BIOLOGICAL ACTIVITY AND ENZYMATIC PRODUCTIVITY OF TWO THERMOPHILIC ACTINOMYCETES ISOLATED FROM THE UHUD MOUNTAIN

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Abstract. Actinomycetes represent an enormous reservoir of bioactive compounds and enzymes. Most of the produced novel compounds are useful in medicine, industry and agriculture. This study was carried out to isolate thermophilic actinomycetes strains that have potential to produce commercially important metabolites from unexplored regions and yet to be investigated for unknown, rare actinomycetes (the Uhud mountain, Madinah, Saudi Arabia). Two thermophilic isolates MA-1 and MA-2 were selected and tested for antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa*. Results showed that they were effective against *B. cereus* and *S. aureus*. No antibacterial activity was recorded against *P. aeruginosa*. Antifungal activity of the isolates was also tested against *Aspergillus niger*, *Penicillium* sp, *Alternaria alternate* and *Fusarium solai*. MA-2 had antifungal activity against *A. niger*, *Penicillium sp* and *A. alternata*. The isolates were able to produce protease, amylase, catalase and lipase. They also exhibited capability to degrade keratin and lignocelluloses. Additionally, plant growth promoting abilities of the isolates were assessed under laboratory conditions. The effect of the isolates on wheat (*Triticum aestivum*) growth was studied. According to the results, the isolates increased the number of seminal roots and the dry biomass of wheat seedlings significantly. Using 16S rRNA gene sequence analysis, they were identified as *Streptomyces macrospores* strain OR916389 (MA-1) and *Streptomyces rameus* strain OR916416 (MA-2). These findings demonstrated that the Uhud mountain has been emerging as a potentially abundant reservoir of novel species/genera of actinomycetes that might be a potential source of bioactive substances of medical, pharmaceutical, industrial and agricultural interests.
Keywords: *antimicrobial agents, enzymes, plant growth enhance, 16S rRNA gene, thermophilic actinomycetes*

Introduction

Actinomycetes are Gram-positive bacteria, mostly soil inhabitants where they produce geosmin; a compound responsible for the earthy-smelling soil. They exhibit both bacterial and fungal characteristics (Das et al., 2008) including the capacity of their hyphae to produce a chain of uninucleate spores and the multinuclear aerial mycelium that regularly produces septa during spore germination (Xia et al., 2020).

The variety of actinomycetes, as well as their ability to create new compounds, distinguishes this group. They produce almost 50% of the bioactive secondary metabolites that have been found (Elmallah et al., 2020). More than 10,000 compounds have been identified from actinobacteria, with *Streptomyces* accounting for around 85% of them and 25% isolated from rare Actinobacteria (Berdy, 2005; Bull and Stach, 2007). Immunosuppressants, antihelminthic, antiaging, anticholesterol, antiprotozoal, antiviral, antifungal, anticancer and antibiotics are some examples of bioactive compounds isolated from actinomycetes (Subathra Devi et al., 2022). They also have the ability to

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release a range of extracellular hydrolytic enzymes outside their cells (Wu et al., 2019; Mihajlovski et al., 2021).

Actinomycetes provide around 75% of antibiotics, primarily antibacterials. Antibacterials come in a wide variety of forms, each with its own set of activities and mechanisms of action. They demonstrated their effectiveness against an extensive range of bacteria, including both Gram positive and Gram negative strains (Hasani et al., 2014). When compared to bacteria and fungi, *Streptomycetes* has historically been the source of the most novel antibiotic medicines (Sharma et al., 2014; Hong et al., 2017). They manufacture a broad variety of antibiotics that are sold on the market, including gentamycin, amphotericin, vancomycin, neomycin, tetracycline, erythromycin and nystatin.

Enzymes produced from actinomycetes including amylase, lipase and protease are crucial in the production of food, textiles, paper and fermentation. Specific enzymes used in pharmaceutical and biomedical industries. They also generate enzymes necessary for the biodegradation of soil and the creation of humus such as chitin, keratin, and lignocelluloses (Das et al., 2021; Javed et al., 2021).

Additionally, actinomycetes have attracted attention due to their capability to encourage plant growth (Reddy et al., 2016). By producing auxins and gibberellin-like compounds, they improve plant growth (Xia et al., 2020). Moreover, they may be used as a source of agroactive chemicals, biopesticides, biocontrol agents, antifungal agents, and biocorrosion.

Thermophilic actinomycetes are relatively poorly studied compared with mesophilic species. They can be found in many different places, including hot springs, deserts, geothermal spots and hydrothermal vents (Al-Dhabi et al., 2020). Their membrane lipids, composed of straight-chains and saturated fatty acids, allow them to survive in high temperatures. Lipids help maintain the membrane's fluidity, thus preserving their membrane's function (Kumar et al., 2021). Thermophilic actinomycetes are useful for developing thermostable enzymes and bioactive compounds with medicinal and industrial properties. While the bacterial's high metabolic activity drives the reaction rate to a peak, elevated incubation temperatures reduce the chance of contamination by extra pathogens (Mehetre et al., 2019; Karadayi et al., 2021). Furthermore, the use of large fermenters in the production process reduces cooling costs and the viscosity of the production medium (Eswari et al., 2019). Difficulty in isolating and preserving thermophilic actinomycetes in a pure culture has resulted in fewer studies of them for the discovery of new metabolites (Ruwandeepika et al., 2022). Moreover, isolation of new bioactive compounds from many natural sources for economic purposes is essential (Kurapova et al., 2012). Hence, it is important to maximize the use of new groups of microorganisms from unexplored or underexploited regions in order to obtain novel bioactive secondary metabolites (Lam, 2006).

