RESEARCH ARTICLE

Isolation, Characterization, and Metabolic Profiling of *Ceratorhiza hydrophila* from the Aquatic Plant *Myriophyllum spicatum*

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ABSTRACT

The goal of the present study was to investigate the antibacterial properties, enzyme production, and metabolic profiling of a new *Ceratorhiza hydrophila* strain isolated from the submerged aquatic plant *Myriophyllum spicatum*. Furthermore, the fungus' morphological characterization and DNA sequencing have been described. The fungus has been identified and submitted to the GenBank as *Ceratorhiza hydrophila* isolate EG19 and the fungus ID is MK387081. The enzyme analyses showed its ability to produce protease and cellulase enzymes. According to the CSLI standard, the ethyl acetate extract of *C. hydrophila* showed intermediate antibacterial activity against *Streptococcus pneumonia*, *Micrococcus luteus*, and *Staphylococcus aureus*. Metabolic profiling has been carried out using 700 MHz NMR spectroscopy. Based on the ^{1}H and ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) NMR data and NMR databases, 23 compounds have been identified. The identified metabolites include 31% amino acids, 9% sugars, 9% amines, 4% sugar alcohols, and 4% alkaloids. This is the first report for the metabolic characterization of *C. hydrophila*, which gave preliminary information about the fungus. It is expected that our findings not only will pave the way to other perspectives in enormous applications using *C. hydrophila* as a new promising source of antimicrobial agents and essential metabolites, but also it will be valuable in the classification and chemotaxonomy of the species.

1. Introduction

Aquatic habitat is a rich environment for the growth of huge number of microorganisms. *Myriophyllum spicatum* L. (F. Haloragaceae) is a submersed aquatic plant that grows in both fresh and brackish water. In weedy situations, it is fast growing, forming dense mats of foliage that interfere with the normal usage of water courses [1]. It was believed that *M. spicatum* was introduced to Egypt by migratory birds [2]. The first record of *M. spicatum* in Egypt was found in Lake Qarun [3]. The introduction of *M. spicatum* to the Delta was detected and started to spread during the past few decades [4]. *M. spicatum* has a key role in producing oxygen, stability of sediments, cycling of nutrition, and providing shelter and habitat for aquatic life [5]. In addition, there are extensive studies which show the use of *M. spicatum* as suitable sorbents of heavy metals from sediment and water. *M. spicatum* plant species may be a useful vehicle for absorbing heavy elements from nutrient-rich sediments [5].

Ceratorhiza hydrophila (*Sclerotium hydrophilum*) is an anamorphic basidiomycetous species that belongs to the family Ceratobasidiaceae. It is a phytopathogen that infects aquatic or semi-aquatic plants of wet meadows and marshes [6]. The fungus has been reported in different areas of the world, including Northeast China [7], Myanmar [8], and in southeastern Australia [9]. It does not produce asexual or sexual spores but produces small sclerotia on the vegetative mycelium that begin white in color, then upon maturation, become darkened [10]. Morphologically, it has a well-defined dolipore septum [11]. The fungus has been reported to infect a wide variety of plants including, *Zizania aquatica* L. [11], *Oryza sativa* L. [12], and *Nymphaea odorata* Aiton [13]. In addition, it was isolated from *Myriophyllum spicatum* L. (or Eurasian water milfoil) in the Afritzer See Lake of Carinthia in Austria [14]. In the past, *Ceratorhiza hydrophila* was called *S. hydrophilum* based on the phenotypes. Recently, based on rDNA analysis and morphology, *S. hydrophilum* was transferred to the genus *Ceratorhiza*, which is the anamorph of the *Ceratobasidium* species [15]. Although, there are no available data about the biotechnological potential of *Ceratorhiza hydrophila*, other related basidiomycetous species showed the ability to produce various metabolic

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products. *Rhizocotonia solani* was found to produce various metabolites including amino acids, fatty acids, sugar alcohols, monosaccharides and carboxylic acids [16]. In addition, *Diplodia zeae* produced many pectolytic and cellulolytic enzymes [17].

Nowadays, it is important to discover new and alternative sources for active metabolites needed in various application fields, with the continuous need for alternatives to the commercial use products. So, in this research, we describe the isolation of *C. hydrophila* from *M. spicatum* from the shore of the river Nile in Helwan, Cairo, Egypt. To our knowledge this is the first report of *C. hydrophila* isolated from this plant in Egypt. Morphological and molecular characterizations of the fungus have been studied. Furthermore, we study its antibacterial activity. We also describe the metabolic profiling of the polar extract of the *C. hydrophila*.

