Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 25(6): 49 – 60 (2021)
www.ejabf.journals.ekb.eg

Antimicrobial and antifouling activities of the cellulase produced by marine fungal strain; *Geotrichum candidum* MN638741.1

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ARTICLE INFO
Article History:
Received: Sept. 22, 2021
Accepted: Nov. 19, 2021
Online: Dec. 15, 2021

Keywords:
Antimicrobial;
Antifouling;
ITS-PCR;
Geotrichum candidum;
Marine fungi;
Cellulase enzyme.

ABSTRACT

Fungi are one of the most important groups of marine microorganisms used to produce important enzymes and antimicrobials. Industrially, the cellulase produced from marine fungi plays an impotent role; however, using it medically is very limited. Hence, this study aimed to use purified and characterized cellulase enzymes produced from *Geotrichum candidum* strain Gad1 to determine their antimicrobial and antifouling activities. The maximum cellulase activity was achieved (55.54 U/ml) with maximum specific activities (325.8 U/mg). The highest antimicrobial activity of cellulase was 42 mm diameter of zone inhibition found against *Vibrio damsel* as antibacterial, and 19 mm diameter of zone inhibition observed against *Aspergillus niger* as an antifungal. By using 600 ul/l of cellulase, the high reduction of the bacterial load in the formed biofilm was shown. The data obtained during the present study confirmed the potential of fungal cellulase. The cellulase enzyme produced by *G. candidum* strain Gad1 demonstrated promising antibacterial, antifungal and antifouling properties.

INTRODUCTION

The fungal community has an amazing effect on the environment and forms a radical source of the ecosystem. It has various functions; one of which is the beneficial antimicrobial activity **(Suleiman, 2020).** In addition, fungi acts as a biocontrol agent **(Suleiman *et al.*, 2019)** and sometimes share in biodiesel production **(Hashem *et al.*, 2020)**. Moreover, they help in the poly unsaturated fatty acid and oil productions **(Hashem *et al.*, 2020; Hashem *et al.*, 2021)**. Furthermore, the fungi aid in the biodegradation of PAHs **(Abdel-Razek *et al.*, 2020)**. On the other hand, the fungi have an antifouling or harmful activity or such as the pathogenesis of fungi, which cause numerous diseases, whether for human, plants or animals. One of the most important groups of marine microorganisms used as sources of producing important enzymes and antimicrobials is the Fungi. Cellulase produced from marine fungi plays an impotent role industrially; however, using the cellulase medically is very little. Due to the prevalence of

antibiotic resistance **(Shawky *et al.*, 2021)**, natural products **(Kamel *et al.*, 2022)** as well as the enzymes can be used as antimicrobials. This would disrupt the microbial cellular machinery **(Thallinger *et al.*, 2013)**. In nature, the anti-bacterial enzymes play an essential role to protect living organisms from microbial attacks **(Sharma & Khan, 2019)**.

Adhesion of fouling microorganisms to the surfaces are the sources of the life problems, especially in submarine hulls and ship **(Uzun *et al.*, 2021)**. Numerous governments and companies spend yearly more than 6 billion dollars to prevent marine biofouling **(Rouhi, 1998)**. The felicitous combination of enzymes with coatings, showing surfaces with the broad antifouling agent and long-range efficacy is considered a challenge **(Cordeiro & Werner, 2011)**.

The current study targeted three main goals. Firstly, the examination of antimicrobial activity by cellulase enzyme produced by marine fungi *Geotrichum candidum* strain Gad1. Secondly, isolation of marine fungi by marine samples isolated from the Egyptian Mediterranean Sea (Alexandria coastline), and subsequently identified depending on routine morphological identification in addition to ITS technique. Those fungi were used as pathogenic fungi during the antifungal examination. Thirdly and lastly, the examination of antifouling activity by cellulase enzyme produced by *Geotrichum candidum* strain Gad1.

MATERIALS AND METHODS

Screening of cellulase producing fungal isolates

In our previous study **(Gad *et al*., 2021)**, *Geotrichum candidum* strain Gad1 was isolated from marine source (sediments) found in the Egyptian Mediterranean Sea (Alexandria coastline), and subsequently identified depending on the routine morphological identification in addition to ITS technique. Additionally, in our previous study we purified and characterized the cellulase produced by *Geotrichum candidum* strain Gad1 **(Gad *et al*., 2022)**.

