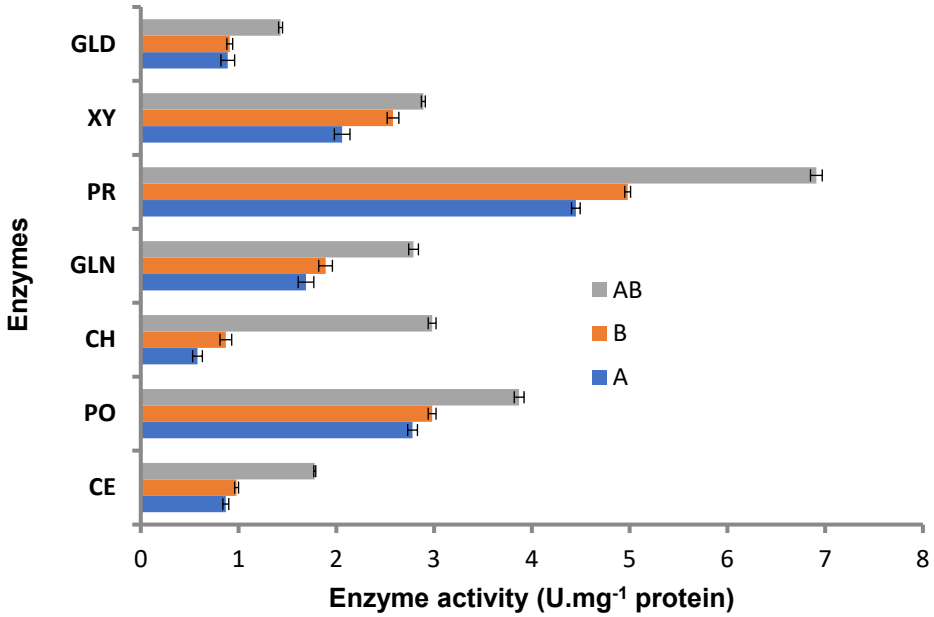

Fig. 4. Hydrolytic enzyme activities of *A. flavus* (A); *A. fumigatus* (B); and the dual fungal culture of *A. flavus* and *A. fumigatus* (AB) cultivated at 30 °C for 10 d (Note: Cellulase (CE), polygalacturonase (PO), chitinase (CH), *β*-1,3-glucanases (GLN), protease (PR), xylanase (ZY), and *β*-glucosidase (GLD))

Aspergillus flavus, A. fumigatus, and the dual culture were incubated at different temperatures to detect the productivity of various enzymes. For example, the optimal temperature of cellulase (as shown in Fig. 5), polygalacturonase (as shown in Fig. 6), and chitinase (as shown in Fig.7) productivity by *A. flavus* and *A. fumigatus* was 30 °C and 40 °C, respectively; the maximum productivity in the dual culture was also observed at 40 °C.

However, the optimal temperature of protease (as shown in Fig. 8) productivity by *A. flavus* and *A. fumigatus* was 20 °C and 30 °C, respectively. The highest temperatures, *i.e.*, 40 °C and 50 °C, were more appropriate than the lowest temperature, *i.e.*, 20 °C, for enzyme productivity by *A. fumigatus* and dual fungal culture. However, *A. flavus* was better suited at 20 °C than 40 °C or 50 °C (as shown in Figs. 5 through 8), which may be the thermophilic/thermotolerant nature of these fungi, as mentioned previously (Abdel-Ghany *et al.* 2019). However, some studies reported that highest amylolytic activity of *A. fumigatus* was at a temperature of 30 °C (Nwagu and Okolo 2010). In another study, a high temperature induced *Trichoderma asperellum* to produce cell wall degrading enzymes for *Pleurotus ostreatus* (Qiu *et al.* 2017).

The high temperature enhanced the enzyme productivity for *A. fumigatus*, at the same time that the highest productivity for the dual culture was observed at a high temperature (as shown in Figs. 5 through 8).