One of the most significant historical locations in the Arab Peninsula is the Uhud mountain, it stands out among the region's mountains because of its rough topography and high altitude. It has a height of 1077 m, a length of 7 km, and a width of 3 km, establishing an east-west chain. It stands out from other mountains in the area because it looks like one solid piece, neither fragmented nor linked to other mountains. The mountain has some of the most remarkable volcanic granite rock formations that are dark red and include many mineral veins of various colors, such as black, blue, white, silver and green. In addition to the wildflowers, the mountain has naturally occurring potholes on rocky formations that collect rainwater, which may be a valuable water supply for wildlife. The type of minerals in the soil determines how well plant life thrives; more vegetation grows in manganese-rich soils than in highly salinized and acidic soils (Obaid

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et al., 2020). Noteworthy, Uhud Mountain is a dry area with summer temperatures reaching 60°C. It is also known for drought. No extensive studies have been conducted on the mountain which is more likely to harbor new thermophilic microorganisms.

Considering these aspects, the current study aimed to isolate, identify and screen of thermophilic actinomycetes from soil samples collected from the Uhud mountain, Madinah, Saudi Arabia and to investigate their ability to produce antimicrobial agents, commercially valuable enzymes and plant growth promoting compounds.

Materials and methods

Sample collection and processing

Samples of soil were taken from the Uhud mountain, Madinah, Saudi Arabia (24° 31' 18" N; 39° 38' 31.1" E) (*Fig. 1*) at 10-15 cm depth, in June 2021. Samples were transferred to the laboratory after being collected into sterile plastic bags and securely sealed. Subsequently, the samples were dried at 120°C for 60 min in order to eliminate bacteria and fungi.

Figure 1. *The Uhud mountain satellite view provided by Maphill; Google earth (Detailed Satellite Map of the Uhud mountain, 2022), with sampling locations indicated*

Isolation and purification of thermophilic actinomycetes

A serial dilution procedure was used to isolate actinomycetes according to Pandey et al. (2011) on starch casein agar (SCA), a selective medium used for the detection of actinomycetes, containing (g/L): 10 starch, 2 K_2HPO_4, 2 KNO_3, 0.3 casein, 0.05 $MgSO_4 \cdot 7H_2O$, 0.02 $CaCO_3$, 0.01 $FeSO_4 \cdot 7H_2O$ and 20 agar in 1000 mL distilled H_2O, pH 7.0 ± 0.2 (Jafari et al., 2014). Amphotericin B (50µg/ml) and nalidixic acid (50µg/ml) were added to the medium as supplements to inhibit the growth of Gram negative bacteria and fungi. The plates were incubated at 50°C for 6 days. After incubation, colonies (rough, chalky) of actinomycetes were selected and purified for further investigation.

Characterization of actinomycetes isolates

To study colonial characteristics, after inoculating the isolates on SCA, the plates were incubated for 3 days. The color of the substrate mycelium, pigmentation and aerial mycelium of the colony were all recorded.

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Molecular identification of actinomycetes isolates

Genomic DNA was isolated from the tested strain with the PrepManPureLink™ Genomic DNA Mini Kit (Invitrogen) under the guidance of Macrogen in Seoul, South Korea. The 16S rRNA gene was amplified using universal bacterial primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3'). The Macrogen Polymerase Chain Reaction (PCR) system cycler protocol was followed, and Axentm H Taq PCR Master Mix (2X) was employed for the amplification process. The PCR process consisted of a 5-min denaturation step at 94°C, and then 31 cycles of 0.5 min each at 95°C for denaturation, 0.5 min at 57°C for annealing and 1.4 min at 72°C for extension. To sequence the PCR products, an ABI 3730 automated sequencer from Macrogen in Seoul, Korea was utilized. The sequences were compared to the GenBank database of known 16S rRNA gene sequences. This comparison was done using the National Center for Biotechnology Information's BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). Then, the percentage of homology was calculated to identify the bacterial isolates. With MEGA version 4.0, a phylogenetic tree was created using the neighbor-joining technique and Jukes-Cantor distance calculation, with 1,000 bootstrap replicates (Hesham et al., 2014).

Screening for antimicrobial activity of actinomycetes isolates

Two stages were involved in antimicrobial activity screening: primary and secondary screening. The primary screening was performed by cross streak method (Ayed et al., 2021) on starch nitrate agar (SNA) containing (g/L): 20 soluble starch, 2 $NaNO_3$, 1 K_2HPO_4, 0.5 KCl, 0.5 $MgSO_4 \cdot 7H_2O$, 2.0 $CaCO_3$ and 20 agar in 1000 mL dH_2O, pH 7.0 ± 0.2 (Swiontek Brzezinska et al., 2019). After adjusting the turbidity of the tested bacteria (equal to 0.5 McFarland), a sterile cotton swab was streaked perpendicular to the actinomycetes isolate after being dipped into the bacterial solution and incubated at 50°C for 3 days. For assessing antifungal activity, each actinomycetes isolate was spread on SNA and incubated at 50°C for 3 days. A disc of the actinomycetes isolate was prepared and aseptically transferred to sabouraud agar plates (SA) having fresh cultures of fungal isolates. For 48 h, plates were incubated at 28°C (Aghighi et al., 2004).

Secondary screening was done by Nathans agar well diffusion method. The isolates were grown on SN broth to produce bioactive substances in an orbital shaker (140 rpm/min) at 50°C for 30 days. After incubation, cultures broth was filtrated, and the filtrates were used for antimicrobial assays. The test organism was swabbed on nutrient agar (NA) plates (for antibacterial activity) and SA (for antifungal activity). NA and SA were prepared as ready-made manufacturer's direction (HIMEDIA). Wells were filled with 200 μL of the actinomycetes filtrate on the test organism swabbed plates. To test the antibacterial and antifungal activities, the plates were incubated for 24 h at 37°C and 48 h at 28°C, respectively (Nathan et al., 1978).

The inhibition zone was measured in order to assess the antimicrobial activity. Each test was carried out in triplicate. The test bacterial isolates included were *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* and the fungal isolates were *Aspergillus niger, Penicillium* sp, *Alternaria alternate* and *Fusarium solai*. They were obtained from Biology Department, Taibah University, Saudi Arabia. For negative control sample, the tested strains were grown in the same culture medium without inoculation with actinomycetes.