Estimation of total protein

In order to measure the antimicrobial activities of purified cellulase enzyme, the extracellular protein was estimated following the method of **Lowry *et al.* (1951)** and using bovine serum albumin as a standard protein. Afterwards, 200 U of purified cellulase was mixed with one ml of alkaline solution. After 10 min, one hundred microliters of diluted folin-ciocalteau reagent was added. After twenty min, the color developed was measured at 750 nm by using the spectrophotometer.

Cellulase activity assay

In order to measure cellulase activity, the reaction was prepared according to the dinitrosalicylic acid method (DNS). The purified cellulase (0.5 ml) and 0.5 ml of 2% carboxy methyl cellulose salt (CMC) substrate concentration were incubated at 45°C for 105 minutes. After incubation, one ml of DNS acid was added to the solution, boiled for

ten minutes and cooled for approximately ten minutes at room temperature to be read with a spectrophotometer at λ 570 nm. One unit (IU) of cellulase activity was defined as the amount of cellulase releasing 1.0 uM of reducing sugar per minute.

Isolation and identification of pathogenic fungi

Morphological characterization and microscopic examination

Based on the macroscopic and microscopic examinations, the cultures of pathogenic were identified, following the arrangement of taxonomic proposed in the sixth edition book of Ainsworth and Bisby's dictionary of the fungi **(Anisworth, 1971)**.

Molecular identification

DNAs of the pathogenic fungal isolates were extracted and the PCR amplifications were carried out using the primers ITS1 (**5`** TCC GTA GGT GAA CCT GCG G **3`**) and ITS4 (**5`** TCC TCC GCT TAT TGA TAT GC **3`**) **(White *et al.*, 1990)**. Amplified DNA was purified using purification Kit (K-3034-1, Bioneer Corporation, South Korea). The PCR products were purified, and Sanger sequencing with PCR primers was performed at Macrogen Incorporation (South Korea). The sequences were assembled using the DNA STAR SeqMan (DNA STAR, Wisconsin, USA). Firstly, generated sequences were submitted to the GenBank database. Sequence analyses were obtained by BLASTN similarity search at (http://www.ncbi.nlm.nih.gov/BLAST) and were secondly multiply aligned, and the phylogenetic tree was constructed by maximum-likelihood method using MEGA_X_10.1.6 software.

Antimicrobial activities of characterized cellulase

Microbial pathogens

Antibacterial activity was determined against Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Klebsilla pnuumenia* ATCC 13883, *vibrio damsela*, *Salmonella typhi*, *Pseudomonas flurescence* DCM 50090, *Aeromonas hydrophila* NRRL 914, and *Salmonella typhi*) and Gram-positive bacteria (*Bacillus subtillis* ATCC 6633, *streptococcus agalactiae* CCM 6187, *Staphylococcus aureus* ATCC 2592, and *Entrococcus faecalis* ATCC 29212). These pathogens were kindly provided by the Microbiology Lab and Marine Biotechnology Lab collections at the National Institute of Oceanography and Fisheries (NIOF), Egypt.

Antibacterial activity

The biological properties were performed using an agar well diffusion method. Antimicrobial activities of cellulase were performed against both Gram-negative bacteria (*E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *K. pneumonia* ATCC 13883, *V. umiga*, *S. Typhi, P. flurescence* DCM 50090, and *A. hydrophila* NRRL 914) and Gram-positive bacteria (*B. subtillis* ATCC 6633, *S. agalactiae* CCM 6187, *S. aureus* ATCC 2592, and *E. faecalis* ATCC 29212). The antibacterial activity was done by modified Kirby-Bauer

well diffusion test. Briefly, the pure cultures of the pathogenic bacteria were subcultured in nutrient broth (Oxoid Co., UK) at 37±2°C on a rotary shaker at 120 rpm. Afterwards, 100 ml fresh culture of nutrient broth containing pathogenic bacteria from each organism was prepared by spreading on nutrient agar plates with the help of a sterile L-rod spreader. After 15 min of inoculation, plates were left aside to permit the absobtion of the culture. Using a micropipette, 50, 75, and 100 U of the cellulase was poured onto each well (8 ±1 mm wells were punched into the plate of nutrient agar) on all plates. After an overnight incubation at 37±2°C, the different levels of zone of inhibition were measured. Ciprofloxacin was used as control **(Soliman *et al.*, 2021; Suleiman & Helal 2022)**.