2. Materials and methods

2.1. Plant material

The submerged aquatic plant *Myriophyllum spicatum* was collected from the banks of the river Nile in Helwan, Cairo, Egypt and authenticated by Prof. Tarek Galal, Professor of Plant taxonomy, Botany and Microbiology Dept. Helwan University.

2.2. Fungus isolation

For fungus isolation, the plant was washed gently under tap water. Surface sterilization was carried out using 70% ethanol for 1 min, then washed with sterile distilled water for 15 min. The stems and leaves of the *Myriophyllum spicatum* plant were cut into cubes (1 cm) and then were incubated on wet sterilized filter paper in 15 cm diameter Petri dishes at 25 °C for five to seven days [18]. The appearance of white like crystals on the surface of the plant parts indicated the growth of the fungus which was further isolated and incubated for a week at 25 °C on potato dextrose agar plates. The fungus was purified and kept on potato dextrose agar slants at 4 °C.

2.3. Fungus identification

2.3.1. Morphological identification

The morphological characteristics of the fungus were studied by observing the color and texture of the colonies, as well as the color and shape of the sclerotia produced. Half cm diameter agar disk from 7-day-old fungal culture inoculated at the center of 9 cm diameter Petri dish using different culture media including Czapek-dox agar [19], Potato dextrose agar (PDA) [20], and Malt agar [21], then incubated for 7–10 days at 25 °C, all the used

chemicals were purchased from (Sigma-Aldrich, St. Louis, MO, USA). Colony texture, the shape, and color of sclerotia were examined with the visual inspection and recorded after four to six days of incubation. Moreover, the fungus' microscopic characterization, including branching patterns and septation of mycelium, was also examined under a light microscope.

2.3.2. Molecular identification

DNA isolation. The fungus was cultured for seven days onto potato dextrose broth medium. The mycelium was separated and ground and the total genomic DNA was isolated from the fungal mycelium according to [22]. The isolated DNA was resolved on 1% agarose gel (Sigma-Aldrich) prepared in 1 X TAE (Tris-acetate-ethylenediaminetetra acetic acid) buffer containing $0.5 \mu g \ ml^{-1}$ ethidium bromide (Sigma-Aldrich). Ethidium bromide-stained gel was visualized using a UV-transilluminator (Vilber Lourmat, Collégien, France).

PCR reaction and sequencing. Polymerase chain reaction was performed using Mytaq Red DNA polymerase master mix (BIOLINE Cat. No. BIO-21108, London, UK) according to the manufacturer's instructions. Briefly, the reaction contained 1 X PCR red master mix buffer, $2.0 \mu l$ of $10 \text{ pmol } \mu l^{-1}$ of ITS1 and ITS4 primers which were designed according to White et al. [23], $1.0 \mu l$ of DNA template ($\sim 30 \text{ ng}$), and $0.25 \mu l$ of MyTaq^{TM} DNA polymerase ($5 \text{ U} \mu l^{-1}$). The total volume was adjusted to $50 \mu l$ using sterile distilled, demineralized water. The amplification reactions were performed using a Thermal Cycler (Biometra, Göttingen, Germany) as follows: 1st cycle of 3 min at 95 °C for initial denaturation, followed by 35 cycles of 20 s at 95 °C (denaturation), 20 s at 55 °C (annealing), 30 s at 72 °C (extension), and then a final extension was carried out for 10 min at 72 °C, after which the reaction was held at 4 °C. The PCR products were separated on 1% agarose gel. The PCR products were purified using PCR-M clean up system (VIOGENE Cat. No. PF1001, Taipei, Taiwan) according to the manufacturer's protocol. The purified samples were sequenced by GATC Company using ABI 3730xl DNA sequencer and ITS4 primer.

Sequencing data analysis. Blast was performed for the obtained nucleotide sequences using Geneious pro software 11.1.5, where the retrieved sequence from NCBI that has high similarity was aligned using MUSCLE tools and the phylogenetic tree was built using maximum likelihood methods [24,25].

2.4. Enzyme production

C. hydrophila was investigated for the production of various industrial enzymes, including protease, asparaginase, cellulase, and amylase. All media were inoculated with a 0.5 cm fungal disk from 7 days old fungal culture and incubated at 25 °C for 72 h. All measurements were made in triplicates.

2.4.1. Protease

Analysis of proteolytic activity was determined using gelatin agar medium [26] containing g l^{-1}: 20.0 gelatin; 20.0 agar in 0.2 M phosphate buffer pH = 7. Protease production was shown by a clear zone formation after the addition of acidic mercuric chloride solution [27].