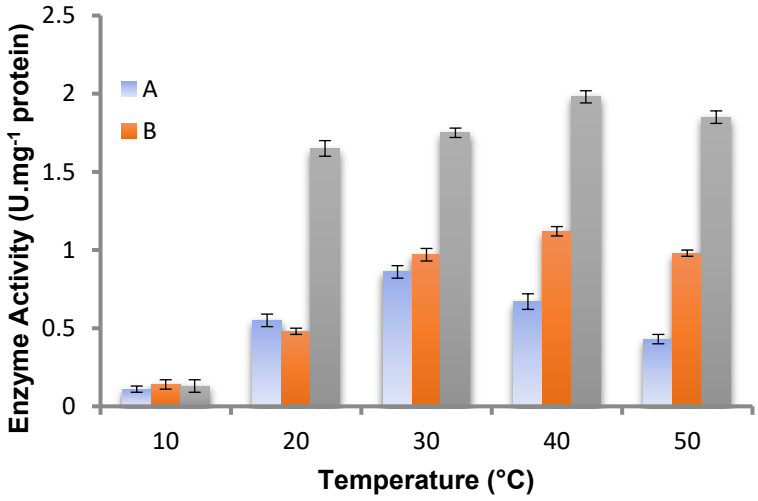

Fig. 5. Effect of the temperature on the cellulase productivity: *A. flavus* (A); *A. fumigatus* (B); and the dual fungal culture of *A. flavus* and *A. fumigatus* (AB)

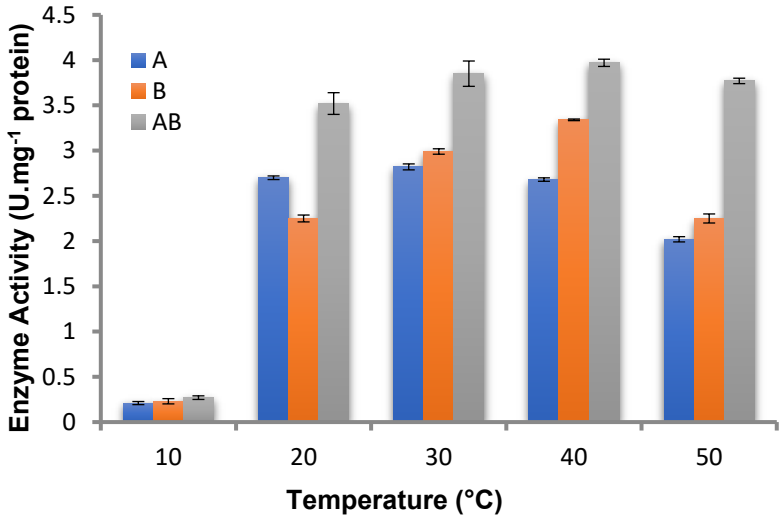

Fig. 6. Effect of the temperature on the polygalacturonase productivity: *A. flavus* (A); *A. fumigatus* (B); and the dual fungal culture of *A. flavus* and *A. fumigatus* (AB)

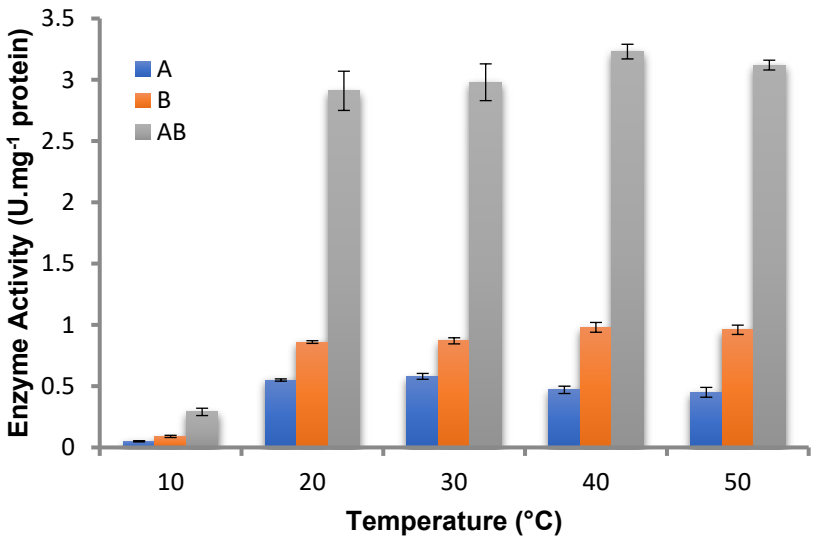

Fig. 7. Effect of the temperature on the chitinase productivity: *A. flavus* (A); *A. fumigatus* (B); and the dual fungal culture of *A. flavus* and *A. fumigatus* (AB)