Antifungal activity

For the antifungal assay, 100 µl fresh culture pathogenic fungi were inoculated in the center of sterile petri-dishes, and after that the potato dextrose agar medium (PDA) **(Baka *et al.*, 2015)** was poured into inoculated petri-dishes. Approximately, 25 µl of different concentrations (100 µg/disc, 75 µg/disc, and 50 µg/disc) of cellulase enzyme were immersed with sterile filter paper discs (Whatman 6 mm: No.1). Zone of inhibition was observed and measured after 72 hr of incubation at room temperature (30°C). The inhibition zone was observed and compared to negative and positive control (Amphotericin B, 100 units/disc) **(Gad *et al.*, 2016)**.

Antifouling activity

Antifouling activity was estimated according to the method of **Kumaran *et al.* (2011)**. Briefly, 100 ml of seawater was mixed separately with the enzyme in a conical flask containing cover glass and incubated overnight at 30°C followed by dying with 0.4% crystal violet solution for 10 minutes. It was then washed with water, left to dry at room temperature, and checked under the microscope. One flask without enzyme was kept as control **(Amer *et al.*, 2019)**.

RESULTS AND DISCUSSION

1. Cellulase production and protein assay

The cellulase enzyme achieved maximum activity with 2% CMC salt and pH of 7.0 at 45°C after 105 minutes of incubation using *Geotrichum candidum* strain Gad1 presented in Table (1). Previous studies reported that fungal species have the ability to produce high cellulase enzyme activity **(Al-kharousi *et al.*, 2015; Marco *et al.*, 2017; Pachauri *et al.*, 2017**) using *Trichoderma longibrachiatum*, *Aspergillus flavus* (TISTR 3637) and *Chaetomium* sp., respectively. The maximum cellulase activity was achieved (55.54 U/ml) with maximum specific activities (325.8 U/mg). This result is higher than that (38.25 U/ml) of **Nehad *et al.* (2019)** who used *Penicillium decumbens*. While, it was lower than that (103.67 U/mL) obtained in the study of **Potprommanee *et al.* (2017)** using *Geobacillus* sp. HTA426.

Table 1: Values of cellulase activity and protein assay produced by *Geotrichum candidum* strain Gad1

Parameter	Value
Cellulase activity (U/ml)	55.4 ± 0.82
Protein assay (mg/ml)	0.17 ± 0.06
Specific activity (U/mg)	325.8

2. Antimicrobial activities of cellulase

2.1. Antibacterial Activity

The antibacterial activities of cellulase and standard ciprofloxacin were determined against both Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Klebsilla pneumonia* ATCC 13883, *vibrio damsela*, *Salmonella typhi*, *Pseudomonas fluorescence* DCM 50090, and *Aeromonas hydrophilia* NRRL 914) and Gram-positive bacteria (*Bacillus subtillis* ATCC 6633, *streptococcus agalactiae* CCM 6187, *Staphylococcus aureus* ATCC 2592, and *Enterococcus faecalis* ATCC 29212).

The results of the antibacterial activity of cellulase enzyme are presented in Table (3). The highest activity of cellulase was 42 mm diameter of zone inhibition found against *vibrio damsela* followed by 28 mm diameter of zone inhibition against *Enterococcus faecalis*. On the left hand, the lowest activity of cellulase was 15 mm diameter of zone inhibition observed against *Staphylococcus aureus*, *Escherichia coli*, *Klebsilla pneumonia* and *Pseudomonas fluorescence*, in comparison with the reference standard ciprofloxacin (5 µg/disc). The antibacterial activities of the cellulase are linear with the increase in the concentration of cellulase (µg/well). Therefore, it is possible to apply cellulase to control pathogens which cause fatal diseases via breaking off the cyst wall and controlling the pathogen **(Jayasekara & Ratnayake, 2019)**. A previous study reported that cellulase enzyme produced from *Coptotermes ceylonicus* has antibacterial activity against *Escherichia coli*, but did not show any activity against *B. subtilis* **(Lucky *et al.*, 2021**).