2.4.2. Asparaginase

Modified Czapek Dox medium [28] was prepared, containing g l^{-1}: 6.0 Na_2HPO_4; 2.0 KH_2PO4; 0.5 NaCl; 0.2 $MgSO_4.7H_2O$; 0.005 $CaCl_2.2H_2O$; 20.0 L-asparagine in dist. H_2O adjusted to pH 5.5. This medium was supplemented with 2% agar and 0.009% phenol red (Sigma-Aldrich). A change in the color of the medium from yellow to red indicated asparaginase production.

2.4.3. Cellulase

The ability to produce cellulase was tested by using cellulose medium [29], containing g l^{-1}: 1.0 carboxymethylcellulose; 0.5 $NaNO_3$; 1.0 K_2HPO_4; 0.5 $MgSO_4 \cdot 7H_2O$; 0.001 $FeSO_4 \cdot 7H_2O$; 1.0 yeast extract; 15.0 agar (Sigma-Aldrich). After incubation, plates were flooded with 0.2 g l^{-1} potassium iodide for 5 min. Clear zone formation indicated cellulase production.

2.4.4. Amylase

Amylase activity was assayed using starch agar medium [30], containing g l^{-1}: 2.0 starch; 1.0 peptone; 20.0 agar (Sigma-Aldrich). After incubation, the plates were flooded with 0.1 g l^{-1} potassium iodide solution. Clear zone formation indicated amylase production.

2.5. Assessment of antibacterial activity

2.5.1. Preparation of crude fungal extracts

For preparation of crude fungal extracts, *C. hydrophila* was cultured into Potato dextrose broth media for seven days at 25 ± 2 °C. Extraction was carried out for both mycelium and filtrate using ethyl acetate by the ratio 1:1 V/V for 24 h and then filtered using Whatman filter paper followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The ethyl acetate extract was evaporated under reduced pressure using rotary evaporator (IKA *RV10* Staufen, Germany) to obtain the crude extract [31].

2.5.2. Test bacteria

Five bacterial strains were used including *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (NBRC102203), which obtained from our lab., whereas the other strains in *Streptococcus pneumonia*, *Micrococcus luteus* and *Proteus* sp. are clinical pathogens which were kindly provided from Pharmaceutical Control Authority, Giza, Egypt. All strains were inoculated on nutrient agar medium and incubated at 37 °C for 24 h. After incubation, bacteria were suspended in sterile nutrient broth media and the cell suspensions were adjusted to a concentration of 0.5 McFarland $(1.5 \times 10^8$ CFU $ml^{-1})$.

2.5.3. Antibacterial activity by well diffusion method

The antibacterial activity of the ethyl acetate extract from *C. hydrophila* against the five tested bacteria was determined by the well diffusion method [32]. About100 µl of bacteria inoculum was streaked onto nutrient agar plates (NA) using sterile swabs. Wells were made on the agar surface with sterilized 6 mm cork borer, then 100 µl of the extract (55.0 mg ml^{-1} dissolved in ethyl acetate) was inoculated into the well. The bacteria seeded plates were placed in the refrigerator for 8 h at 4 °C before being incubated at 37 °C for 24 h. Clindamycin (2.0 µg/disk) and ciprofloxacin (5.0 µg/disk) were used as the positive control antibiotics according to the Clinical Laboratory Standards Institute (CLSI) [33,34], whereas ethyl acetate was used as the negative control. The plates were observed for the inhibition zone formation around the wells. The inhibition zone (mm) was determined by measuring the diameter of the inhibition zone around the well and expressed as means ± SD. All measurements were recorded in triplicates.

2.6. Statistical analysis

Data were analyzed using Minitab 17 software by one-way ANOVA. Fisher least significant difference (LSD) method with 95% confidence level.

2.7. Metabolic profiling using NMR spectroscopy

2.7.1. Fungal culturing and sample collection

C. hydrophila suspention culture was initiated by inoculating 0.5 cm disk of seven days old culture in 50 ml Potato dextrose broth medium and incubating at 25 °C for a week. The mycelia were collected (six replicates were collected from six different culturing flasks, i.e. $n = 6$). and immediately immersed in

liquid nitrogen then stored at $-80\,^{\circ}$C for 24 h. For water removal from the fungal tissues, samples were lyophilized for 24 h. The dried mycelial samples were homogenized and ground to a fine powder, then 50 mg was used for metabolites extraction.

