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Isolation, screening and selection of *Aspergillus* and *Penicillium* strains from wheat rhizosphere for multienzyme production and antifungal properties

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Abstract

Objective

Microbial enzymes are crucial for developing industrial bioprocesses due to their economic relevance in various bioindustry sectors. Therefore, the search for new hyper producing strains is very meaningful for meeting industrial needs. This study aimed to isolate and screen *Aspergillus* and *Penicillium* strains with high potential for the production of multiple biotechnologically important enzymes and biocontrol of some phytopathogens.

Materials and methods: Thirty fungal strains were isolated from the rhizospheric soils of durum wheat crops in central Algeria and identified based on their phenotypic characteristics. Twelve strains from the targeted genera were screened for their ability to produce eight hydrolytic and lignocellulolytic enzymes on specific solid media. The hyper producer strains were tested for simultaneous enzyme production under solid-state fermentation using wheat bran as substrate and antagonism effect against some wheat pathogens (*Fusarium graminearum, F. culmorum, F. verticillioides,* and *Alternaria alternata*) by dual culture assay.

Results: The findings revealed high production rates among screened strains: 100% for amylases, 91.67% for chitinases and cellulases, 83.33% for laccases, 75% for pectinases and lipases, and lower rates for proteases and gelatinases. Among the most efficient strains, *A. niger* S2 exhibited the highest amylase (5.46 IU/mL), protease (29.80 U/mL), and laccase (0.072 IU/mL) activities. Milk-clotting activities were notable for *Penicillium* sp. S1 (7.06 SU/mL) and *A. niger* S9 (46.60 SU/mL). Additionally, the strains produced significant chitinase levels (1.27–1.50 IU/g) within only 48 hours. The antagonistic activity of highly chitinase producers against *Fusarium* and *Alternaria* pathogens showed strong inhibition rates ranging from 50.3% to 73.33%, with *A. niger* S9 being the most effective.

Conclusion: These findings revealed the potential of these locally isolated strains as promising candidates not only for the production of industrially relevant enzymes but also as effective biocontrol agents, paving the way for their application in both industrial biotechnology and sustainable agricultural practices.

Keywords: antagonistic activity, chitinases, durum wheat, hydrolytic enzymes, rhizospheric fungi

Paper type: Research paper.

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Introduction

Microorganisms are the most significant sources of various enzymes. They provide environmentally friendly consumer products by reducing energy, water, and raw material consumption and generating a smaller amount of residual waste. Microbial enzymes have attracted more attention than other sources because of their active and stable nature (Ahlawat et

al., 2018), wide variety of catalytic activities, relatively high yields, ease of genetic manipulation, rapid production in inexpensive media, and activity under mild conditions of temperature and pH (McKelvey & Murphy, 2018). Among the industrial enzymes, 50% are produced by fungi and yeasts, 35% are from bacteria, and the remaining 15% are from plants (Liu & Kokare, 2023). Fungi are considered the organism of choice for enzyme isolation since their biology is well characterized, and they are normally recognized as GRAS (generally recognized as safe) (Kavanagh, 2011; Souza et al., 2015). The principal fungi used in this regard are members of the *Aspergillus*, *Penicillium,* and *Rhizopus* genera (Kavanagh, 2011; Londoño-Hernández et al., 2020). Fungal hydrolases are the most important class of enzymes with applications in various fields (Money, 2016), of which proteases and amylases represent more than 65% and approximately 30% of all industrial enzymes worldwide, respectively (Gimenes et al., 2019; Liu & Kokare, 2023). Many *Aspergillus* and *Penicillium* species are economically, biotechnologically, and medically important, and have major social impacts. These filamentous fungi are also important for their strong degradative abilities, which have been exploited for the production of enzymes (Tsang et al., 2018). Moreover, species of both genera have received much attention for their ability to reduce phytopathogen populations, and their use for the management of soil-borne phytopathogens is considered an effective, low-cost, eco-friendly, and sustainable alternative strategy (Boughalleb-M'Hamdi et al., 2018; Khan & Javaid, 2021). *Aspergillus* and *Penicillium* species have the ability to combat pathogens by exerting antagonism in the form of extracellular enzymes (such as chitinases, glucanases, proteases, cellulases, lipases, etc.) and bioactive compounds that degrade the cell wall of phytopathogens and suppress plant pathogens (Nicoletti & De Stefano, 2012; Khan & Javaid, 2021). The primary objective of this study was to isolate and screen filamentous fungi from the rhizospheric soils of wheat crops, with a specific focus on species from the *Aspergillus* and *Penicillium* genera, for their ability to produce a broad spectrum of hydrolytic (chitinases, amylases, proteases, gelatinases, pectinases, and lipases) and lignocellulolytic (cellulases and laccases) enzymes. The selected strains were further utilized to develop a solid-state fermentation (SSF) process for simultaneous enzyme production. Additionally, the antagonistic potential of these strains was evaluated against key soil-borne phytopathogens, providing insight into their biocontrol capabilities.

Materials and Methods

Chemicals: Chitin from shrimp shells, bovine serum albumin (BSA), Folin & Ciocalteu's phenol reagent, bromophenol blue, tannic acid, starch, Tween 80, guaiacol, sodium carbonate, starch, D-glucose, and 3,5-dinitrosalicylic acid (DNS) were obtained from Sigma-Aldrich (Steinheim, Germany). Agar and both microbial media, Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar, were purchased from Liofilchem srl (Roseto d. Abrazzi (TE), Italy). All other chemicals used in this work were of analytical grade and were procured from BIOCHEM Chemopharma (ZA Cosne Sur Loire, France), Loba Chemie Pvt. Ltd. (Mumbai, India), and TM Media (Titan Biotech Ltd., Rajasthan, India).

Sample collection and isolation of fungal strains: The rhizospheric soils of durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.)) obtained from different regions of the Bouira (Ain

Bessem, Ain Turk, and Aomar) and Medea (Sedraia) provinces (center of Algeria) were used as sources of the fungal strains exploited in this study. Soil samples were collected from the surface layers (0–15 cm deep) surrounding the roots of durum wheat, a depth typically recommended for isolating rhizospheric fungi. Prior to sampling, the soil surface was scraped to remove dead leaves and plant debris. Approximately 500 g of root-bound soil was collected from each site into sterile plastic bags and transported to the laboratory for immediate analysis. All instruments used during sampling were disinfected by flaming with 96° ethyl alcohol to prevent cross-contamination between samples. The soil stock solutions were prepared by suspending 10 g of each representative soil sample (obtained after mixing samples from the same wheat field) in 90 mL of sterile normal saline solution in Erlenmeyer flasks. The mixtures were then homogenized by stirring for 15–30 min (Velp Scientifica srl, Usmate (MB), Italy) to achieve good particle disaggregation and maximum release of microorganisms (Rapilly, 1968). Two replicates were prepared for each dilution of the soil stock solutions (up to 10^{-5}), which were then inoculated in triplicate. One hundred microliters of each dilution were spread on Sabouraud agar medium plates (Acharya & Hare, 2022) supplemented with chloramphenicol (0.05 g/L) and amoxicillin (0.5 g/L) to prevent bacterial growth. After incubation at 28±2°C for 5–7 days (UNB 200, Memmert GmbH+Co.KG, Schwabach, Germany), several colonies with different aspects, colors, and textures were observed in the same Petri dish, requiring a series of successive subcultures until pure isolates were obtained. Once purified, each isolate was identified by a specific code number and then maintained on PDA slants at 4°C until further use.

Identification of the isolated fungi: The purified fungal strains were identified on the basis of their phenotypic characteristics through both macroscopic and microscopic observations. Identification was guided by the keys provided by Pitt & Hocking (2009), Campbell et al. (2013) and Kidd et al. (2022). The macroscopic appearance of the colonies was examined directly on PDA medium after 5-7 days of incubation. The key characteristics used for identification included colony texture, color, growth pattern, reverse coloration, and any soluble pigments produced in the medium. Microscopic identification of the fungal strains was conducted via two techniques. (i) The top layer of the fungal mycelium was aseptically removed with a sterile inoculation loop, spread on a clean glass slide with a drop of sterile normal saline, and covered with a coverslip for observation under an optical microscope (×400, OPTICA Axiom 2000, Italy). (ii) For the second technique, a mycelial sample from a sporulating mature colony was adhered to adhesive tape, stained with a few drops of 1% methylene blue solution (w/v), and observed at the same magnification (×400). The characteristics recorded included the color, appearance, and size of the conidiophores, the presence or absence of septa on the hyphae, the shape of the spores and conidia, the morphology of the head and hyphae, and the presence of metulae and vesicles, etc.

Screening of isolates producing enzymes of interest: The ability of the purified strains of *Aspergillus* and *Penicillium* genera to produce the enzymes of interest (chitinases, amylases, proteases, gelatinases, pectinases, lipases, cellulases and laccases) was screened using specific media. The use of solid media in Petri dishes to detect the production of various extracellular enzymes, first proposed by Hankin and Anagnostakis (1975), was the method used in this part of our investigation. This technique allows a rapid screening of large fungal populations for the

absence or presence of specific enzymes. The qualitative test is based on the ability of the isolated fungal strains to grow on culture media containing the specific substrate for the desired enzyme, which acts as a production inducer. This substrate usually represents either the sole source of carbon or nitrogen in the medium. Extracellular enzyme production is determined directly by the formation of a clear zone of hydrolysis, the development of a specific color around the colony, or after the addition of chemical reagents to detect residual test substrate. After the preparation of the solid media, the prepoured plates were inoculated with a pinpoint inoculum of the test fungus and incubated at 28±2°C. The colony radial growth rates and substrate-clearing zones were measured for each fungal species when appropriate. All screening assays were performed in triplicate on specific media supplemented with antibiotics (chloramphenicol 0.05 g/L, amoxicillin 0.5 g/L) to prevent bacterial growth. Enzyme activity was determined by measuring the diameter of the clear zone (mm) or the intensity of the color zone around the colonies.