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Primary screening of exoenzymatic activities of actinomycetes isolates

Catalase

A drop of 3% hydrogen peroxide was applied on a microscope slide to perform the catalase test. The actinomycetes colony was contacted with an applicator stick, and the tip was then spread over the hydrogen peroxide drop. The emergence of bubbles because of the presence of catalase enzyme that broke down the hydrogen peroxide into water and oxygen indicated a positive result (Bruno, 2019).

Lipase

The tested isolates were cultured onto minimal basal media MBM containing (g/L): 1 $(NH_4)_2SO_4$, 0.8 K_2HPO_4, 0.2 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.1 $CaCl_2 \cdot 2H_2O$, 0.005 $FeSO_4 \cdot 7H_2O$ and the medium's pH was adjusted to 7.0 ± 0.2 (Hesham et al., 2014). 2% of olive oil (*v/v*) was supplemented to the medium for lipase production. Then, the plates were incubated at 50°C for 4 days. Following incubation, a 20% copper sulphate solution was flowed over the plates, and the emergence of a blue precipitate was monitored (Soleymani et al., 2017).

Amylase

All isolates were individually subjected for primary screening to determine their amylase production capability. Briefly, each isolate was streaked onto SNA plates and incubated for 50°C for 2 days. The plates were soaked with iodine solution after incubation. Amylase positive isolates were identified and recorded based on the clear zone formation around the actinomycetes growth (Dutta et al., 2016).

Protease

Isolates of actinomycetes were cultured on skim milk medium containing (g/L): 50 dry milk instant non-fat, 5 pancreatic digests of casein, 2.5 yeast extract, 1 glucose and 12.5 agar. One liter of H_2O was used to dissolve these ingredients, and the pH was then adjusted to 7.0 ± 0.2. After streaking the plates with the isolates, they were cultured for 2 days at 50°C. Positive results were shown by the creation of a clean zone around actinomycetes colonies (Song et al., 2020).

Keratinase

MBM culture medium was used to measure keratinolytic activity. Pigeon feather and human hair were used as nitrogen sources in the tests. To minimize particle size, pigeon feathers, from a local farm, and hair were washed many times and chopped into tiny bits. Before being used to prepare the culture media, chopped feathers and hair were dried for 24 h at 60°C. To ascertain if these isolates successfully broke down the keratin in hair and feathers, 2 g of starch was added to the culture media as a carbon source, ammonium sulphate was eliminated from the culture media and replaced with feather or hair as nitrogen sources. After sterilization, a disc of the tested isolates was inoculated in 250 mL Erlenmeyer flask with 50 mL of MBM with 0.05 g of feather or hair. The flasks were incubated at 50°C and 130 rpm for 30 days. Using pre-weighed filter paper, the culture broth was filtered after incubation (Macherey-Nagel grade MN 615,125 mm diameter). The filter paper and insoluble particles were dried at 60°C for 24 h, or until the weight remained constant. Based on the difference between the end weight of the

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control (medium with hair or feathers) and the weight of the analytical culture, the percentage of degradation of hair and feathers was computed (González et al., 2020). Additionally, 100 µL from the culture and negative control was spread on a SNA and incubated for 48 h at 50°C.

Determination of lignocellulolytic capability

Palm fronds were collected from a local date farm in Madinah, Saudi Arabia. They were cut into small pieces, washed and dried at 60°C overnight in an oven before use. MBM was prepared and palm fronds were supplemented to culture media. Then, a disc of the tested isolates was inoculated in 250 mL Erlenmeyer flask with 50 mL of MBM and 0.5 g of palm fronds as a sole carbon source. The flasks were incubated for 30 days at 50°C and 130 rpm. After incubation, the culture broth was filtered using pre-weighed filter paper (Macherey-Nagel grade MN 615, 125 mm diameter). The filter paper and insoluble particles were dried at 60°C for 24 h, or until the weight remained constant. The difference between the final weight of the control culture (medium containing palm fronds and without isolate) and the weight of the analytical culture was used to determine the percentage of palm frond degradation. Additionally, 100 µL from the culture and negative control was spread on SNA and incubated for 48 h at 50°C.

Effect of thermophilic actinomycetes on plant growth

The effect of selected actinomycetes isolates on plant growth was investigated using wheat seeds (*Triticum aestivum*) landrace Qasimi. After 10 min of sterilizing the seeds with 5% sodium hypochlorite, they were triple washed with sterilized water. After sterilization, 15 seeds were placed using sterilized forceps on a cotton mesh in sterilized plastic boxes and soaked with 15 ml of 2% concentration of the filtrates of the tested isolates. For negative controls, one box was soaked with SNA and the other one with H_2O. Then, the plastic boxes were covered and after 7 days the following measurements were recorded: biomass of the grown seedlings, radical length, coleoptile length, longest seminal root and number of roots (Sreevidya et al., 2016).

Scanning electron microscopy (SEM)

After 30 days of incubation at 50°C with shaking settings of 130 rpm, samples of hair, feathers, and palm fronds from inoculated and control (actinomycetes-free) cultures were removed and subjected to SEM examination to observe the deterioration. Samples were placed on stubs and gold sputtered. A JEOL JSM 600 scanning electron microscope (Tokyo, Japan) was used to get the images. The scanning electron micrographs were captured at magnification of 50-650x (Kootker et al., 2020).

Statistical analysis

The mean ± standard deviations (SD) were used to represent all values. Using Minitab®19 statistical software, one-way analysis of variance (ANOVA) with multiple comparison tests (Tukey's) was used to assess the data for statistical significance. At $P \leq 0.05$, statistical significance was determined.

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Results

Isolation and characterization of thermophilic actinomycetes isolates

In this study, two thermophilic isolates, designated as MA-1 and MA-2, were purified and used for further analysis. The isolates were characterized on SNA in terms of mycelium morphology and pigmentation. In general, colonies of MA-1 and MA-2 have irregular shape, filamentous margins, and a cretaceous appearance. The color of aerial and substrate mycelia of the isolates was white, however, pigmentation was light brown and grey for MA-1 and MA-2, respectively (*Fig. 2b, d*).