2.7.2. NMR metabolite extraction

The extraction was carried out based on the ratio between dry mass and water loss [35,36] using 2:2:1 methanol: chloroform: water according to Kim et al. [37]. The hydrophilic fraction was removed from the extract and dried under vacuum using vacuum concentrator (Labconco, Kansas, USA) for 24 h.

2.7.3. NMR sample preparation, data collection, and metabolites identification

The dry polar fraction was dissolved in 620 μl of NMR buffer (1.0 mM TMSP (internal standard (3-trimethylsilyl)-2, 2′, 3, 3′-tetradeuteropropionic acid, 100 mM sodium phosphate buffer at pH 7.3 and 0.1% sodium azide, in 99.9 atom % D_2O). NMR data collection and processing were carried out according to Abdelsalam et al. [38]. Briefly, a 700 MHz Bruker AvanceTM III spectrometer (BrukerBioSpin, Billerica, MA) was used for the 1 D and 2 D NMR measurement. The data were collected with a spectral width of 16.0 ppm and 64 K points, providing a collection duration of 2.9 s. The first increment of the presat-noesy spectra for solvent suppression was collected using 120 scans, 4 dummy scans, 3 s relaxation delay, and on-resonance pre-saturation at the residual water frequency. The 90° pulse widths for each sample were measured using the automatic pulse calculation experiment (pulsecal). At 700 MHz, the Bruker hsqcedetgpsisp2.2 pulse sequence was utilized to collect two-dimensional ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Correlation) data.

The ^{1}H was detected with a spectral width of 11 ppm in the F2 channel, whereas the ^{13}C was detected in the F1 channel with a spectral width of 180 ppm.

The metabolites were identified using the Chenomx Suite library of compounds (Chenomx Inc., Edmonton, Alberta, Canada). Coupling constants (J value) were measured using Mnova software. Compounds identification was verified by comparing the measured chemical shifts (δ), multiplicities, and the coupling constants (J value) of the ^{1}H and ^{1}H-^{13}C (HSQC) NMR data with those previously published and with the values of standard compounds reported in the Human Metabolome Database (HMDB) (https://hmdb.ca/).

3. Results

In the present study, *C. hydrophila* was isolated from the aquatic plant *Myriophyllum spicatum* growing along the banks of the river Nile in Helwan, Cairo, Egypt (Coordinates: 29.864° N and 31.291° E). Morphological examination of the fungus carried out on different growth media, including Czapeks-dox agar, Potato dextrose agar and Malt agar media. On the potato dextrose agar, the colonies initially appeared as white fluffy mycelium within the first four days of incubation then gradually large numbers of small and globose scattered sclerotia were observed on the surface of the colonies. The sclerotia were initially white in color, then turned brown to black over time. On Czapeks-dox agar medium, the colonies appeared as white mycelium without sclerotia until the sixth day of incubation. Starting from the eighth day, few white sclerotia were gradually developed, which acquired dark brown color over time. In addition, on malt agar medium, the fungus appeared as white mycelium until the end of the incubation. Sclerotia were absent on this growth medium. Microscopic examination for the fungus showed a septate and branched mycelium while the sclerotia were globose in shape (Figure 1).

In addition, the resulting nucleotide sequence of fungal DNA was analyzed by GenBank database using advanced BLAST (Megablast) searches from the National Center for Biotechnology Information
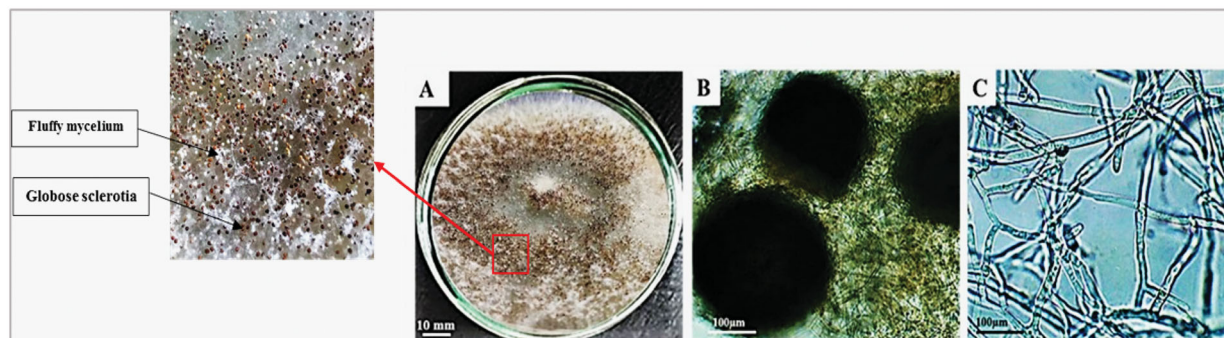

Figure 1. Morphological and microscopic examination of *Ceratorhiza hydrophila*. (a) growth on Potato dextrose agar media after seven days of incubation at 25°C, (b) brown globose sclerotia (4X), (c) septate branched mycelium (40×).