Chitinases: For chitinase production, the detection medium consisted of (g/L): $(NH_4)_2SO_4$ 3.0, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.30, citric acid 1.0, agar 15, colloidal chitin 4.50, bromocresol purple 0.15, and 0.2 mL of Tween 80. The pH was adjusted to 4.70 before sterilization (121°C, 15 min). In the case of chitinase production, the color of the medium around the fungal colony changes from bright yellow to purple. This is induced by the breakdown of chitin to N-acetylglucosamine, which causes a corresponding shift in pH toward alkalinity and thus changes the pH indicator dye color (bromocresol purple) (Agrawal & Kotasthane, 2012).

Amylases: The ability of the isolated fungal strains to produce amylolytic enzymes was assayed by growing the fungi on a medium composed of starch (2.0 g), peptone (1.0 g), yeast extract (1.0 g), agar (20 g), and distilled water (1 L). After incubation, the plates were flooded with an aqueous solution of IKI at 1% (w/v). A yellow to clear zone around the colony in otherwise blue medium was considered a positive test for starch hydrolysis (Abdel-Raheem & Shearer, 2002).

Proteases: The synthesis of proteases by the selected strains was evaluated on two different culture media. (i) Modified *Czapek-Dox Agar* (CDA) medium with the following composition (g/L): sucrose 30, $NaNO_3$ 3, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.50, KCl 0.50, $FeSO_4.7H_2O$ 0.01, casein 1, and agar 15 (pH = 6.0) (Agrawal et al., 2005). (ii) *Casein Agar Medium* (CAM) containing (g/L): potato dextrose 30, casein 5, and agar 24 (Sattar et al., 2019). After incubation, clear zones were observed surrounding colonies that were able to degrade casein.

Pectinases: The medium described by Kabir and Tasmim (2019) was used to detect the production of pectinases. The fungal strains were inoculated on modified MS medium consisting of (g/L): KH_2PO_4 3.0, Na_2HPO_4 6.0, NH_4Cl 2.0, NaCl 5.0, $MgSO_4.7H_2O$ 0.10, pectin 2.0, and agar 15. Pectin hydrolysis was detected by flooding the Petri dishes with a freshly prepared iodine-potassium iodide solution (1 g of iodine and 5 g of potassium iodine in 330 mL of distilled water). This reagent precipitates intact pectin in the medium; therefore, a translucent zone around a colony in an otherwise opaque medium indicates depolymerization of the pectin.

Lipases: To test lipolytic enzyme production, we used the medium described by Pham et al. (2021) containing Tween 80 (sorbitan monooleate, Sigma) as the primary source of carbon. The basal medium was composed of peptone (10 g), NaCl (5.0 g), $CaCl_2.2H_2O$ (0.1 g), agar (18 g),

and distilled water (990 mL). Ten milliliters of Tween 80 were autoclaved separately from the rest of the medium. After mixing under aseptic conditions at 45°C, the medium was poured into Petri dishes, inoculated and then incubated. A positive test was confirmed by the visual precipitation of calcium salts around the colony caused by the fatty acids released from the hydrolysis reaction.

Gelatinases: The medium used for gelatinolytic enzyme screening consisted of PDA medium plus 0.4% (w/v) gelatin at pH 6. A solution of gelatin in distilled water (8%, w/v) was sterilized separately and then added to the PDA medium at a rate of 5% (v/v). After incubation, the culture plates were flooded with a saturated aqueous solution of ammonium sulfate to precipitate the protein. A clear zone around the colonies indicated the production of gelatinases (Gopinath et al., 2005).

Cellulases: The production of cellulases by the selected strains was tested on CDA medium supplemented with 1% (w/v) carboxymethylcellulose (CMC) (Bellaouchi et al., 2021). After incubation, the presence of cellulase activity on Petri plates was revealed by the addition of Lugol's solution (5% (w/v) iodine and 10% (w/v) potassium iodide) for 10 min. In the case of a positive result, clear zones around the colonies were obtained after treatment.

Laccases: The production of laccases was qualitatively assessed using two culture media. (i) Bromophenol blue dye medium: 0.05 g of the dye was added to 250 mL of potato dextrose agar, followed by sterilization at 121°C for 20 min. After inoculation and incubation, laccase production was confirmed by dye discoloration from the medium (formation of a yellow zone around the colonies) (Kumar et al., 2016). (ii) Tannic acid medium: after sterilization, 1 g of tannic acid was dissolved in a small volume of distilled water and then added to 200 mL of PDA medium. The formation of reddish-brown zones indicated positive laccase secretion (Kiiskinen et al., 2004).

Simultaneous enzyme production by SSF: According to the screening results, the selected fungal strains were exploited for the production of multiple enzymes (amylases, proteases, laccases and milk clotting enzymes) at the same time. Solid-state fermentation (SSF) was adopted as the production process. To achieve our objective, wheat bran supplemented with mineral solution was used as the fermentation substrate, but no inducer was added for enzyme production.

Inoculum preparation: Fungal mycelia of the three most efficient strains were grown on PDA medium at 28°C for 5-7 days. Spores from each culture were collected separately by adding 10 mL of a sterile solution of Tween 80 (0.1%, v/v) to Petri dishes (Agrawal et al., 2005). The agar surface was slightly scratched to suspend the fungal spores, which were then filtered under aseptic conditions using filter paper №4 (Macherey-Nagel GmbH & Co. KG, Düren, Germany). One milliliter of the suspension containing 8×10^7 spores/mL was used as the inoculum. The size of the inoculum was estimated by direct counting of spores under an optical microscope (×400) (OPTICA Axiom 2000, Italy) using a Malassez cell (Poly-Optik GmbH, Bad Blankenburg, Germany).

Medium preparation and fermentation: Ten grams of wheat bran purchased from a local market were introduced into 250 mL Erlenmeyer flasks and then supplemented with the mineral solution M-9 (g/L: NaH_2PO_4 12.8, KH_2PO_4 3.0, NaCl 0.5; NH_4Cl 1.0, $MgSO_4.7H_2O$ 0.5,

$CaCl_2.2H_2O$ 0.01, pH = 5) (Tunga et al., 1998) to reach a moisture content of 54.6%. This preparation was used to induce the production of proteases, amylases, milk-clotting enzymes, and laccases. After sterilization (121°C for 20 min), each flask was inoculated with one mL of 8×10^7 spore suspension and incubated at 28°C for 72 h.

Chitinase production: In addition, chitinase production was carried out with 5 g of wheat bran supplemented with 0.05 g of chitin powder as an inducer and moistened to a level of 69% with another mineral solution (MSO) (g/L): NH_4NO_3 5.0, KH_2PO_4 5.0, $MgSO_4.7H_2O$ 1.0, and NaCl 1.0, with 0.1% (v/v) trace element solution (w/v): 0.08% $MnSO_4$, 0.17% $ZnSO_4.7H_2O$, and 0.25% $FeSO_4.7H_2O$ (Binod et al., 2005). The pH of the mineral mixture was adjusted to 5.0. The medium was autoclaved at 121°C for 20 min, then inoculated with 1 mL of the spore suspension of each isolate, and incubated for 48 h at 28±2°C.

Extraction of enzymes: At the end of the incubation period, the released enzymes in the fermentation medium were extracted by adding distilled water or Tween 80 (0.1%, v/v) to each Erlenmeyer flask (1:5, w/v), followed by a horizontal shaking step at 200 rpm for 2 h at 30°C (Stuart SSL2 reciprocating shaker, Staffordshire, UK). The fermentation musts were filtered and then clarified by centrifugation (4500 ×*g*, 20 min, 4°C) (MPW-352R, MED. Instruments, Warszawa, Poland). The recovered supernatants, representing the crude enzymatic extracts, were fractioned into aliquots and then stored at -18°C until the assay step and estimation of the various enzymatic activities.

Analytical methods

Estimation of protein concentration: The protein content of the crude extracts was determined according to the method of Bradford (1976). One hundred microliters of each enzyme extract were added to 3 mL of Bradford's reagent. After homogenization and standing in the dark for 5-10 min, the absorbance was measured at 595 nm against a blank containing distilled water instead of the enzyme extract. Bovine serum albumin (BSA) (1 mg/mL) was used as a standard.