2.2. Antifungal Activity

- **Identification of pathogenic marine fungal isolates**

Four fungal isolates were identified based on macroscopic, microscopic (Fig. 1), and molecular identification as *Aspergillus terreus* GAD12, *Aspergillus niger* GAD13, *Aspergillus fumigatus* GAD14, *Aspergillus cristatus* GAD. The sequence was submitted to Gen Bank and the accession number was provided as MN638752.1, MN638753.1, MN638754.1 and MN871428.1, respectively (Table 2).

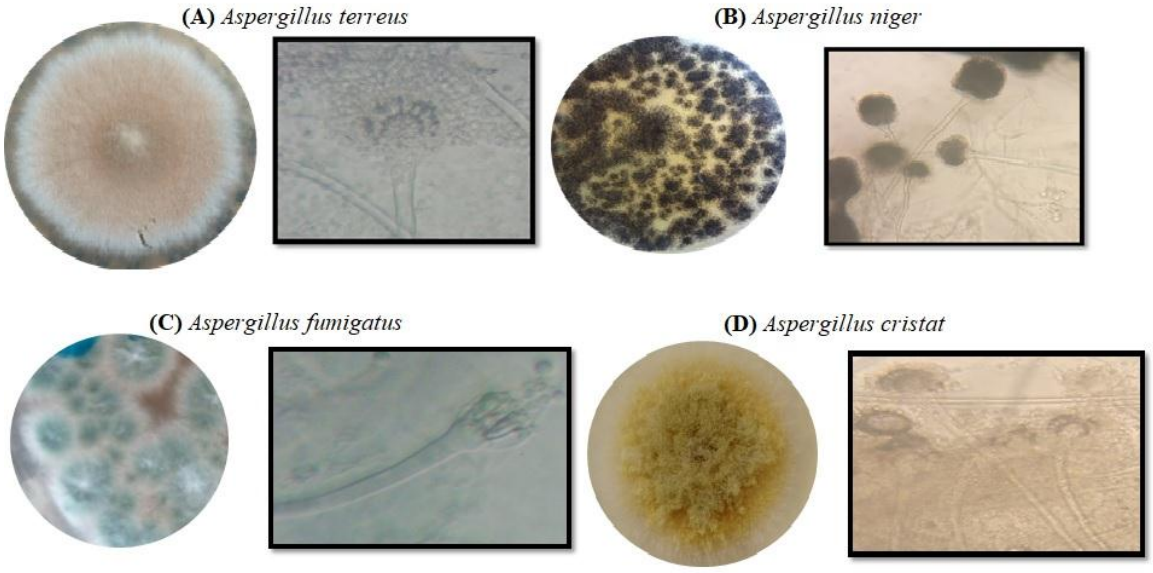

Fig. 1. Macrograph showing the macroscopic (left) and microscopic (right) of (A*) Aspergillus terreus*,(B) *Aspergillus niger*, (C) *Aspergillus fumigatus*, and (D) *Aspergillus cristatus.*

Table 2. Highest identity sequence from GenBank and closest neighboring organism using ITS sequence data

Highest identity sequence from GenBank			**Closest neighboring organism**
Accession number	**Partial ITS sequence (**bases**)**	**Identity (%)**	
MN638752.1	554	97.11	*Aspergillus terreus*
MN638753.1	590	99.49	*Aspergillus niger*
MN638754.1	622	100	*Aspergillus fumigatus*
MN871428.1	513	99.42	*Aspergillus cristatus*

The obtained sequence showed 97.11%, 99.4%, 100%, and 99.42% similarity with *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Aspergillus cristatus*, respectively (Fig. 2). *Aspergillus* is a genus of asexual fungi that produces compounds as enzymes and secondary metabolites; however, some *Aspergillus* fungi also cause numerous diseases to humans and lead to death as Aspergillosis **(Thornton, 2020)**. **Fernandez *et al.*** (**2021**) reported that, aspergillosis may be an important complication of coronavirus disease 2019 (COVID-19).