Molecular genetic characterization

The sequences of the 16S rRNA of MA-1 and MA-2 demonstrated a high degree of similarities with *Streptomyces macrosporus* (97%) and *Streptomyces rameus* (98%), respectively (*Fig. 2a, c*). The sequences of 16S rRNA of the strains were determined and submitted to GenBank database with accession numbers OR916389 (MA-1) and OR916416 (MA-2).

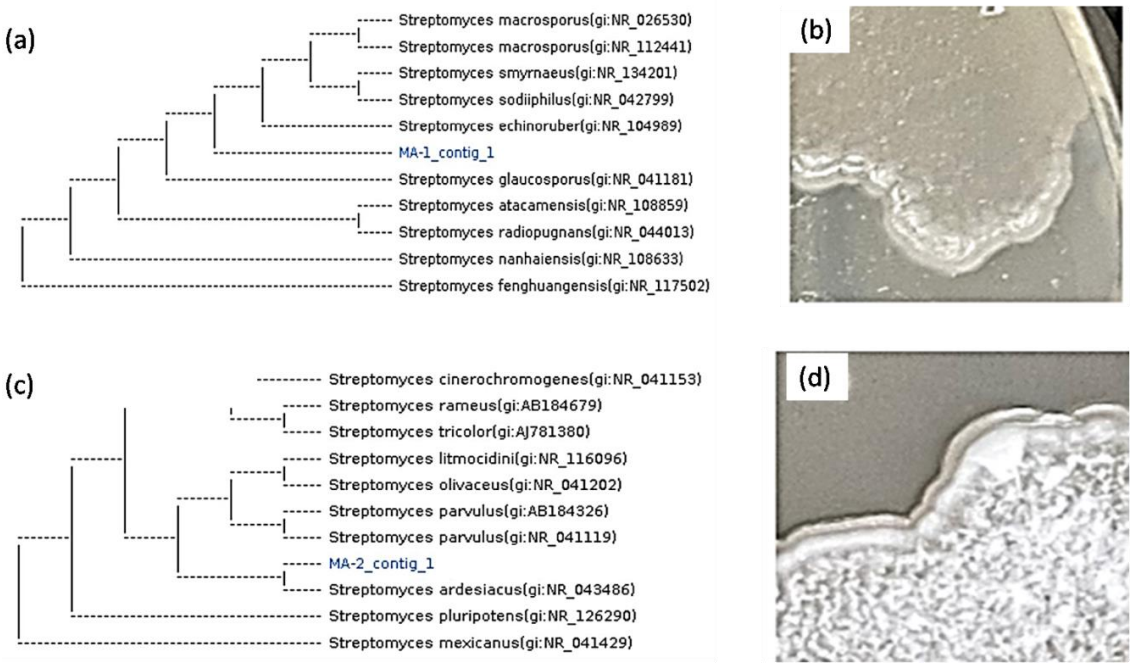

Figure 2. *The maximum likelihood phylogenetic tree constructed based on 16S rRNA gene sequences showing the relationship of MA-1 (a) and MA-2 (c) with the closely related strains within the genus Streptomyces. Cultural characteristics of (b) MA-1 and (d) MA-2 collected from the Uhud mountain, Madinah, Saudi Arabia*

Antibacterial activity of actinomycetes isolates

In primary screening the antibacterial activity against *B. cereus* showed best results with isolates MA-1 and MA-2, with inhibition zones of 16.3 ± 1.5 and 14.7 ± 0.6, respectively. The highest antibacterial activity against *S. aureus* was shown by MA-1 with inhibition zones of 12.0 ± 1.0, while less antibacterial activity was shown with isolate MA-2 with inhibition zone diameter of 10.3 ± 0.6 (*Fig. 3; Table 1*).

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Secondary screening of antibacterial activity showed results against *B. cereus* with MA-1 recorded inhibition zone of 28.3 ± 1.5, followed by MA-2 with inhibition zone of 25.3 ± 1.5. Regarding *S. aureus*, MA-1 exhibited inhibition zone of 21.7 ± 1.2, while MA-2 showed inhibition zone of 25.3 ± 1.5. No antibacterial activity was detected against Gram negative (*P. aeruginosa*) in both primary and secondary screening (*Fig. 3; Table 1*).

Antifungal activity of thermophilic actinomycetes isolates

Antifungal activities of the MA-1 and MA-2 were studied using disc diffusion method on SA against four fungal species: *F. solani*, *A. niger*, *Penicillium* sp and *A. alternata*. MA-2 had antifungal activity against *A. niger* with inhibition zones 31.3 ± 1.2 and 19.7 ± 1.5 in the primary and secondary screening, respectively. MA-2 also inhibited the growth of *A. alternate* with inhibition zones 36.3 ± 1.5 and 22.3 ± 1.2. Growth of *Penicillium* sp also inhibited by MA-2 with inhibition zones of 28.7 ± 1.2 and 26.3 ± 1.5. However, the isolates did not show any antifungal activity against *F. solani* (*Fig. 3; Table 1*).

Table 1. *Antibacterial and antifungal activity of the thermophilic actinomycetes isolates MA-1 and MA-2*

	Zone of inhibition (mm)							
Bacteria	***B. cereus***		***S. aureus***		***P. aeruginosa***			
	MA-1	MA-2	MA-1	MA-2	MA-1	MA-2		
1ry screening	16.3 ± 1.5	14.7 ± 0.6	12.0 ± 1.0	10.3 ± 0.6	0	0		
2ry screening	28.3 ± 1.5	25.3 ± 1.5	21.7 ± 1.2	25.3 ± 1.5	0	0		
Fungi	***A. niger***		***A. alternata***		***Penicillium* sp**		***F. solani***	
	MA-1	MA-2	MA-1	MA-2	MA-1	MA-2	MA-1	MA-2
1ry screening	0	31.3 ± 1.2	0	36.3 ± 1.5	0	28.7 ± 1.2	0	0
2ry screening	0	19.7 ± 1.5	0	22.3 ± 1.2	0	26.3 ± 1.5	0	0

± Standard deviation of the three replicates

Utilization of different wastes by thermophilic actinomycetes isolates

MA-1 and MA-2 had the capability to produce the hydrolytic enzymes tested including protease, amylase, catalase and lipase. In order to perform the keratinolytic assay, feathers or hair were used as the only supply of nitrogen in culture flasks. MA-1 exhibited 21.2% for feather degradation and 18.4% for hair degradation. MA-2 showed 20.8% of feather degradation and 18.7% of hair degradation. MA-1 and MA-2 were able to grow on SNA compared to control. The lignocellulolytic enzymes production was also assessed in culture flasks with palm frond as a sole source of carbon. The difference between the final weight of the control culture (medium with palm frond and without isolates) and the weight of the analytical culture (with isolates and palm frond) was used to calculate the percentage of palm frond degradation. MA-1 was the highest in palm frond degradation followed by MA-2 with a degradation percentage of 49.2% and 46.9%, respectively. MA-1 and MA-2 were able to grow on SNA compared to control (*Fig. 4*).