Protease assay: Proteolytic activity (ProA) was determined from the effect of the enzyme on casein (substrate) as described by Anson (1938) with slight modifications. The enzymatic reaction consisted of 2.5 mL of casein (2.5%, w/v) in citrate/sodium phosphate buffer (0.1 M, pH 5.2) as the substrate, 0.5 mL of the same buffer, and 0.5 mL of the crude extract. The reaction mixtures were incubated at 40°C for 15 min. The reaction was stopped by adding trichloroacetic acid (TCA) solution (4%, w/v), which precipitated the non hydrolyzed proteins and proteolytic enzymes produced by the strains. After centrifugation (8000 ×*g*, 5 min) (Sigma 3-16 L, Osterode am Harz, Germany), the supernatant was used to estimate the proteolytic activity using Folin & Ciocalteu's phenol reagent (diluted 10 times) and sodium carbonate solution (2%, w/v). The color developed after 30 min of incubation in the dark at room temperature was measured with a UV-VIS spectrophotometer (Optima, SP-3000 nano, Tokyo, Japan) at 750 nm. The blank was prepared under the same conditions, except that the enzyme extract was added after the addition of TCA. Tyrosine (0.1 mg/mL) was used as a standard to establish the calibration curve for the following concentrations: 0, 20, 40, 60, 80, and 100 µg/mL. Proteolytic activity (U) is defined as "the amount of enzyme that releases the equivalent of 1 µg of tyrosine per min under the standard assay conditions".

Milk-clotting activity: The milk-clotting activity (MCA) of the crude extracts was determined by visual evaluation of the first flake appearance of coagulated milk (Arima et al., 1970). It represents the amount of enzyme (V_2) that coagulates skim milk (10%, w/v) prepared in 0.01 M $CaCl_2.2H_2O$ (pH = 6.4) (V_1) and pre-incubated at 35°C for 15 min. The clotting time (T) corresponds to the time that elapsed between the addition of the enzyme and the appearance of the first curd particles on the inner wall of the test tube immersed in a water bath and subjected to slow rotation (6-8 rpm). The MCA of the crude enzymatic extracts was calculated according to Eq. 1:

$$MCA\ (SU/mL) = \frac{2400 \times V_1}{T \times V_2} \qquad (1)$$

Amylase assay: Amylase activity (AmyA) was evaluated according to the method of Bernfeld (1955). Briefly, 500 µL of a starch solution prepared at 1% (w/v) in phosphate buffer (0.1 M; pH 5) were mixed with 500 µL of the diluted enzyme extract. The substrate was pre-incubated at 40°C for 15 min before the enzyme extract was added (Murado et al., 1993). The reaction mixture was then kept at the same temperature for 30 min. The hydrolysis reaction was blocked by adding 1 mL of the DNS reagent. This causes precipitation of the enzyme and ensures dosing of the reducing sugars released after an oxidation-reduction reaction. After heating at 100°C for 5 min and cooling, 10 mL of distilled water were added. Since wheat bran naturally contains starch, a negative control was prepared as previously described using an enzyme extract denatured at 100°C for 15 min and treated in the same way as the samples. The absorbance was measured at 540 nm against a blank prepared under the same conditions. The amount of reducing sugars released was determined via a standard glucose curve (0-1.4 mg/mL). One unit of enzymatic activity (IU) is defined as the amount of enzyme that allows the release of reducing sugars, equivalent to 1 µmole of glucose per min under the test conditions.

Laccase assay: Laccase activity (LccA) was assayed following the protocol described by Abd El Monssef et al. (2016), with guaiacol used as a substrate. The color developed due to its oxidation by laccases was used to measure the enzyme activity at 450 nm. The reaction mixture was prepared as follows: 1 mL of 2 mM guaiacol solution, 3 mL of sodium acetate buffer (10 mM, pH 5), and 1 mL of crude enzyme extract. A blank was also prepared using 1 mL of distilled water instead of the enzyme extract. The mixture was incubated at 30°C for 15 min, and the absorbance was read at 450 nm using a UV-VIS spectrophotometer (Optima, SP-3000 nano, Tokyo, Japan). The enzyme activity, expressed in International Unit (IU), represents the amount of enzyme required to oxidize 1 µmol of guaiacol/min. Eq. 2 was used to calculate laccase activity:

$$LccA\ (IU/mL) = \frac{A \times V}{t \times e \times v} \qquad (2)$$

Where A: absorbance at 450 nm, V: total volume of the mixture, v: volume of the enzyme extract, t: incubation time (15 min), e: extinction coefficient for guaiacol (0.6740 $\mu M^{-1}\ cm^{-1}$).

Chitinase assay: For the measurement of chitinolytic activity (ChiA), colloidal chitin prepared at 0.5% (w/v) in citrate-phosphate buffer (0.05 M, pH 6.6) was used as a substrate

(Monreal and Reese, 1969). The reaction mixture containing 200 µL of the crude enzymatic extract and 200 µL of the substrate was incubated for 1 h at 37°C in a water bath (WNB 14, Memmert GmbH+Co.KG, Schwabach, Germany). After that, the reaction was stopped by adding 1 mL of DNS reagent, followed by heating at 100°C for 5 min. After centrifugation at 4000 ×*g* for 5 min, the absorbance was measured at 540 nm. The calibration curve was prepared by serial dilution of N-acetylglucosamine as a standard (1 mg/mL). One unit of chitinase activity represents the amount of enzyme required to release 1 µmole of N-acetylglucosamine from chitin per min and is expressed as IU/g of substrate.

Dual culture assay to assess antagonistic effects: The antagonistic activity of the fungal isolates characterized by the highest chitinase activity was evaluated *in vitro* via a direct confrontation test in dual culture against four phytopathogenic fungi: *Fusarium culmorum, F. graminearum, F. verticillioides,* and *Alternaria alternata.* Pathogens were isolated from infected durum wheat plants (leaves and seeds) and identified on the basis of their macroscopic and microscopic characteristics. Molecular analysis was performed for *F. culmorum* (GenBank accession number: PX843723) and *F. verticillioides* (GenBank accession number: OR610687) by sequencing the ITS region. The phytopathogens were kept at 4°C in PDA slants and subcultured on the same medium before subsequent experiments on *in vitro* antagonistic activity. The confrontation tests were carried out in Petri dishes previously poured with PDA medium supplemented with chloramphenicol (0.05 g/L). Two small mycelial plugs (6 mm in diameter) cut from the periphery of each fungus species colony (7 days at 28°C) were transferred aseptically and inoculated at the same time in the opposite direction at 25 mm from the center of the Petri dish. The controls were monocultures of each of the phytopathogens placed 20 mm from the edge of the Petri dish. Mycelial growth was measured every 24 h for one week after incubation in an oven at 28±2°C (UNB 200, Memmert GmbH+Co.KG, Schwabach, Germany). The percentage inhibition of pathogen growth (I) was calculated using Eq. 3:

$$I\ (\%) = \frac{DC - DT}{DC} \times 100 \qquad (3)$$

Where DC represents the radial growth (cm) of the control-pathogen, and DT is the radial growth (cm) of the test-pathogen in the dual culture plate (Saravanakumar & Wang, 2020). The interaction type between two fungi (antagonist and phytopathogen) was evaluated using numerical score as described by Yazid et al. (2023): mutual intermingling (1/1) (without inhibition), mutual antagonism upon contact (2/2) (space between colonies < 2 mm), mutual antagonism at a distance (3/3) (space between colonies > 2 mm), dominance of one species upon contact (4/0) (strong inhibition), and dominance of one species at a distance (5/0) (strong inhibition).

Statistical analysis: The results are expressed as mean ± SD, and the measurements were repeated three times (n=3). Statistical analysis of the collected data was performed using JMP® Pro 13.2.1 software (SAS Institute Inc., USA). The difference between the enzymatic activities was considered statistically significant when the *p*-value was ≤0.05 according to the Tukey-Kramer HSD test.

Results and Discussion

Culture and microscopic characteristics of the isolated strains: Among the microbiota colonizing the rhizosphere, rhizospheric fungi hold a prominent position but are less explored than rhizobacteria. They have a wide range of applications in different fields, such as biomedicine, pharmaceuticals, agriculture, environmental sustainability, and industry, as biocatalysts, thanks to their ability to secrete an arsenal of different enzymes (Pattnaik & Busi, 2019). In our study, thirty fungal isolates were isolated from rhizospheric soil samples of durum wheat collected from four local crops in central Algeria using serial dilution followed by spread plating on Sabouraud medium. The isolates were identified based on their cultural and morphological characteristics and assigned to the following genera: *Penicillium* (Figure 1A-F'), *Rhizopus* (Figure 1G-H'), *Aspergillus* (section *Nigri* and *Flavi*) (Figure 1I-M'), *Cladosporium* (Figure 1N-N'), *Alternaria* (Figure 1O-O'), and *Fusarium* (Figure 1P-R'). The morphological characteristics of the other isolates were insufficient for precise identification. *Penicillium* and *Aspergillus* isolates were the most prevalent, highlighting their widespread presence and adaptability. Their versatile metabolism and advanced physiology enable them to utilize a diverse range of substrates, allowing them to thrive in various environments (Pitt, 2014; Plascencia-Jatomea et al., 2014). *Cladosporium* and *Alternaria* isolates emerged specifically for a particular region, probably induced by its environmental conditions.

Screening of hydrolytic and lignocellulolytic enzymes: Several researchers still insist that screening programs for the selection of microorganisms able to produce bioactive molecules remain an important aspect of biotechnology, despite the progress in genetics and microbial physiology. Screening using a specific agar medium containing an enzyme inducer is a key step in the process's development (Ogawa & Shimizu, 1999; Gopinath et al., 2005; Niyonzima, 2019). Twelve isolates of the *Penicillium* and *Aspergillus* genera obtained from different regions were selected to screen for their ability to produce extracellular enzymes. The incubation period required for the growth and development of the hydrolysis halo or colored precipitate varied depending on the strain and screening test. The diameter of this zone and the intensity of the color are related to the amount of enzyme produced by the fungus.