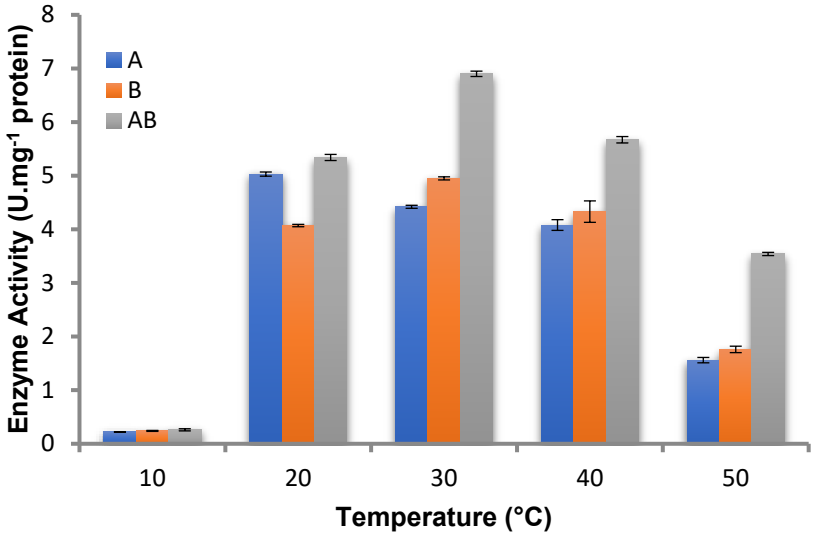

Fig. 8. Effect of the temperature on the protease productivity: *A. flavus* (A); *A. fumigatus* (B); and the dual fungal culture of *A. flavus* and *A. fumigatus* (AB)

These results show that a high temperature can improve the growth of *A. fumigatus*, which is unlike *A. flavus*; therefore, the highest enzyme activity may be attributed to *A. fumigatus*. Another explanation for the current finding is that a high temperature may alter the cell wall components of *A. flavus*, and these alterations can stimulate *A. fumigatus* enzyme activity.

The antibiosis activity of the fungal cultures was studied separately as well as jointly with the two cultures against both bacteria and fungi. *A. flavus* and *A. fumigatus* appeared to have antibacterial activity against *S. aureus*, *E. coli*, and *B. subtilis* (as shown in Fig. 9). The antibacterial activity of *A. fumigatus* was greater than *A. flavus*, particularly against *S. aureus* and *E. coli*. Antifungal activity for *A. flavus* and *A. fumigatus* was observed against *F. moniliforme*, while negligable inhibition was recorded against *M. circinelloides*. The antifungal activity was also tested against *A. fumigatus*; the extract of

the growth medium of *A. flavus* showed inhibition of *A. fumigatus*, while the extract of the growth medium of *A. fumigatus* showed no inhibition against itself. It is clear that the dual culture provided more antimicrobial activity against the tested bacteria and fungi (the inhibition zones were 3.13, 3.47, 2.27, 1.77, 1.03, and 2.0 mm, respectively) than either *A. fumigatus* (inhibition zones were 2.80, 3.10, 1.83, 0.0, 0.87, and 1.03 mm) or *A. flavus* (inhibtion zone was 2.40, 1.43, 1.57, 1.33, 0.0 , and 0.77 mm, respectively) alone with the test organisms *S. aureus*, *E.coli*, *B. subtilis*, *A. fumigatus*, *M. circinelloides*, and *F. moniliforme*, respectively. All results were compared with the positive control (antibiotic and antifungal agent) and the negative control well ammended with the solvent extract (as shown in Table 1).

Table 1. Antimicrobial Activity of the Fungus Separately and in a Dual Culture

Test Organism	A	B	AB	G	K	C
S. aureus	2.40 ± 0.17	2.80 ± 0.17	3.13 ± 0.06	2.60 ± 0.10	-	0.0 ± 0.0
E.coli	1.43 ± 0.06	3.10 ± 0.14	3.47 ± 0.12	3.30 ± 0.17	-	0.0 ± 0.0
B. subtilis	1.57 ± 0.06	1.83 ± 0.06	2.27 ± 0.12	1.37 ± 0.06	-	0.0 ± 0.0
A. fumigatus	1.33 ± 0.12	0.0 ± 0.0	1.77 ± 0.06	-	1.70 ± 0.12	0.0 ± 0.0
M. circinelloides	0.0 ± 0.0	0.87 ± 0.12	1.03 ± 0.06	-	0.83 ± 0.06	0.0 ± 0.0
F. moniliforme	0.77 ± 0.06	1.03 ± 0.21	2.00 ± 0.10	-	0.87 ± 0.12	0.0 ± 0.0
Note: culture filtrate extract of *A. flavus* (A); *A. fumigatus* (B); and the dual culture (AB); Gentamicin (G) and Ketoconazole (K) are the antibiotic and antifungal agents, respectively; and C is the negative control extracted solvent						