(NCBI). The fungal isolate was identified as *Ceratorhiza hydrophila* (synonym: *Sclerotium hydrophilum*) and submitted to the Genbank as *Ceratorhiza hydrophila* isolate EG19 with the fungus ID MK387081. Furthermore, the designed phylogenetic tree of the partial sequence with corresponding sequences from other fungi in GenBank showed that *Ceratorhiza hydrophila* isolate EG19

(MK387081) have high similarity toward *Ceratorhiza hydrophila* isolate SR6 (JQ359765) of 99% (Figure 2).

3.1. Enzyme production

The production of protease, asparaginase, cellulase, and amylase enzymes from *C. hydrophila* has been

KT269527 , 91.4% , *Ceratobasidiaceae sp.*
KT269493 , 91.7% , *Ceratobasidiaceae sp.*
JQ070603 , 91.2% , *fungal sp. acwVH17_17*
KX290847 , 91.1% , *Ceratobasidium sp. AG-W*
AF200514 , 85.3% , *Rhizoctonia bicornis*
DQ279044 , 87.8% , *Ceratobasidium sp. MLC1*
AY927329 , 87.8% , *Rhizoctonia sp. AG-G*
AB478784 , 87.8% , *Ceratobasidium sp. AG-G*
MT177263 , 87.6% , *Rhizoctonia solani AG-G*
KC354146 , 95.1% , *Sclerotium hydrophilum*
MK213723 , 90.5% , *Rhizoctonia solani*
GU356400 , 94.8% , *Sclerotium hydrophilum*
FJ231390 , 92.9% , *Sclerotium hydrophilum*
MK043069 , 92.1% , *Sclerotium hydrophilum*
FJ231396 , 94.8% , *Sclerotium hydrophilum*
MK387081 , 100.0% , *Ceratorhiza hydrophila isolate EG19*
JQ359765 , 99.0% , *Ceratorhiza hydrophila*
KX181457 , 98.7% , *Sclerotium hydrophilum*
MH367053 , 98.8% , *Sclerotium sp. (in: Thelephorales)*
KT362098 , 98.8% , *Sclerotium hydrophilum*
EU152866 , 98.6% , *Sclerotium hydrophilum*
KF673644 , 98.3% , *fungal endophyte*
EU152864 , 98.4% , *Sclerotium hydrophilum*
KX674534 , 98.0% , *Sclerotium hydrophilum*
KT362104 , 96.8% , *Rhizoctonia solani*
KT362095 , 91.4% , *Rhizoctonia solani*
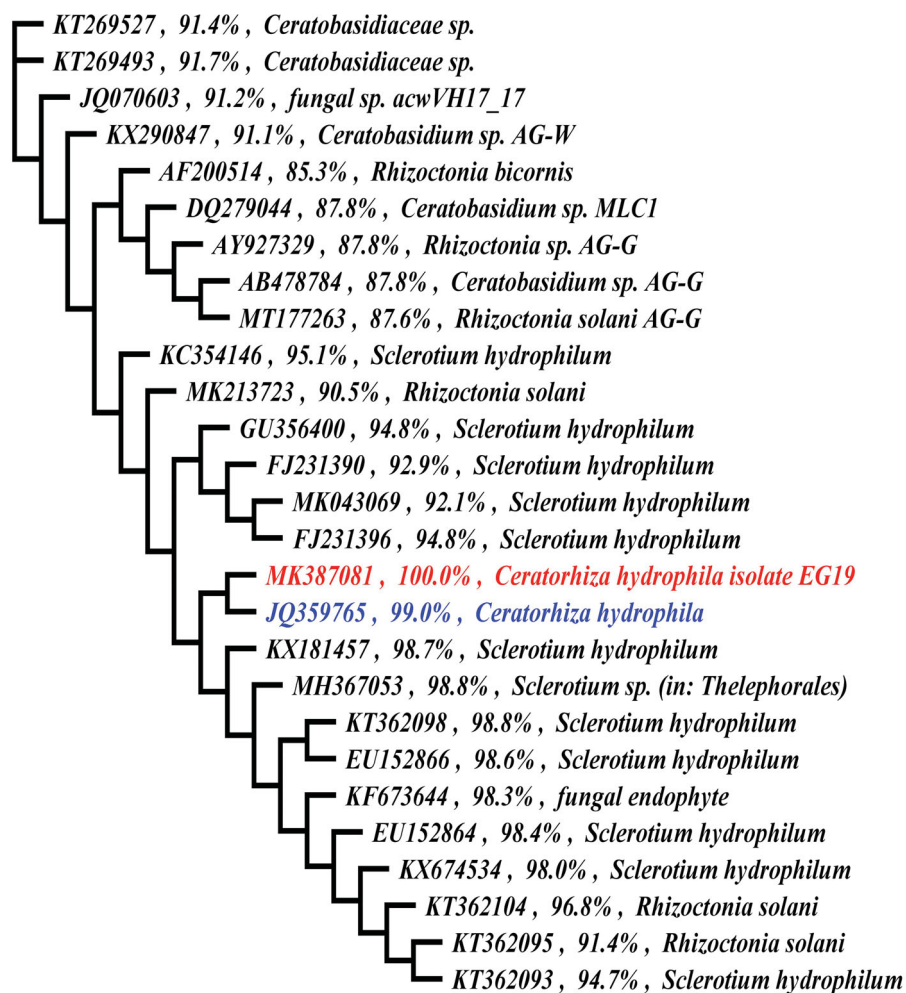KT362093 , 94.7% , *Sclerotium hydrophilum*