Amylases: As shown in Table 1, all the strains were able to produce amylases after 72 h of incubation. These results were confirmed by the formation of a yellow to clear zone surrounding the colony after the breakdown of starch (Figure 2A-D). The highest amylase activity observed was achieved by the *Penicillium* sp. S1 strain (1.933±0.13 cm). This result is consistent with findings by Shruthi et al. (2020), who reported that various *Aspergillus* and *Penicillium* species were also capable of producing amylases. Our findings align with those of previous studies indicating that *A. niger* isolates derived from different date by-products, exhibited hydrolytic zones ranging from 2.60±0.58 to 10.60±0.58 mm (Bellaouchi et al., 2021). These values are comparable to those observed in our study, suggesting that the *Penicillium* sp. S1 strain is a potent amylase producer. These findings underscore the potential of *Penicillium* and *Aspergillus* species in industrial applications where high amylase activity is desirable and highlights the importance of ongoing screening efforts to identify strains with superior enzyme production capabilities.

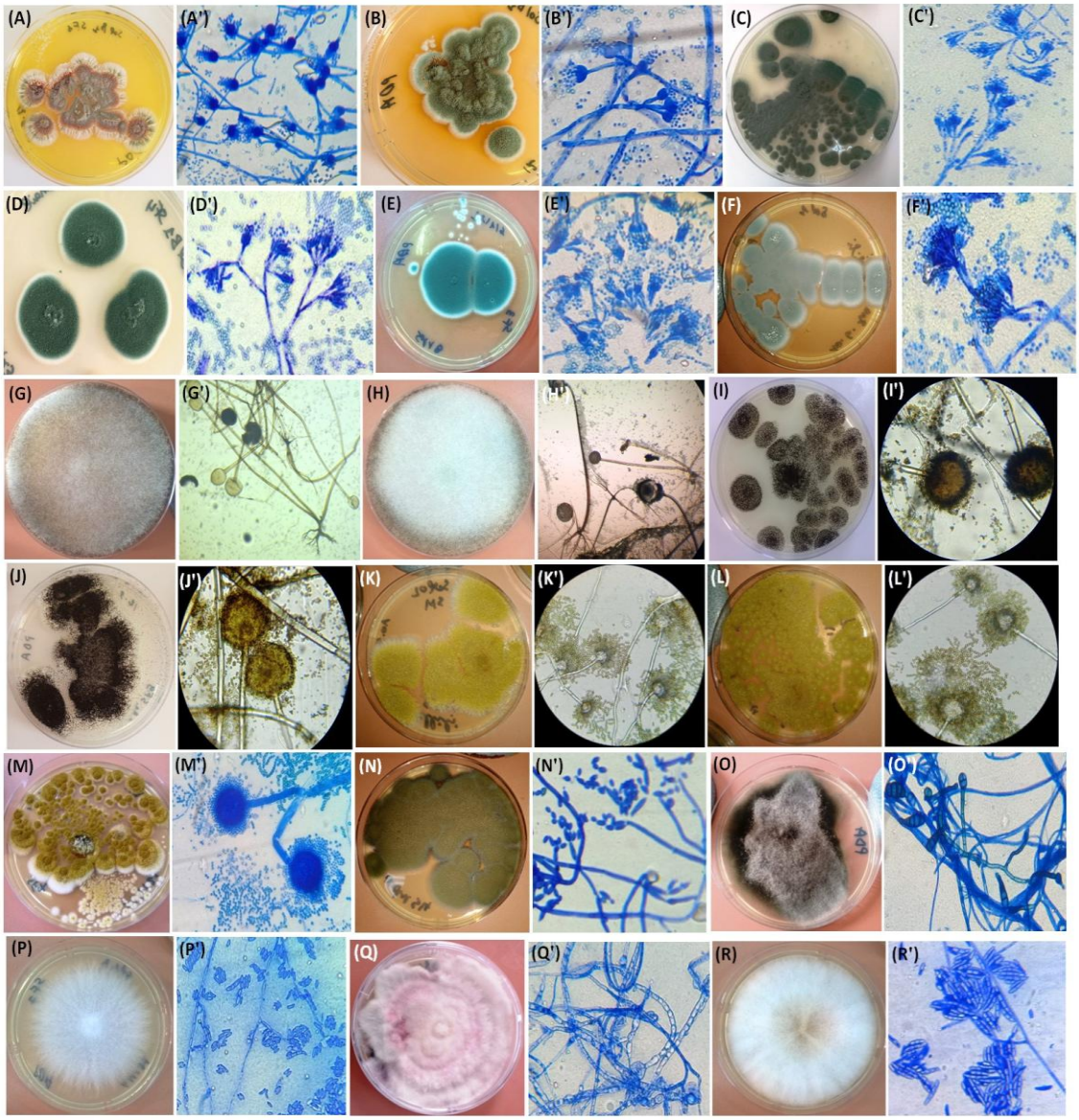

Figure 1. Macroscopic and microscopic (×400) appearances of some fungal strains isolated from the rhizosphere of durum wheat grown on PDA medium after 5-7 days at 28±2°C. The strains were identified based on their phenotypic properties: *Penicillium* (A-F'), *Rhizopus* (G-H'), *Aspergillus* (section *Nigri* and *Flavi*) (I-M'), *Cladosporium* (N-N'), *Alternaria* (O-O'), and *Fusarium* (P-R')

Table 1. Screening of *Aspergillus* and *Penicillium* isolates for different extracellular enzymes

	Amyl (cm)	Prot (cm)		Pect (cm)	Lip a	Cellu (cm)	Gela (cm)	Chit (cm)	Lacc	
		CDA	CAM						TM	BM
***Penicillium* strains**										
PS1	**1.933 ± 0.13**^{a}	-	0.97± 0.06^{c}	1.333 ± 0.115^{b}	+++	1.60± 0.076^{c}	-	4.00 ± 0.20^{d}	+	-
PS2	0.87± 0.125^{e}	-	1.133 ± 0.13^{c}	1.833 ± 0.21^{a}	+++	1.067 ± 0.07^{ef}	1.10± 0.10^{d}	2.00 ± 0.15^{f}	++	-
PS3	1.333 ± 0.11^{c}	-	-	0.67± 0.115^{d}	+	0.816 ± 0.03^{f}	-	7.90 ± 0.0^{a}	+	-
PS4	1.57± 0.057^{b}	-	-	0.683 ± 0.104^{d}	++	1.45± 0.05^{cd}	-	3.20 ± 0.40^{e}	-	-
PS5	1.833 ± 0.06^{a}	-	0.70± 0.08^{d}	01.10 ± 0.10^{c}	++	-	-	-	+	-
PS6	0.70± 0.10^{f}	-	-	1.233 ± 0.06^{bc}	+	1.267 ± 0.12^{de}	-	3.50 ± 0.10^{e}	+	+
***Aspergillus* strains**										
A. flavus S5	0.77± 0.04^{ef}	-	-	0.533 ± 0.06^{d}	+	1.96± 0.05^{b}	-	7.90 ± 0.0^{a}	+	-
A. flavus S6	1.133 ± 0.16^{d}	-	-	0.67± 0.115^{d}	+	**2.60± 0.153**^{a}	-	6.30 ± 0.50^{b}	++	-
A. niger S1	0.77± 0.08^{ef}	2.067 ± 0.06^{c}	1.70± 0.11^{b}	-	-	1.67± 0.115^{c}	2.40± 0.20^{b}	6.60 ± 0.30^{b}	+	++
A. niger S2	1.37± 0.067^{c}	**2.733 ± 0.18**^{a}	**2.17± 0.152**^{a}	-	-	1.30± 0.10^{de}	2.133 ± 0.115^{c}	7.90 ± 0.0^{a}	+++	+++
A. niger S9	1.067 ± 0.05^{d}	2.370 ± 0.20^{b}	2.07± 0.145^{a}	-	-	1.24± 0.08^{de}	**3.067 ± 0.06**^{a}	7.90 ± 0.0^{a}	++	+++
Aspergillus SF7	1.30± 0.13^{c}	1.533 ± 0.09^{d}	-	**1.933 ± 0.12**^{a}	+++	1.17± 0.152^{e}	-	5.40 ± 0.25^{c}	-	+
Pourcentage (%)	100	33.33	50	75	75	91.67	33.33	91.67	83.33	41.67

Amyl: amylases, Prot: proteases, Pect: pectinases, Cellu: cellulases, Lipa: lipases, Gela: gelatinases, Chit: chitinases, Lacc: laccases, CDA: Czapek-Dox agar, CAM: casein agar medium, TM: tannic acid medium, BM: bromophenol blue dye medium. +++: high activity; ++: moderate activity; +: low activity; -: not detected. Values not connected by the same letter in the same column are significantly different ($p \leq 0.05$) as determined by the test of Tukey.