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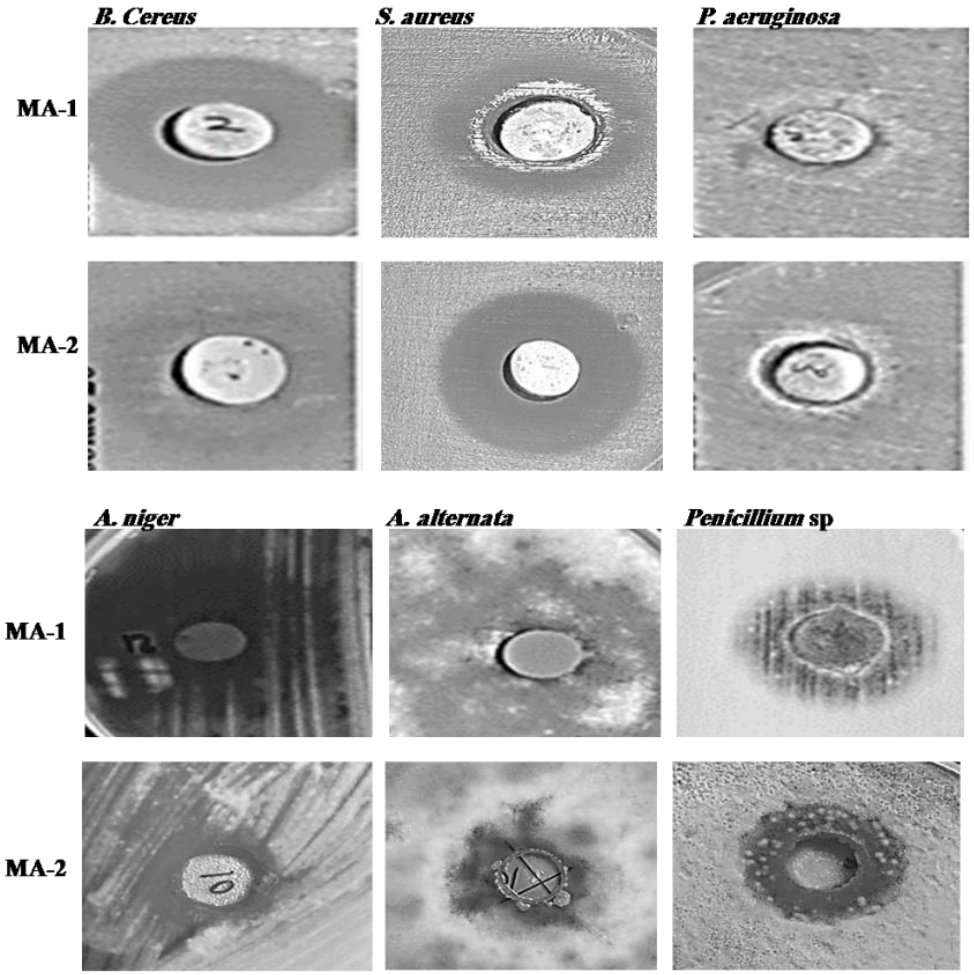

Figure 3. *Antimicrobial screening of the thermophilic actinomycetes isolates MA-1 and MA-2 against pathogenic bacteria and fungi*

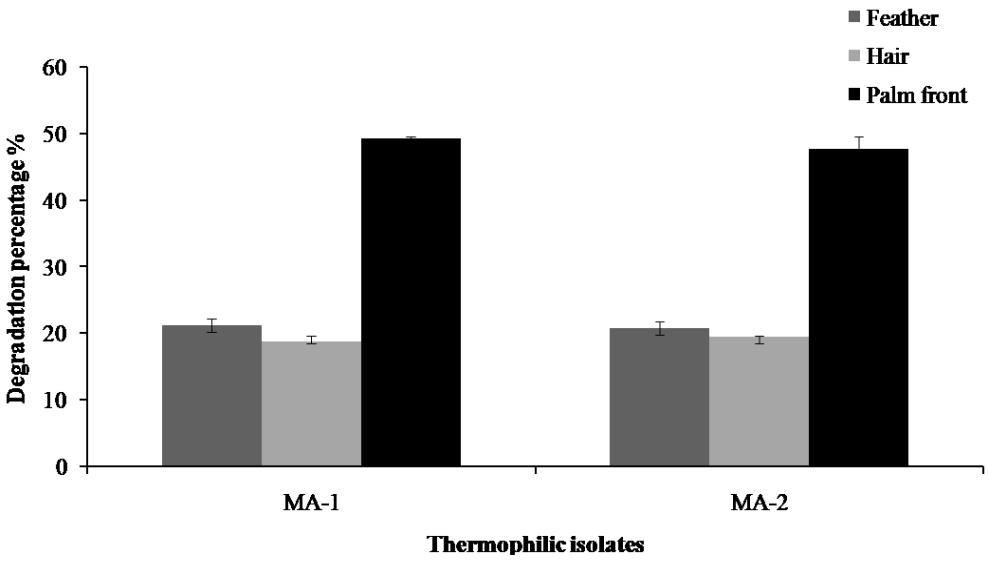

Figure 4. *Degradation percentage of feather, hair and date palm frond due to activity of thermophilic MA-1 and MA-2 after 30 days of incubation at 50°C and 130 rpm*

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SEM images

The results of SEM images showed that both thermophilic isolates exhibited alteration in the morphology of the substrates. Regarding feathers, the results showed that the MA-1 and MA-2 could weaken and crack the feather barbules. The barbules became hyaline, weak and soft when compared to the control sample as illustrated in *Figure 5a*. For hair, the thermophilic MA-1 and MA-2 showed damage in the cortex and medulla (*Fig. 5b*). On the other hand, the cavitation and some pores appeared on the frond of date palm due to degradative enzyme of MA-2.