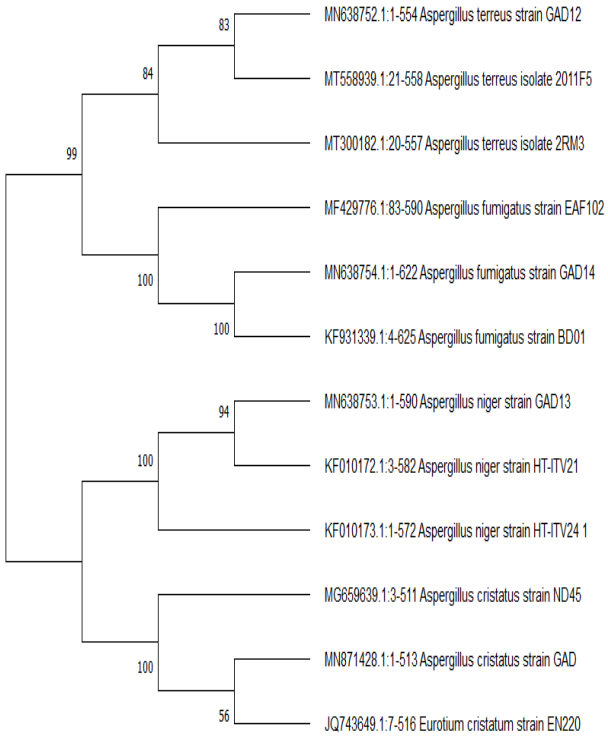

Fig. 2. Phylogenic tree of marine pathogenic fungi using ITS sequence data

The antifungal activities of cellulase and standard amphotericin B (100 U/well) were determined against four pathogenic fungi (Table 3). The highest recorded activity was 19 mm diameter of zone inhibition observed against *Aspergillus niger*, followed by 18 mm diameter of zone inhibition against *Aspergillus terreus* and *Aspergillus cristatus*.

Whereas, on the left hand, the lowest activity was 11 mm diameter of zone inhibition, and it was found against *Aspergillus fumigatus*. Overall, the cellulase showed significant activity against all the tested pathogenic fungi. The antifungal activities of the cellulase were linear with the increase in the concentration of cellulase (μg/well). The variable degrees against all the tested pathogenic fungi depended on the microbial species **(Gad *et al.*, 2016)**. Positive results of lysis pathogenic fungi with cellulase activity have been previously reported **(Downer *et al.*, 2001)**. Mechanism of suppression pathogenic fungi has occurred by lysis of the enzyme to sporangial and hyphal walls **(Richter *et al.*, 2011)**.

Table 3: Antibacterial and antifungal activities of cellulase enzyme, mean zone of inhibition (mm) at 30°C

Microorganism	Purified cellulase concentration of the well (µg /well)			CIP (100 U/disc)	AMP
	100	75	50		
Staphylococcus aureus	18± 0.05	17 ± 0.1	15± 0.1	27 ± 0.1	ND
Escherichia coli	18 ± 0.12	16 ± 0.1	15± 0.09	27 ± 0.12	ND
Pseudomonas aeruginosa	-	-	-	26 ± 0.05	ND
Bacillus subtilis	-	-	-	25 ± 0.04	ND
Enterococcus faecalis	28 ± 0.07	22 ± 0.09	19± 0.07	27 ± 0.13	ND
Klebsiella pneumoniae	20 ± 0.1	17 ± 0.06	15± 0.14	27 ± 0.06	ND
Vibrio damsela,	42 ± 0.13	38 ± 0.07	35± 0.09	27 ± 0.05	ND
Salmonella typhi	26 ± 0.12	21 ± 0.06	18± 0.08	27 ± 0.05	ND
Pseudomonas flurescence	19 ± 0.09	17 ± 0.07	15± 0.08	27 ± 0.1	ND
streptococcus agalactiae	21 ± 0.07	18 ± 0.13	16± 0.1	27 ± 0.1	ND
Aeromonas hydrophila	25 ± 0.1	22 ± 0.12	18± 0.07	27 ± 0.1	ND
Aspergillus terreus	18 ± 0.09	16 ± 0.1	14± 0.1	ND	16 ± 0.08
Aspergillus niger	19 ± 0.08	17 ± 0.07	15± 0.05	ND	15 ± 0.05
Aspergillus fumigatus	15 ± 0.05	13 ± 0.08	11± 0.08	ND	16 ± 0.09
Aspergillus cristatus	18 ± 0.07	16 ± 0.1	13 ± 0.07	ND	16 ± 0.07