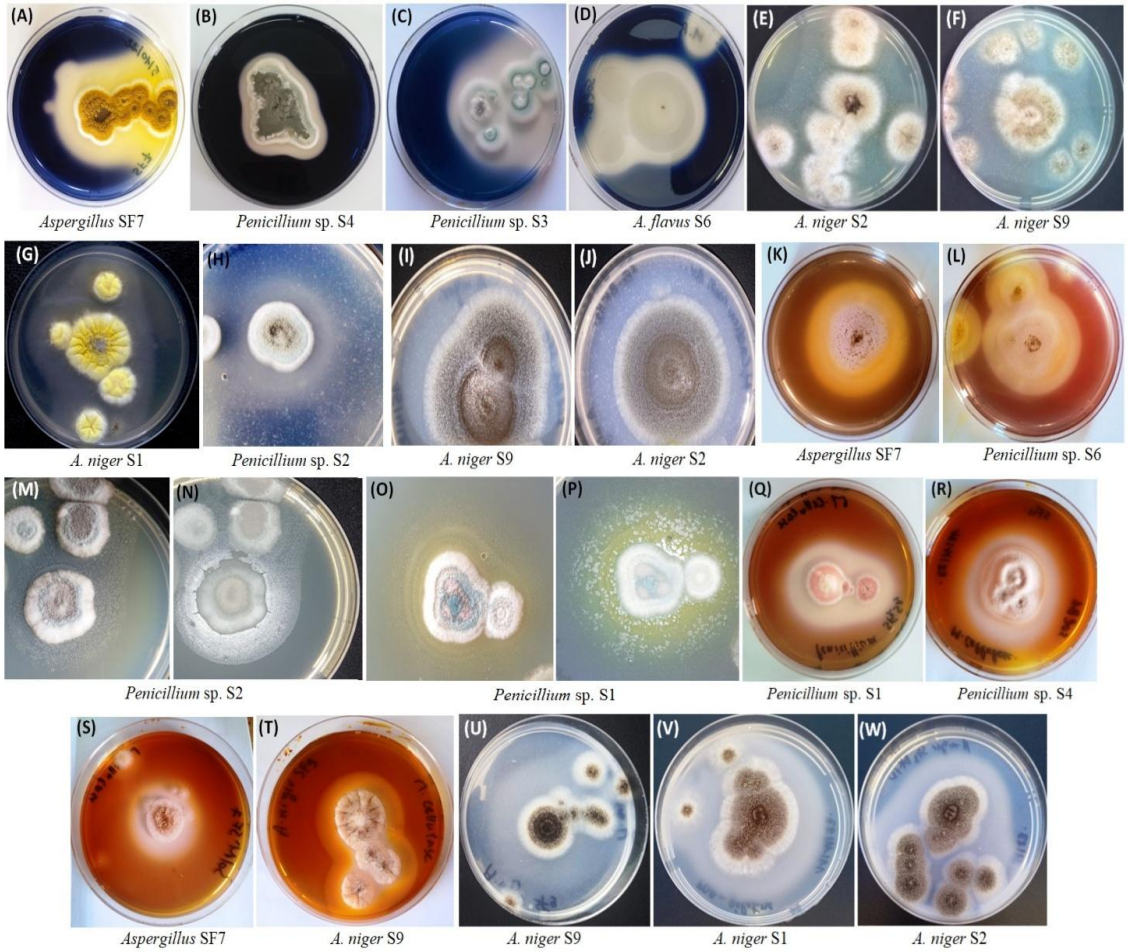

Figure 2. Examples of hydrolysis zones obtained during the screening of amylase (A-D), protease (E-J), pectinase (K-L), lipase (M-P), cellulase (Q-T), and gelatinase (U-W) activities of some isolated fungal strains. The presence of these zones confirmed the ability of the tested strains to produce the targeted enzymes. The diameter of the hydrolysis zone is directly related to the amount of extracellular enzyme secreted in the medium. For lipases, exposing the area around the colony to light improves the visibility of precipitated fatty acid crystals

Proteases: The two solid media used to screen protease production gave different results, where only four strains (33.33%) were able to hydrolyze casein compounds in CDA medium (Figure 2E-G). However, 50% of the selected strains were positive for CAM (Figure 2H-J). The best zone of hydrolysis (2.733±0.18 cm) was recorded using the first medium with *A. niger* S2 (Table 1). There are several possible regulatory mechanisms involved in enzyme production processes, including enzyme induction. Therefore, the presence of an inducing substance in the culture medium in sufficient quantity is essential to induce the synthesis of the target enzyme during the screening step, which can explain the difference in the level of results obtained for each strain. Usman et al. (2021) reported that 38% of the strains isolated from soil samples of dairy and grape farms could form a clear zone of hydrolysis on skim milk agar plates (6.70±1.5-21.30±1.5 mm), with strain Z1BL1, identified as *Aspergillus* sp., being the best protease producer.

In a similar study, 15 fungal isolates from soil showed significant protease activity (8-24.75 mm) after 72 h of incubation. The most efficient strains belong to the *Aspergillus*, *Rhizopus*, *Fusarium*, and *Mucor* genera (Maitig et al., 2018).

Pectinases and lipases: The pectin hydrolysis test revealed that 75% of the isolated strains developed a clear zone on agar plates after 96 h of incubation at 28°C, as shown in Figure 2K-L and Table 1. The same isolates were also positive when tested for lipase production. The clear crystal zone that developed around the fungus was an indication of lipolytic activity (Figure 2M-P). Among the fungi tested, *Penicillium* strains were characterized by good potential to secrete lipases and pectinases into the medium, whereas *A. niger* strains (S1, S2, and S9) could not hydrolyze pectin or Tween 80 during the incubation period. Bellaouchi et al. (2021) demonstrated that most of the isolated *A. niger* strains (62.5%) were unable to excrete lipases in the medium containing Tween 20 as a lipase inducer.

Cellulases: After 5 days of incubation at 28°C, cellulase production was confirmed for 11 fungal strains (91.67%) by the formation of clear to yellow zones on CMC agar after the addition of Lugol's solution (Figure 2Q-T). This treatment was sufficient to make the hydrolysis zone more visible without the need for additional steps as required in other protocols that use Congo red solution (2%, w/v) for staining, thus simplifying the protocol used. As given in Table 1, *Aspergillus* strains showed a good potential to hydrolyze cellulose compared with *Penicillium* strains. *A. flavus* S6 was characterized by the highest cellulase activity, with a clear zone of 2.60±0.153 cm. Our results concurred with those reported by Shruthi et al. (2020), who reported that all isolated *Aspergillus* and *Penicillium* strains were able to release cellulases into the medium.

Gelatinases: All the strains tested grew well on PDA medium supplemented with gelatin, but the hydrolysis of this substance was detected for only four strains (33.33%) after the incubation period (72 h at 28°C) (Figure 2U-W). According to Abdel-Raheem and Shearer (2002), such a result could mean that the enzyme is not produced, that it is produced but not released from the mycelium, or that it is produced and released but the medium inhibits its detection. Therefore, the absence of a reaction is not an absolute confirmation of a species' inability to produce a specific enzyme. The strain *A. niger* S9 was the most efficient in producing gelatinolytic enzymes, with a hydrolysis zone of approximately 3.067±0.06 cm (Table 1). However, no gelatinase activity was detected for forty strains of *A. niger* isolated by Bellaouchi et al. (2021).

Chitinases and laccases: The ability to secrete chitinases in the medium supplemented with colloidal chitin was found to be greater for *Aspergillus* strains than for *Penicillium* strains with respect to the diameter and color of the zone produced around the colonies after 7 days of incubation (Table 1). The addition of bromocresol purple, a color-changing indicator dye, improved visibility during the screening process. The peak activity was noted after only 3 days of incubation for *A. niger* S9, where the medium was characterized by an intense purple color (Figure 3A-D).