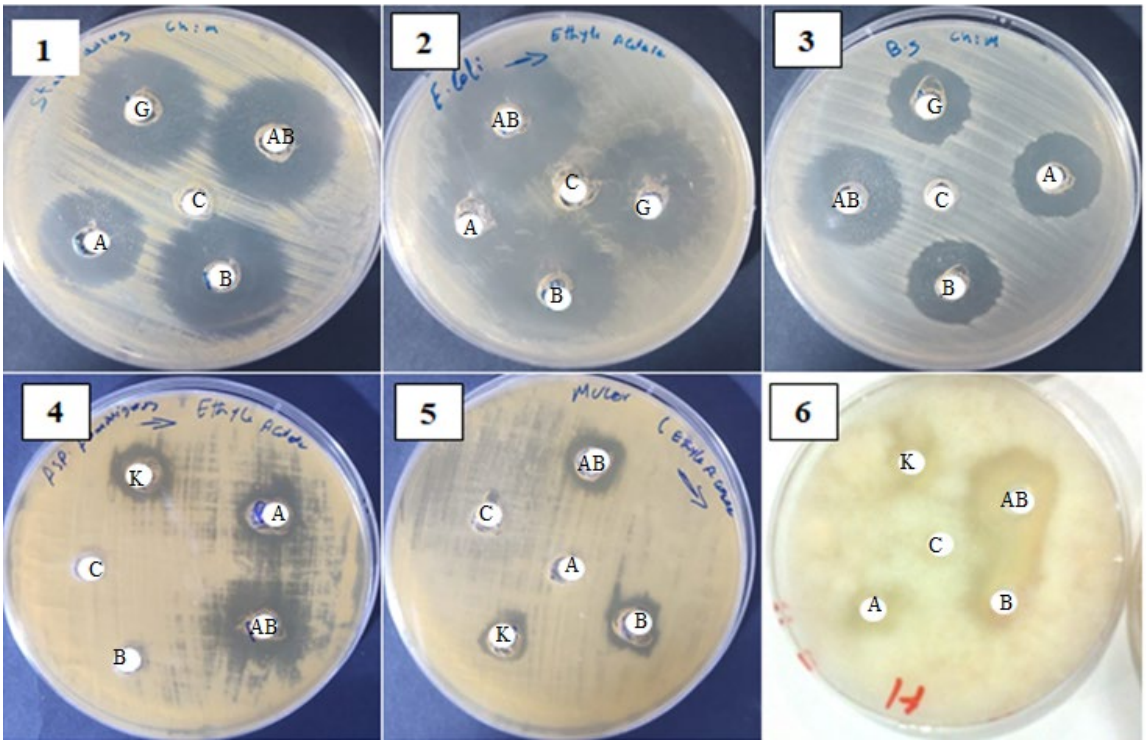

Fig. 9. Antibiosis of the culture filtrate extract of *A. flavus* (A); *A. fumigatus* (B); and the dual culture (AB) against the following *Staphylococcus aureus* (1); *Escherichia coli* (2); *Bacillus subtilis* (3); *Aspergillus fumigatus* (4); *Mucor circinelloides* (5); and *Fusarium moniliforme* (6) (Note: Gentamicin (G) and Ketoconazole (K) are the antibiotic and antifungal agents, respectively; and C is the negative control extracted solvent)

The antimicrobial activities of *Aspergillus* spp. were previously studied and are still under study. Ethyl acetate from endophytic fungus *Aspergillus clavatonanicus* extract exhibited antimicrobial potential against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococccus luteus*, and *C. albicans* (Mishra *et al.* 2017). Earlier, Bertagnolli *et al.* (1996) studied the effect of the culture filtrate of *Rhizoctonia solani* against microorganisms, who observed the inhibitory activity of filtrate against *Bacillus megaterium* and *Trichoderma harzianum* isolate *via* exoenzymes, *i.e.*, glucanase, endoproteinase, exochitinase, and phospholipase, which disrupt the microbial cell walls and membranes. The combination of the two fungal species in biotechnological applications was believed to be advantageous because these two species have evolved to function in the same habitats in natural environments.

CONCLUSIONS

1. The dual fungal culture induced hydrolytic enzymes production and antibiosis.
2. *Aspergillus flavus* and *A. fumigatus* are promising hydrolase producers that can enable the bioconversion of woody biomass.
3. A high temperature is suitable for *A. fumigatus* growth and the production of hydrolytic enzymes.
4. Human pathogenic bacteria were inhibited by the fungal secondary metabolites.

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