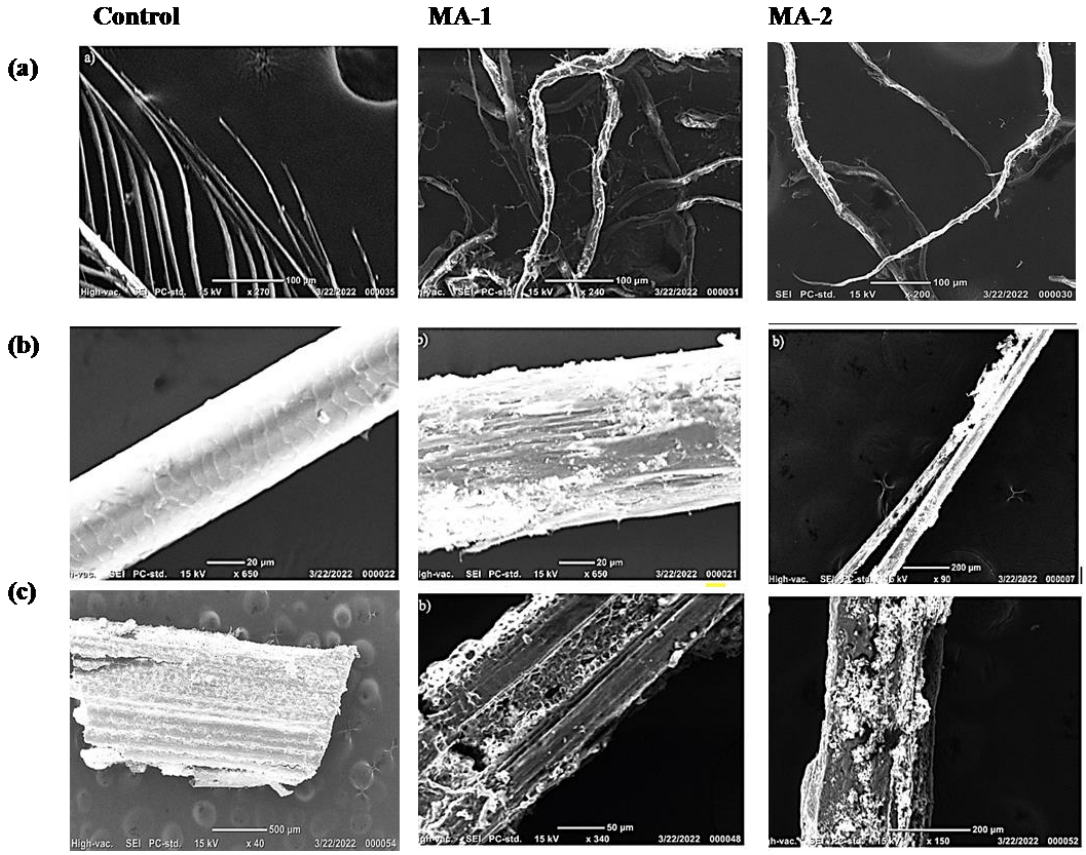

Figure 5. *SEM- images of MA-1 and MA-2 grown on MBM supplemented with feather, hair or palm fronds after 30 days at 50°C. (a); feather, (b); hair (c); palm frond. The control samples were not inculcated with actinomycetes*

Impact of thermophilic actinomycetes on plant growth

The effect of the concentrated culture filtrates of MA-1 and MA-2 on wheat (*Triticum aestivum*) was tested, however, a complete inhibition of grain germination was observed. Therefore, different dilutions of the filtrate were examined, and a 2% concentration which showed the best effect was selected to be tested on wheat growth. The dry biomass of wheat seedlings treated with the different isolates was also evaluated, and the results indicated significant differences ($P \leq 0.05$) between the isolates and the negative controls, MA-1 being the highest followed by isolate MA-2 with a dry biomass of 0.56 ± 0.03 and 0.53 ± 0.04, respectively. The two isolates increased the number of seminal roots significantly ($P \leq 0.05$), with MA-1 having the

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highest number MA-2 with seminal roots number of 6.13 ± 0.8 followed by MA-2 (4.73 ± 0.7). Regarding radical length, coleoptile length and longest seminal root, the isolates had no effect in comparison with the negative controls which showed the best results (*Fig. 6*).