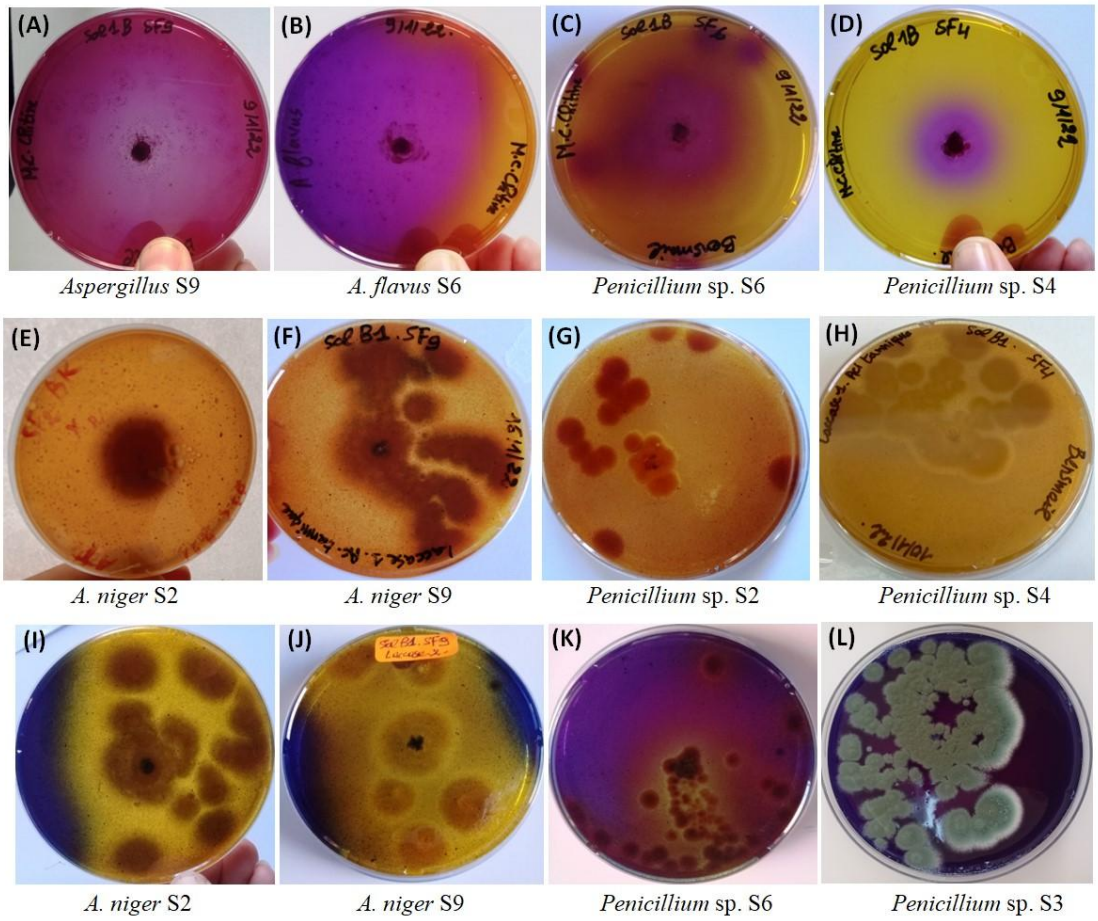

Figure 3. Screening of some fungal strains for chitinase and laccase production. **A-D: production of chitinolytic enzymes on medium supplemented with colloidal chitin after 3 days of incubation at 28°C (the increase in diameter and intensity of the purple color is directly related to the amount of chitinases in the medium), E-G: fungal strains showing positive results (appearance of reddish-brown color) for laccase production on PDA medium supplemented with tannic acid, I-K: laccase production on PDA medium containing bromophenol blue dye (the medium turns yellow around the colonies). H and L: absence of laccase activity (absence of modification)**

Laccase production was screened using PDA medium amended with 5 g/L tannic acid (TM) or 0.2 g/L bromophenol blue (BM) dye. According to the findings (Table 1), about 83% of the fungal strains oxidized the tannic acid contained in the first medium to form a reddish-brown to dark-brown precipitate below the colonies after 3 days of incubation (Figure 3D-H). The degradation of bromophenol blue dye confirmed laccase production by only five strains, most of which were *Aspergillus* strains (Figure 3I-L). These strains were capable of making the medium colorless within 48 h of incubation. As a result of the screening step, the three strains identified as *A. niger* S2, *A. niger* S9, and *Penicillium* sp. S1 were found to be the most potent among all the isolates and were selected for further work to produce the target enzymes by SSF.

Multi-enzyme production by the selected strains: The production of multiple extracellular enzymes (amylases, proteases, milk-clotting enzymes, and laccases) by the selected strains *Penicillium* sp. S1, *A. niger* S2, and *A. niger* S9 is summarized in Table 2.

Table 2. Protein content, protease, milk-clotting, amylase, laccase, and chitinase activities of the crude enzymatic extracts obtained by SSF on wheat bran

	***Penicillium* sp. S1**	***A. niger* S2**	***A. niger* S9**
Proteins (mg/mL)	0.230±0.015^{c}	0.318±0.018^{b}	0.368±0.030^{a}
ProA (U/mL)	23.851±0.437^{b}	29.80±1.350^{a}	13.83±0.182^{c}
MCA (SU/mL)	7.06±0.009^{b}	0	46.602±1.240^{a}
AmyA (IU/mL)	3.340±0.144^{b}	5.46±0.237^{a}	1.56±0.070^{c}
LccA (IU/mL)	0.058±0.005^{b}	0.072±0.005^{a}	0.020±0.002^{c}
ChiA (IU/g sub)	1.27±0.060^{b}	1.50±0.020^{a}	1.30±0.061^{b}

ProA: proteolytic activity, MCA: milk-clotting activity, AmyA: amylase activity, LccA: laccase activity, ChiA: chitinase activity. Values not connected by the same letter in the same row are significantly different ($p \leq 0.05$) as determined by the test of Tukey.

Proteolytic and milk-clotting activities: The crude extracts produced by the selected strains using wheat bran as a substrate exhibited both proteolytic and milk-clotting activities, except for the crude extract of *A. niger* S2, where a total absence of the MCA was noted even after 2 h of interaction between this extract and skim milk under the standard conditions of the assay (Table 2). The highest ProA was provided by the crude extract of *A. niger* S2 (29.80±1.35 U/mL), followed by that of *Penicillium* sp. S1 (23.851±0.437 U/mL). Weak ProA (≤ 9.279±3.2 U/mL) were reported by Marques Nolli et al. (2022) for various extracts of *Aspergillus*, *Fusarium,* and *Pleurotus* species produced by SSF on wheat bran. However, a higher trend in the enzyme production by nine fungal isolates was noted by Usman et al. (2021), with ProA ranging between 31.7±1.7-78.2±1.5 U/mL. The milk-clotting activity (MCA) of *A. niger* S9 crude extract (46.602±1.240 SU/mL) was notably higher than that of *A. flavo furcatis* strains DPUA 1623, 1539, and 1608, which exhibited activities of 19.05, 30.04±0.38, and 36.36 SU/mL, respectively (Alecrim et al., 2014). Additionally, *A. niger* S9 demonstrated superior MCA compared with *A. niger* FFB1 (35.70±3.80 SU/mL) (Bensmail et al., 2015). Remarkably, the MCA of *A. niger* S9 was approximately 5.5 times greater than the purified protease activity produced by *Rhizopus oryzae* NBRC 4749 (Chen et al., 2009). Thus, it seems that the MCA of *A. niger* S9 is very interesting and can be improved by optimizing the fermentation and enzyme extraction conditions. It is possible that the conditions of the SSF process were not favorable for milk-clotting enzyme production by the strain *A. niger* S2, since it is not a general rule that a proteolytic enzyme can coagulate milk unless it has a specific activity on the Phe_{105}-Met_{106} bond of κ-casein.

Amylase activity: As shown in Table 2, the three isolates possessed a high potential for amylase production, where the crude extract of *A. niger* S2 yielded the highest AmyA (5.46±0.237

IU/mL). This value was almost 4.23 and 20.22-fold higher than those of partially purified extracts of *A. clavatus* (1.29±0.01 IU/mL) and *P. citrinum* (0.27±0.015 IU/mL), respectively (Shruthi et al., 2020). The three fungal strains presented greater AmyA than did the crude extracts of *A. flavus* NSH9 (1.055±0.03 U/mL) (Mustafa et al., 2016), *A. niger* FAB-211 (1.241 U/mL) (Arsat & Girma, 2018), *Penicillium* spp. (0.87 IU/mL) (Jakheng et al., 2020), and *Penicillium* sp. L1 (1.91 IU/mL) (Silva et al., 2022), which were produced after optimization of several factors affecting the fermentation process (SmF or SSF).

Laccase activity: In the present study, the assessment of laccase production by SSF on wheat bran was estimated by the method of Abd El Monssef et al. (2016), which is based on the oxidation of guaiacol. The reddish-brown color developed during the assay was very light, reflecting the low laccase activity of the crude extracts for the three fungal strains (Table 2). We found that the capacity of *A. niger* S2 to secrete laccase was superior than that of *Penicillium* sp. S1 and *A. niger* S9. The maximum LccA (0.061 U/mL) of *Trichoderma longibracheatum* ITCC 8996.13 was achieved after 3 days of incubation (Mathur et al., 2013). This activity is in agreement with that determined in our study for *Penicillium* sp. S1 crude extract and very close to that of *A. niger* S2, but it remained lower than that of *A. niger* (1.66 U/mL) grown on corn cob waste for 12 days (Abdel Ghany et al., 2020). Kiiskinen et al. (2004) screened the extracellular laccase production of 11 fungal strains on a liquid medium containing soybean meal. The best activity obtained with an unidentified haploid Basidiomycete was 2.40 IU/mL. A similar trend of LccA (2.551 IU/mL) was recorded for *A. niger* IBP2013 grown on wheat straw (Hasan et al., 2023). From the obtained results, we conclude that the selected strains were able to produce the target enzymes simultaneously but in different quantities. We can explain the ability of the strain *A. niger* S2 to produce more enzymes by its enzymatic properties and specificity with respect to the substrate (wheat bran) and the effect of SSF conditions on each strain, where it is quite possible that we were far from the optimal production conditions for *A. niger* S9 and *Penicillium* sp. S1.

Chitinase production: The chitinase activity of each isolate obtained after 48 h of incubation is presented in Table 2. The three strains were determined to be good chitinase producers, with a ChiA between 1.27±0.060-1.50±0.020 IU/g of substrate, which is equivalent to 0.254±0.012-0.30±0.004 IU/mL of extract. Among the three strains, *A. niger* S2 was more active than were *A. niger* S9 and *Penicillium* sp. S1. The maximum ChiA (0.14 U/mL) was detected in *Aspergillus niveus* LH0306 crude extract obtained using 1% shrimp shells as the sole carbon source in the fermentation medium (Ornela et al., 2024). However, the ChiA of the crude enzymatic extracts of *T. aureoviride* and *T. virens* were 0.036 U/mL (Wasli et al., 2009) and 0.147 U/mL (Agrawal & Kotasthane, 2012), respectively, after the SmF process using colloidal chitin as the fermentation substrate.