CIP: ciprofloxacin antibacterial standard; AMP: amphotericin-B antifungal standard; ND: Not determined.

3. Antifouling activity

Collectively, the antifouling activity of the produced cellulase by *Geotrichum candidum* strain Gad1 is presented in Fig. (3). However, data in Fig. (3C) show the high reduction of the bacterial load in the formed biofilm by the action of cellulase, while Fig. (3B) shows less reduction compared to the control (biofilm formed without addition of the enzyme, Fig. 3A).

Knowingly or unknowingly, pathogenic microorganisms usually form biofilms by secreting extracellular compounds that are composed of 1,3- or 1,4-β-linked hexose residues and found in cellulose polymer. Usually, they are found on a wide range of surfaces, including living tissues and indwelling medical devices. Therefore, cellulases can be further studied for their efficient application in removing this type of biofilms from medical devices **(Jayasekara & Ratnayake, 2019)**.

The antimicrobial properties of cellulase allow it to exhibit an antifouling activity because the fouling phenomenon starts initially by forming a bacterial film on the substratum. Actually, the previous investigations performed to detect the efficacy of the fungal cellulase against biofilm and/or microbial fouling are rather few. Consequently, the current study can be considered a valuable work investigating the antifouling the effect of the cellulase produced by *G. candidum* strain Gad1.

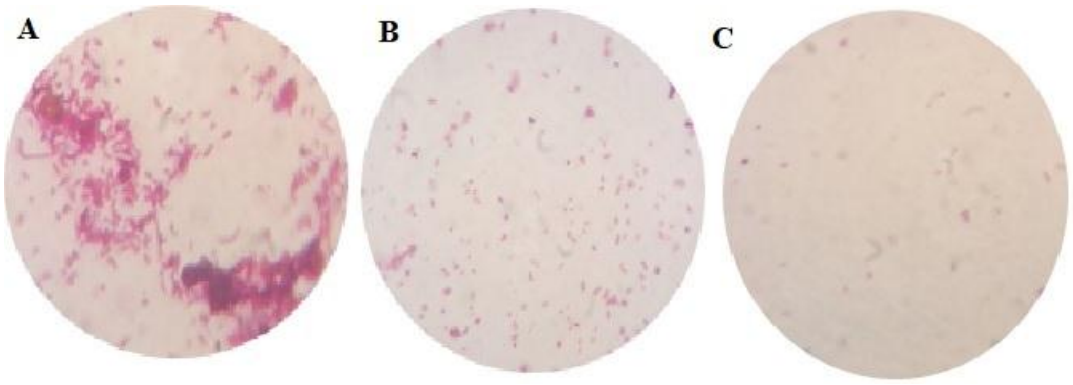

Fig. 3. Macrographs illustrating the antifouling effect of the cellulase produced by *G. candidum* strain Gad1 using 600 ul/l (C), 300 ul/l (B), and the control (A).

CONCLUSION

The current progress in microbial cellulase applications is truly remarkable and is attracting worldwide attention. It has already conquered the global market in an unbeatable way. Advances in emerging fields such as biotechnology, microbiology, and molecular biology will open up novel strategies to magnify the still-unlocked potential of these enzymes. In addition, a large number of approaches have been employed in recent decades to detect anti-infective substances as antifouling or antimicrobial, on the basis of their different modes of action over microorganisms rather than a different end effect that remains, in both cases, the prevention of biofilm formation. On the other hand, the data obtained during the present study confirmed the potential of fungal cellulase in such a manner. The cellulase enzyme produced by *G. candidum* strain Gad1 demonstrated promising antibacterial, antifungal, and antifouling properties.

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