Figure 2. Maximum likelihood tree showed that *Ceratorhiza hydrophila* isolate EG19 (MK387081) had a high similarity of 99% toward *Ceratorhiza hydrophila* isolate SR6 (JQ359765).
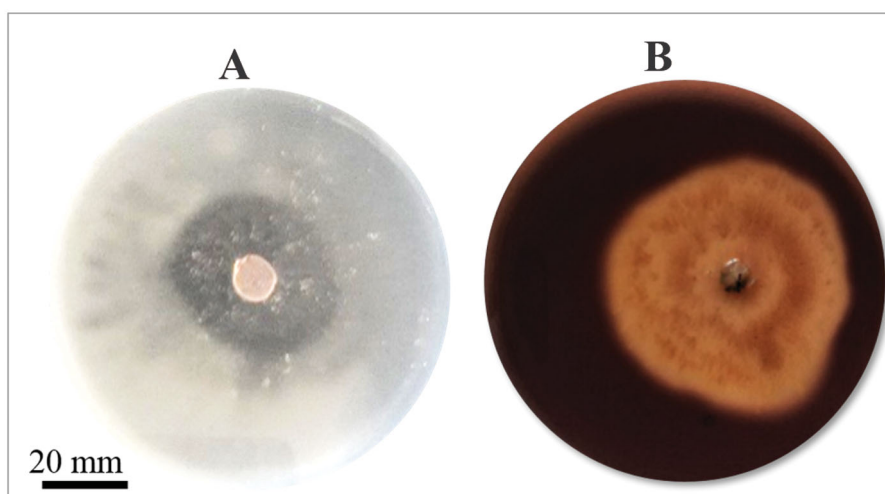

Figure 3. *Ceratorhiza hydrophila* enzymatic activities after incubation for 72 h at 25°C. (a) protease, (b) cellulase.

investigated. The results showed that the isolated fungus was capable to produce only protease and cellulase (Figure 3 and Table 1) but not asparaginase, and amylase.

3.2. Assessment of antibacterial activity

The ethyl acetate extract of *C. hydrophila* was assayed for its antibacterial activity against the tested bacteria (Table 2) according to CLSI standards. The results reveal that the fungal extract had an intermediate inhibitory response against the Gram-positive bacteria *Streptococcus pneumonia*, *Staphylococcus aureus*, and *Micrococcus luteus*, recording inhibition zones 18 ± 1.4 mm, 20 ± 0.7 mm and 16 ± 0.7 mm, respectively. On the other hand, resistance response was shown by the tested Gram-negative bacteria; *Escherichia coli* and *Proteus* sp. toward the fungal extract with inhibition zones of 17 ± 2.1 mm and 0.0 mm, respectively.