Antagonistic activity of *Aspergillus* strains: Chitinolytic microorganisms may provide an alternative to chemicals and could be used as natural methods to control fungal diseases. Among chitinolytic fungi, the fungistatic properties of the genus *Trichoderma* are the most studied (Brzezinska & Jankiewicz, 2012; Boughalleb-M'Hamdi et al., 2018; El-Debaiky, 2017). However, recent studies have demonstrated that *Aspergillus* species can effectively inhibit the

growth of various plant pathogenic fungi, positioning them as potent biocontrol agents. They are regarded as among the most potent emerging antagonists in recent years (Khan & Javaid, 2021).

Direct confrontation tests carried out *in vitro* between *Aspergillus* isolates with high chitinase activity (*A. niger* S2, *A. niger* S9, and *A. flavus* S5) and four phytopathogens highlighted an inhibitory action in the dual culture plates. The selected strains caused statistically significant reductions in the mycelial growth of the test pathogens (Figure 4). For all the isolates, after 4 days of confrontation, the inhibition rates increased rapidly and the growth rate of the phytopathogenic fungi was essentially abolished. After 7 days, *A. niger* S9 was the most efficient against *F. culmorum, F. graminearum,* and *A. alternata*, with inhibition rates of up to 56.20%, 58.30%, and 73.33%, respectively. Compared to *A. niger* S2 (52.41%) and *A. niger* S9 (55.75%), *A. flavus* S5 had the greatest antagonistic effect on *F. verticillioides*, resulting in 60% of growth suppression. The inhibitory effect of these two strains was significantly inferior ($p \leq 0.05$) to that induced by *A. flavus* S5. The growth of *A. alternata* was strongly inhibited by *A. niger* S9 (73.33%), while the lowest inhibition value was given by *A. niger* S2 against *F. graminearum* (50.30%). These results demonstrated the significant antifungal activity of *Aspergillus* strains against wheat phytopathogens and their potential as biological control agents. Our results are in accordance with other studies using different species of the genus *Aspergillus*. The antagonistic effects of *Aspergillus* spp. from section *Nigri* against many soil-borne phytopathogens (*F. oxysporum* f. sp. *melonis, F. solani*, *Pythium aphanidermatum*, *Sclerotinia sclerotiorum*, *S. cepivorum A. alternata*, *A. solani*, *Botrytis cinerea*, and *Rhizoctonia solani*) (Patibanda & Sen, 2007; Seema & Devaki, 2012; El-Debaiky, 2017), and spoilage fungi (*Hyphopichia burtonii, Paecilomyces variotii, Aspergillus tamari,* and *Eurotium chevalieri*) (Fendiyanto & Satrio, 2020) have been established. Boughalleb-M'Hamdi et al. (2018) reported that the growth of *F. oxysporum* f. sp. *niveum*, *F. solani* f. sp. *cucurbitae* and *F. solani* f. sp. *melonis* was strongly inhibited by strains of *A. flavus*, *A. niger* and *A. terreus.* Similarly, Khan and Javaid (2021) demonstrated that *A. flavipes, A. niger,* and *A. flavus* significantly restrained the growth of *Macrophomina phaseolina*, with inhibition rates ranging from 37 to 53%. The interaction scores and indices of dominance (ID) between the selected *Aspergillus* strains and the four phytopathogens of wheat are presented in Table 3. This quantitative evaluation can be used to evaluate the nature of the interaction between the antagonist and the phytopathogen strain, as well as the possible mechanism of antagonism (Yazid et al., 2023). Antagonists are considered highly competitive and dominant when they score 4/0 or 5/0 against the phytopathogen, but not competitive when they score 1/1, 2/2, or 3/3. Furthermore, a score of 4/0 indicates strong inhibition of the antagonist against the pathogen "upon contact", suggesting that it produces cell wall-degrading enzymes to inhibit the growth of the pathogen fungus (the possible mechanism is mycoparasitism). The 5/0 score also signifies a strong inhibition of the phytopathogen but "at a distance," possibly through the synthesis of antifungal compounds by antagonists that hinder pathogenic growth (Yazid et al., 2023). The growth of *F. verticillioides* was mutually inhibited by *A. niger* S2 and *A. niger* S9 at distances greater than 0.2 cm, while *A. niger* S2 exhibited antagonistic activity through contact against *F. graminearum*. The strain *A. flavus* S5 showed high antagonism upon contact against all the tested phytopathogens.

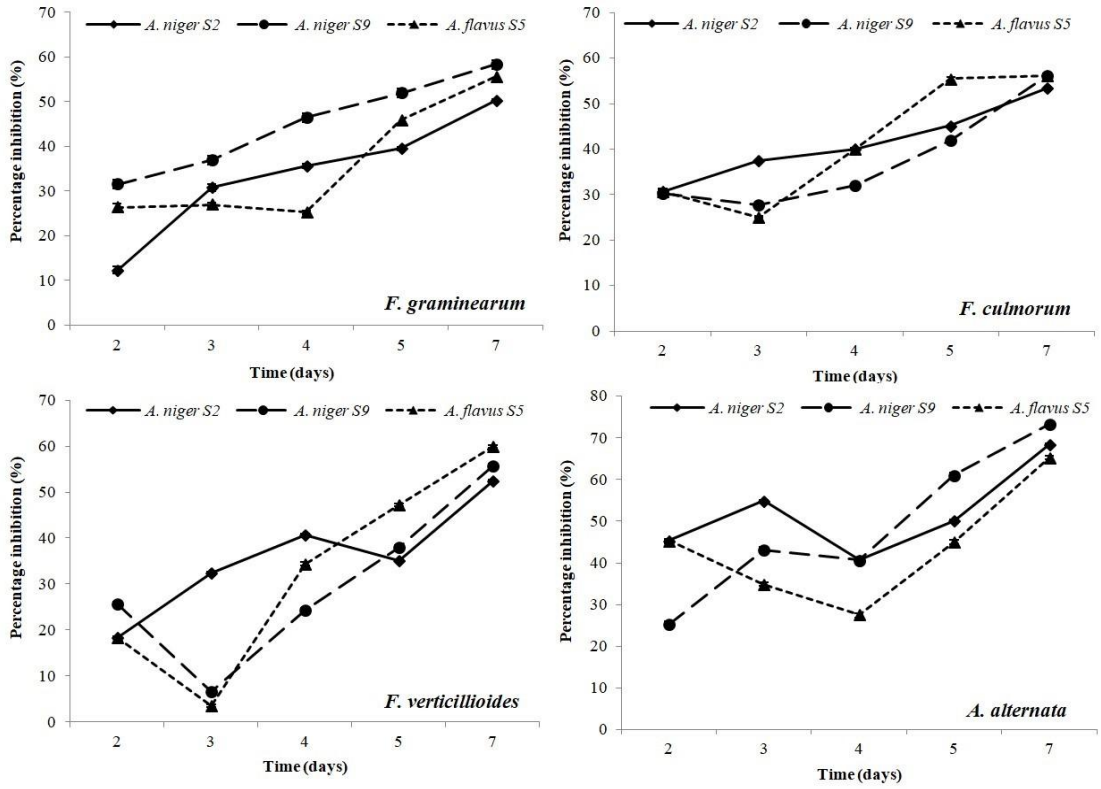

Figure 4. Inhibition of phytopathogen growth induced by interaction with the newly isolated *Aspergillus* strains in dual cultures. The tested fungal strains inhibited the growth of *Fusarium* and *Alternaria* phytopathogens from the second day of confrontation, with an increased effect observed after four days. The maximum inhibition rates were achieved after 7 days for all antagonists tested

Table 3. Interaction scores and Index of Dominance of wheat rhizospheric *Aspergillus* spp. towards four phytpathogenic fungi after 7 days of confrontation

Antagonist	Phytopathogenic fungi				Index of Dominance (ID)
	F. graminearum	*F. culmorum*	*F. verticillioides*	*A. alternata*	
A. niger S9	4/0	4/0	3/3	4/0	15/3
A. flavus S5	4/0	4/0	4/0	4/0	16/0
A. niger S2	2/2	4/0	3/3	5/0	14/5

ID: represents the cumulative of different interaction scores from similar antagonist against phytopathogens.

The same interaction type characterized the antifungal activity of *A. niger* S2 against *F. culmorum* and *A. niger* S9 towards the four phytopathogenic fungi, with the exception of *F. verticillioides*. Only *A. niger* S2 was able to inhibit the growth of *A. alternata* at a distance (score of 5/0). Moreover, *A. flavus* S5 presented the highest ID (16/0), whereas the lowest ID was associated with *A. niger* S2 (14/5) (Table 3). As shown in Figure 5 and from the interaction scores

for each antagonist (Table 3), the isolated strains adopted different biocontrol mechanisms against the tested phytopathogens, such as antibiosis, competition for space/nutrients, and mycoparasitism in most cases. In certain experiments, *Aspergillus* strains were observed to overgrow the pathogen's mycelia and alter the pigmentation of *Fusarium* colonies as well as the surrounding medium in dual culture setups. Since mycoparasitism is the dominant mechanism by which *Aspergillus* strains inhibit the growth of the tested phytopathogenic fungi, further work will investigate the effects of enzymatic extracts from the three strains on phytopathogens *in vitro* and *in planta* before their mass production.