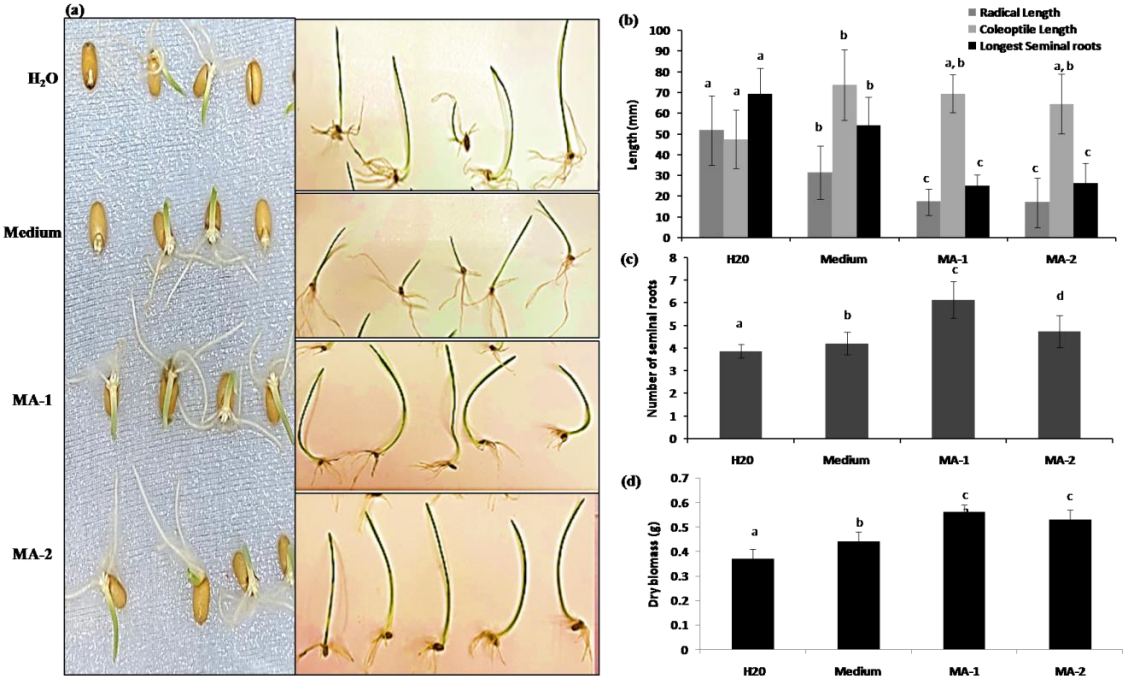

Figure 6. *Impact of culture filtrates (2%) of thermophilic actinomycetes isolates at on wheat seed germination (a) and seedlings (Triticum aestivum) growth parameters (b, c, d). Plotted values are the means of triplicate treatments ± standard deviation of the mean. Means with different letters are significantly different ($P \leq 0.05$)*

Discussion

Much attention has been paid to actinomycetes occurring in harsh habitats due to the production of various natural products and their specialized mechanisms for adaptation to extreme environments (Selim et al., 2021). The main challenge for thermophilic actinomycetes is their ability to survive and produce active molecules at higher temperatures. The Uhud mountain constitutes the appropriate environment to isolate these thermophilic microbes. It is an underexplored region with a high potential for discovering new microbes with novel metabolic properties, isolating these compounds from these unexplored habitats would be useful and cost-effective due to their high thermostability. Moreover, biotechnological processes carried out at elevated temperatures reduce the risk of contamination from common mesophilic bacteria and contribute significantly to bioavailability and solubility. Several novel primary and secondary metabolites have been discovered from extraordinary populations of extremophilic actinomycetes, highlighting the potential value of these microorganisms for the discovery of new natural products. Additionally, the demand for bioactive compounds has increased tremendously in recent times due to the increased incidence of naturally resistant pathogens against existing antibiotics. In relation to the number of expensive antibiotics used to treat pathogens, it is critical to isolate new bioactive

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compounds for therapeutic use. Moreover, it is imperative to develop new anti-microbial agents since many bacteria, viruses, and other microbes lack effective and safe antibiotics (Katti et al., 2022).

Twenty morphologically different thermophilic actinomycetes isolates were isolated from soil samples collected from the Uhud mountain. All the isolates were subjected to preliminary screening for antimicrobial and enzymatic activities, where two potential bioactive strains were selected for further analysis. Significant antibacterial activity against *B. cereus* and *S. aureus* was shown by the two isolates. The growth of Gram negative *P. aeruginosa*, however, was unaffected. These results in accordance with previous studies, which showed that *Streptomyces* spp. had activity against Gram positive bacteria (Thakur et al., 2007; Euanorasetr et al., 2010). The research conducted by Baskaran et al. (2011) revealed that the most significant antagonistic actions were directed against *S. aureus* and *B. subtilis*. A different study found that actinomycetes have antibacterial action against *S. aureus*, but not against *P. aeruginosa* (Selim et al., 2021). They also agreed with the findings of a study conducted by Gozari et al. (2019) in which significant antibacterial efficacy of *Streptomyces* spp. against *P. aeruginosa*, *Vibrio cholera* and *S. aureus* was observed. The structural distinctions between Gram positive and Gram negative bacteria may account for their varying susceptibilities; Gram negative bacteria have an exterior polysaccharide membrane that contains the structural lipopolysaccharide components (Hasani et al., 2014; Gozari et al., 2019). This makes the cell wall impermeable to lipophilic compounds; Conversely, Gram positive bacteria will be more vulnerable since they only have an exterior layer of peptidoglycan, which is insufficient as a permeability barrier (Zgurskaya et al., 2015). Actinomycetes produce glycopeptides, a class of antibiotics that target the cell wall (Yushchuk et al., 2020). They are glycosylated cyclic or polycyclic non-ribosomal peptides. By binding to D-alanyl-D-alanine (D-Ala-D-Ala) within Gram positive bacteria cell walls, glycopeptides prevent the addition of units to the peptidoglycan and inhibit its synthesis (De Simeis and Serra, 2021). Among the most important glycopeptide antibiotics are vancomycin, teicoplanin, and telavancin. Vancomycin is used to treat infections brought on by *S. aureus* strains resistant to methicillin (Binda et al., 2014). The presence of toxigenic *S. aureus* strains in food poses an actual threat to public health (Grasso et al., 2016). The study's findings indicated that the inhibitory potential discovered during primary screening was not the same as that discovered during secondary screening. According to Robinson et al. (2001), a possible explanation might be cultures on solid and liquid media may produce different active secondary metabolites. This might be explained by variations in the inoculum size, substrate composition, and incubation period between submerged fermentation and primary screening.