3.3. Metabolic profiling using NMR spectroscopy

NMR spectroscopic analysis of the polar extract of *C. hydrophila* led to the identification of 23 metabolites. The chemical shifts, multiplicities, and coupling constants were retrieved from the ^{1}H NMR spectra using Topspin and MestReNova software for spectral analysis, processing, and peak annotation (Table 3, Figure 4). By defining the ^{1}H-^{13}C correlation, the HSQC spectra verified the structure identification of the metabolites (Table 3, Figure 1 in Appendices). A variety of chemical classes have been recognized, including 31% amino acids, 9% sugars, 9% amines, 4% sugar alcohols, and 4% alkaloids. The presence of acetate, pyruvate, dimethylamine, trimethylamine, and betaine was revealed by

singlet signals integrating for three protons, *i.e.* CH_3, in the upfield section of the ^{1}H NMR spectrum at δ_H 1.92 (δ_C 25.6), 2.38 (δ_C 29.3), 2.72 (δ_C 37.0), 2.90 (δ_C 47.4), and 3.28 (δ_C 56.2). The anomeric protons of α-, β-glucose, and trehalose have also been observed at δ_H 5.24 (d, $J = 3.67$), 4.64 (*d*, $J = 7.88$), and 5.20 (*d*, $J = 3.73$), respectively. Analysis of the cross peaks in the HSQC spectrum was used to correlate carbons to these protons (Table 3, Figure 1 in the appendices). The remaining protons of this sugars were defined in the region δ_H (δ 3.0–5.5 ppm). Furthermore, the amino acid glycine was identified by the methylene protons resonating at δ_H 3.55 (2H, *s*, δ_C 44.3), whereas alanine traced by its corresponding signals at $\delta_{H\alpha}$ 3.77 (3H, *q*, $J = 7.20$, δ_C 53.5) and $\delta_{H\beta}$ 1.48 (1H, *d*, $J = 7.25$, δ_C 18.6). The relatively deshielded methine protons detected downfield of the spectrum at δ_H 6.52 (1H, s, δ_C 137.3), $\delta_{H\alpha}$ 7.96 (1H, s, δ_C 140.3), and δ_H 8.46 (1H, s) were attributed to the presence of fumarate, xanthine, and formate. Eventually, the assignment of all metabolites was further confirmed using Chenomx NMR suite and verified by comparing the generated spectral data with those reported in HMDB and other earlier literature studies.

4. Discussion

This study was performed on the basidiomycetous fungus *Ceratorhiza hydrophila,* which was isolated from the aquatic plant *Myriophyllum spicatum* in Egypt. The aim of the study was to investigate the antibacterial properties, enzymes production, and metabolic profiling of the fungus as a promising and new source of metabolites, which can be used in various application fields.

To our knowledge, this is the first report of enzyme investigation from *C. hydrophila*, and it was found to have the ability to produce both protease and cellulase enzymes. Similarly, protease production was detected in *Rhizoctonia solani* [39]. In addition, the proteolytic activity was also detected in *Aspergillus tubingensis*, *Talaromyces purpureogenus*, *Acremonium flavum*, *Lichtheimia ramosa* and *Hormographiella aspergillata* [40]. In industrial

Table 1. Enzyme production by *Ceratorhiza hydrophila*.

Enzyme	Clear zone diameter (cm)
Protease	3.6 ± 0.25^b
Asparaginase	0.0^c
Cellulase	5.5 ± 0.1^a
Amylase	0.0^c

In each column means followed by the same letter are un-significantly at $p < 0.05$ based on Fisher test.

Table 2. Antibacterial activity of the crude extracts of *Ceratorhiza hydrophila* against different tested bacterial strains.

Bacterial strain	Inhibition zone (mm)			
	C. hydrophila (55 mg ml^{-1})	Clindamycin (2 m µg) (Positive control)	Ciprofloxacin (5 m µg) (Positive control)	Ethyl acetate (Negative control)
Streptococcus pneumonia	18 ± 1.4^{ab}	15 ± 0.06	ND	0 ± 0
Micrococcus luteus	20 ± 0.7^a	24 ± 0.06	20 ± 0.0	0 ± 0
Staphylococcus aureus	16 ± 0.7^b	22 ± 0.06	30 ± 0.0	0 ± 0
Escherichia coli	17 ± 2.1^b	ND	$25 \pm .1$	0 ± 0
Proteus sp.	0 ± 0^c	ND	22 ± 0.1	0 ± 0

In each column means followed by the same letter are un-significantly at $p < 0.05$ based on Fisher test. Antibacterial activity is presented as means of measurements of inhibition zones (mm) ± SD. ND means not detected.

Table 3. The identified compounds in the polar extract of *Ceratorhiza hydrophila* with their chemical formulas, chemical shifts, multiplicity and coupling constants.