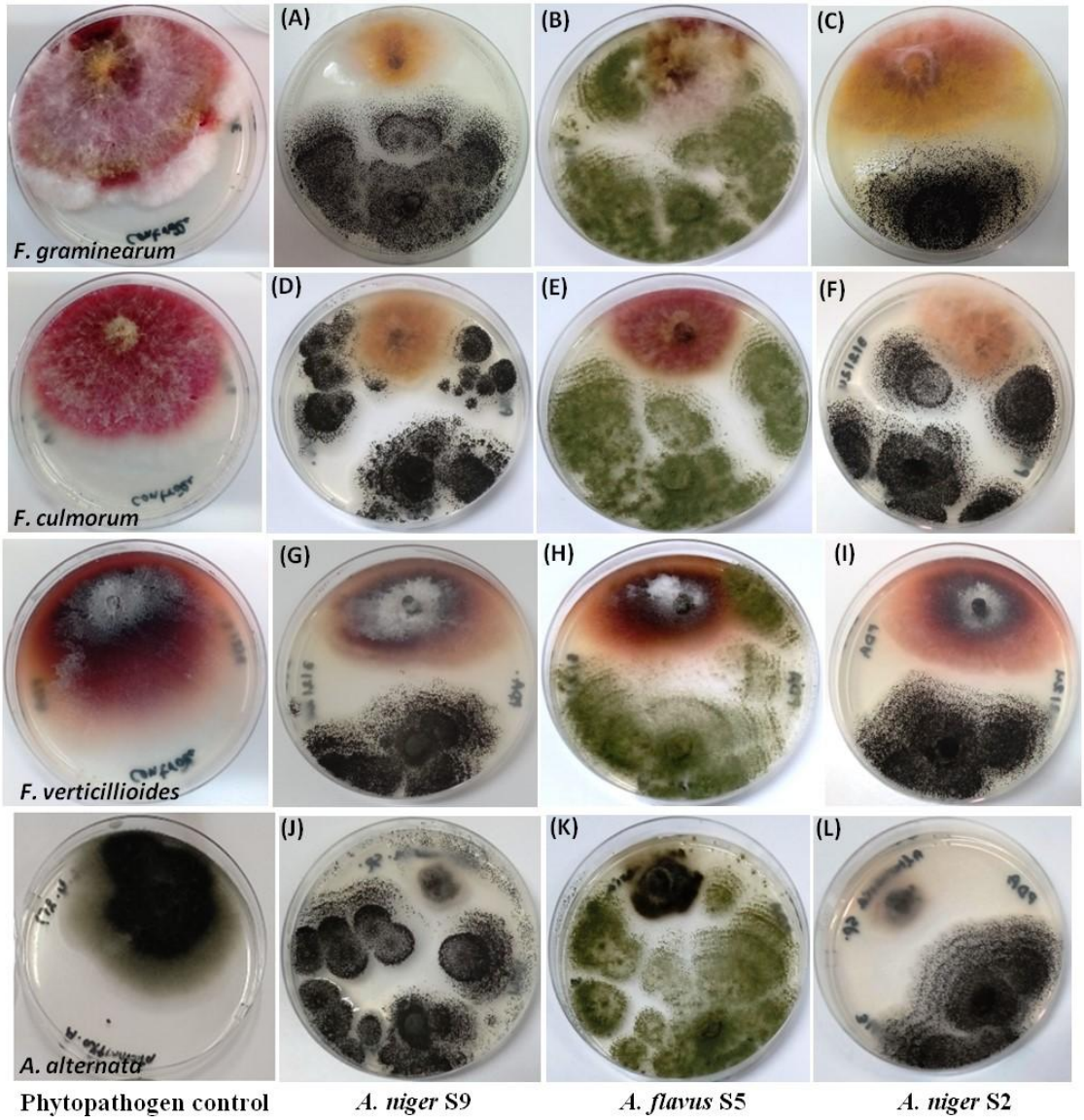

Figure 5. Antagonistic activity of *A. niger* S9, *A. flavus* S5 and *A. niger* S2 strains against *F. graminearum* (A-C), *F. culmorum* (D-F), *F. verticillioides* (G-I) and *A. alternata* (J-L) on PDA medium after 7 days of confrontation. The three *Aspergillus* strains tested exhibited high antifungal potential against *Fusarium* and *Alternaria* pathogens, as evidenced by their reduced mycelial growth and, in some cases, discoloration of the pathogen mycelium

Conclusions: From the rhizosphere soil of durum wheat crops in various regions of Algeria, thirty fungal strains were successfully isolated and identified on the basis of their macroscopic and microscopic characteristics, with the majority classified under the genera *Penicillium* and *Aspergillus*. These strains were screened for their ability to produce multiple extracellular enzymes on solid media. The screening revealed broad enzymatic potential, with most isolates demonstrating significant chitinolytic, amylolytic, cellulolytic, lipolytic, pectinolytic, and laccase activities. However, only a limited number of strains were capable of hydrolyzing casein and gelatin. Among the tested strains, *A. niger* S2, *A. niger* S9, and *Penicillium* sp. S1 emerged as the most efficient, exhibiting the highest levels of amylase, protease, laccase, and chitinase activities under solid-state fermentation on wheat bran within a relatively short period. Notably, *in vitro* dual culture assays demonstrated the strong biocontrol potential of *A. niger* S2, *A. niger* S9, and *A. flavus* S5 against four wheat-associated plant pathogens through various antagonistic mechanisms. The isolated strains hold significant potential for further industrial and biotechnological applications.

Author Contributions

Methodology, investigation, data curation, visualization, formal analysis, and software were performed by SB and SB. Writing of original draft, reviewing, and editing by SB, SB and HM. Isolation and identification of phytopathogen strains by MS. Conceptualization, supervision, project administration by FH and SL. All authors commented on previous versions of the manuscript, read and approved the final manuscript.

Data Availability Statement

Data available on request from authors.

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Ethical Considerations

Not applicable.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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جداسازی، غربالگری و انتخاب سویه‌های *Aspergillus* و *Penicillium* از ریزوسفر گندم برای تولید چندآنزیمی و ویژگی‌های ضدقارچی

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چکیده

هدف: آنزیم‌های میکروبی به دلیل اهمیت اقتصادی در بخش‌های مختلف صنایع زیستی، نقش مهمی در توسعه فرایندهای زیستی صنعتی دارند. بنابراین، جستجو برای یافتن سویه‌های جدید با توان تولید بالا، جهت تأمین نیازهای صنعتی، بسیار حائز اهمیت است.

هدف این مطالعه جداسازی و غربالگری سویه‌های *Aspergillus* و *Penicillium* با توان بالای تولید چندین آنزیم مهم زیست‌فناورانه و همچنین قابلیت کنترل زیستی برخی عوامل بیماری‌زای گیاهی بود.

مواد و روش‌ها: تعداد ۳۰ سویه قارچی از خاک‌های ریزوسفری مزارع گندم دوروم در مرکز الجزایر جداسازی و بر اساس ویژگی‌های فنوتیپی شناسایی شدند. دوازده سویه متعلق به جنس‌های هدف از نظر توانایی تولید هشت آنزیم هیدرولیتیک و لیگنوسلولولیتیک بر روی محیط‌های جامد اختصاصی مورد غربالگری قرار گرفتند. سویه‌های با تولید بالا برای بررسی تولید همزمان آنزیم‌ها در شرایط تخمیر حالت جامد با استفاده از سبوس گندم به‌عنوان بستر، و نیز اثر آنتاگونیستی علیه برخی عوامل بیماری‌زای گندم (*Fusarium graminearum, F. culmorum, F. verticillioides,* and *Alternaria alternata*) با آزمون کشت دوگانه ارزیابی شدند.

نتایج: یافته‌ها نشان دادند که میزان تولید آنزیم در میان سویه‌های بررسی‌شده بالا بود؛ به‌طوری‌که ۱۰۰٪ برای آمیلازها، ۹۱/۶۷٪ برای کیتینازها و سلولازها، ۸۳/۳۳٪ برای لاکازها، ۷۵٪ برای پکتینازها و لیپازها و میزان کمتری برای پروتئازها و ژلاتینازها مشاهده شد. در میان کارآمدترین سویه‌ها، *A. niger* S2 بیشترین فعالیت آمیلازی (5.46 IU/mL)، پروتئازی (29.80 U/mL) و لاکازی (0.072 IU/mL) را نشان داد. فعالیت انعقاد شیر در *Penicillium* sp. S1 (7.06 SU/mL) و *A. niger* S9 (46.60 SU/mL) قابل توجه بود. علاوه بر این، سویه‌ها در مدت تنها ۴۸ ساعت مقادیر قابل توجهی کیتیناز (1.27–1.50 IU/g) تولید کردند. فعالیت آنتاگونیستی سویه‌های با تولید بالای کیتیناز علیه عوامل بیماری‌زای *Fusarium* و *Alternaria* نرخ مهار بالایی بین ۵۰/۳٪ تا ۷۳/۳۳٪ نشان داد که در این میان *A. niger* S9 مؤثرترین سویه بود.

نتیجه‌گیری: نتایج این مطالعه پتانسیل بالای سویه‌های بومی جداسازی‌شده را به‌عنوان گزینه‌های امیدوارکننده نه‌تنها برای تولید آنزیم‌های صنعتی، بلکه به‌عنوان عوامل مؤثر کنترل زیستی نشان داد. این یافته‌ها می‌تواند راه را برای کاربرد این سویه‌ها در زیست‌فناوری صنعتی و کشاورزی پایدار هموار سازد.

کلمات کلیدی: آنزیم‌های هیدرولیتیک، فعالیت آنتاگونیستی، قارچ‌های ریزوسفری، کیتینازها، گندم دوروم

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