The isolates showed antifungal activity against *A. nigers*, *A. alternate* and *Penicillium* sp. According to the results, there was no antifungal activity against *F. solani*. Khamna et al. (2009) reported that the crude extract of antifungal compounds produced by actinomycetes was active against *R. stolonifer*, *A. flavus*, *F. oxysporum* and *Alternaria*. Lim et al. (2000) selected 32 actinomycetes isolates, which showed inhibitory activity against mycelial growth of plant pathogenic fungi including *Alternaria mali*, *Colletotrichum gloeosporides*, *F. oxysporum*, *F. cucumerinum*, *Magnaporthe grisea*, *Phytophthora capsici* and *Rhizoctonia solani*. In search for soil actinomycetes having antifungal activity against plant fungal-pathogens, 110 isolates were screened by Aghighi et al. (2004) from which 14 isolates were found active

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against *A. solani, A. alternate, F. solani, Phytophthora megasperma, V. dahlia* and *Sacchromyces cerevisiae*. From 110 soil inhabitant strains that have been isolated from soil samples, 10 strains showed antifungal activity as determined through screening and bioassays by agar disk and well diffusion methods.

As metabolic catalysts, enzymes play a crucial role in a wide range of industries. As a result of the constant research into new enzymes, industrial processes have been improved, which has contributed heavily to the increase in profits. Actinomycetes produce valuable extracellular enzymes capable of decomposing several organic materials. Actinomycetes living in extreme environments are known to produce novel enzymes with great industrial potential (Sheldon and Woodley, 2018). Higher reaction rates, longer half-lives, improved operational stability, a lower chance of contamination during fermentation, and a reduction in viscosity are all shown by thermally stable enzymes (Sharma et al., 2019). Thermostable biocatalysts are greater global requirements than mesophilic biocatalysts (Haki and Rakshit, 2003). The isolates were able to produce lipase. Similarly, Tiwari et al. (2015) reported variable thermophilic actinomycetes isolates from the variable location in India were able to produce many bioactive compounds such as lipases enzyme at elevated temperatures 65°C. According to the results of this study, the isolates were able to produce amylase. This result was with agreement with Salahuddin et al. (2011) who reported the capacity of two thermophilic strains, KS-52 and KS-60 to manufacture α-amylase enzyme by growing on varied quantities of substrates, such soluble starch and maize starch. When soluble starch was present, the activity of α-amylase was at its peak. They were able to produce catalase enzymes. Similarly, two thermophilic actinomycetes isolates from geothermal springs in Armenia capable of producing catalase and oxidase enzymes (Panosyan, 2019). In many biotechnological fields, including bioremediation, catalase has been used as an important enzyme (Kaushal et al., 2018). It is also utilized in food wrappers, where it keeps food from oxidizing. As part of the textile business, catalase eliminates hydrogen peroxide from textiles so that the material is peroxide-free. Several lens cleaning products use hydrogen peroxide to disinfect contact lenses (Onyegeme-Okerenta, 2012). They were tested for keratinolytic activity were able to produce keratin after 30 days of incubation at 50°C. Keratins are the most abundant proteins in mammalian epithelial cells. They are essential components of skin, nails, hair, feathers and wool (Bragulla and Homberger, 2009). Because of their rigid structures, keratins are insoluble and difficult to degrade. As keratins contain a great deal of amino acids and proteins, they have been incorporated into inexpensive animal feeds as feather meal (Qiu et al., 2020). Keratin and keratinase are used in several fields, such as biotechnology, cosmetic industry, pharmaceuticals and medical therapy. The accumulation of excess keratin on the other hand is recognized as a solid waste and a troublesome environmental pollutant (Sypka et al., 2021). One report has shown that *Thermoactinomyces candidus* can digest wool keratin completely. Our findings are consistent with those of Chitte et al. (1999) who found keratinolytic activity in the culture broth of the thermophilic *Streptomyces thermoviolaceus* SD8 that breaks down feathers. For the enzyme to be produced, the ideal pH and temperature were 8 and 55°C, respectively. Up to 65°C and a pH range of 6.5–8.5, the enzyme remained stable. The enzyme may hydrolyze feathers to create leucine, threonine, and tyrosine as well as fibrin, muscle, collagen, nail, and hair.

The isolated thermophilic actinomycetes in this study exhibited protease induction at high temperature. Therefore, these isolates may have various applications in the

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detergent, food, pharmaceutical, leather, chemical reaction and waste treatment industries (Solanki et al., 2021). After growing for 18 h at 55°C, the thermophilic actinomycete *Streptomyces megasporus* SDP4 released several proteases, as described by (Patke and Dey, 1998). The enzyme preparation demonstrated activity between pH 6–12 and 25°C–85°C, respectively, across a wide range of values. Aksoy et al. (2012) reported five *Thermoactinomyces* strains from hot springs and soils of west Anatolia that produce thermostable alkaline protease. Similarly, large concentrations of the enzymes extracellular phosphatase, catalase, tyrosinase, lipase, cellulase, protease, amylase, and L-asparaginase were generated by thermophilic actinomycetes from the Bikaner area of Rajasthan's Thar desert (Begani et al., 2019).

The current MA-1 and MA-2 showed lignocellulolytic activity after 30 days of incubation at 50°C. *Streptomyces thermocarboxydus* strain DF3-3 has been found to degrade lignin or lignocellulose and used them for biofuel production as illustrated by Tan et al. (2022).

The extract of thermophilic actinomycetes in the current study enhanced wheat (*Triticum aestivum*) seedlings growth, mainly increasing number of roots and increased dry biomass. Similarly, Omar et al. (2022) reported that four *Streptomyces* strains isolated from wheat rhizosphere soil caused dry-weight increases in cucumbers and tomatoes. Dicko et al. (2018) examined the impact of actinomycetes that promote plant development on maize growth and yield. *Actinomyces* sp. H7, O19, and AHB12 were chosen as three actinomycetes because of their capacity to generate enzymes, phytohormones, antibacterial compounds, and phosphate-fixing capabilities.

Conclusion

The study's overall findings indicated that the Uhud mountain's unexplored environment may be a potential source of bioactive actinomycetes. The isolated thermophilic actinomycetes may be considered as an essential source of bioactive compound(s). The strains isolated in this study showed good antimicrobial activities and they appeared to have potential agricultural applications. Furthermore, the overproduction of extracellular enzymes suggests that thermophilic actinomycetes from the Uhud mountain may be bioprospected in the future for new, stable, and valuable chemicals and enzymes with industrial and environmental applications.

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