	Compound name	Chemical formula	^{1}H (ppm) (functional group or specific H, multiplicity)	^{13}C (ppm) (functional group or specific C)	J (Hz)
1	Acetate	$C_2H_4O_2$	1.92 (CH_3, s)	25.6 (CH_3)	–
2	Alanine	$C_3H_7NO_2$	3.77 (H^α, q)	53.5 (C^α)	7.20
			1.48 (H^β, d)	18.6 (C^β)	7.25
3	Betaine	$C_5H_{11}NO_2$	3.28 (CH_3, s)	56.2 (CH_3)	–
			3.90 (CH_2, s)	69.2 (CH_2)	–
4	Choline	$C_5H_{14}NO$	4.05 (H^α, m)	58.6 (C^α)	–
			3.21 (H^γ, s)	56.8 (C^γ)	–
			3.50 (H^β, m)	70.4 (C^β)	–
5	Dimethylamine	C_2H_7N	2.72 (CH_3, s)	37.0 (CH_3)	–
6	Formate	CH_2O_2	8.46 (CH, s)	–	–
7	Fumarate	$C_4H_4O_4$	6.52 (CH, s)	137.3 (CH)	–
8	Glucose	$C_6H_{12}O_6$	4.63 ($^{1\beta}$CH, d)	98.9 ($^{1\alpha}$CH)	7.88
			5.24 ($^{1\alpha}$CH, d)	94.7 ($^{1\beta}$CH)	3.67
			3.47 ($^{2\alpha}$CH, m)	74.4 ($^{2\alpha}$CH)	
			3.39 (^{4}CH, m)	72.6 (^{4}CH)	
			3.70 ($^{5\beta}$CH, m)	63.7 (^{6}CH)	
9	Glycerol	$C_3H_8O_3$	3.57 (CH_2, dd)	65.3 (CH_2)	6.25,6.65
			3.65 (CH_2, dd)	65.4 (CH_2)	
10	Glycine	$C_2H_5NO_2$	3.55 (H^α, s)	44.3 (C^α)	–
11	Isoleucine	$C_6H_{13}NO_2$	1.26 (H^γ, m)	26.8 (C^γ)	–
			1.01 (H^γ, d)	17.5 (C^γ)	7.0
			0.92 (H^δ, t)	–	7.1
12	Lactate	$C_3H_6O_3$	1.33 (CH_3, d)	22.1 (CH_3)	6.87
13	Leucine	$C_6H_{13}NO_2$	0.97 (H^δ, t)	24.0 (C^δ)	6.09
			1.07 (H^β, m)	–	–
14	Malate	$C_4H_6O_5$	2.34 (CH_2, dd)	45.0 (CH_2)	15.37,10.2
			2.68 (CH_2, dd)	45.2 (CH_2)	15.37,2.9
15	Malonate	$C_3H_4O_4$	3.12 (H^β, s)	50.2 (C^β)	–
16	O-Phosphocholine	$C_5H_{15}NO_4P$	3.20 (CH_3, s)	56.7 (CH_3)	–
			3.57 (CH_2, m)	68.9 (CH_2)	–
			4.15 (CH_2, m)	60.6 (CH_2)	–
17	Pyruvate	$C_3H_4O_3$	2.38 (CH_3, s)	29.3 (CH_3)	–
18	Succinate	$C_4H_6O_4$	2.41 (CH_2, s)	36.4 (CH_2)	–
19	Threonine	$C_4H_9NO_3$	1.31 (CH_3, d)	22.5 (C^α)	6.49
			3.58 (CH_3, d)	63.4 (C^γ)	5.20
20	Trehalose	$C_{12}H_{22}O_{11}$	5.20 (^{1}CH, d)	95.8 (^{1}CH)	3.73
			3.66 (^{2}CH, dd)	73.6 (^{2}CH)	10,3.97
			3.46 (CH, t)		9.54
21	Trimethylamine	C_3H_9N	2.90 (CH_3, s)	47.4 (CH_3)	–
22	Valine	$C_5H_{11}NO_2$	3.60 (H^α, d)	63.0 (C^α)	4.54
			0.99 (H^γ, d)	20.8 (C^γ)	7.00
			1.04 (H^γ, d)	20.4 (C^γ)	7.00
23	Xanthine	$C_5H_4N_4O_2$	7.96 (CH, s)	140.3 (CH)	–

applications, proteases used as anticlotting agent [41], anticancer agent [42] and in waste management [43]. On the other hand, cellulase which has an important role in biofuel production, minimization of energy crisis, environmental pollution [44], was also reported to be produced by *Pleurotus ostreatus* [45], *Rhizoctonia solani*, *Cephalosporium acremonium* and *Fusarium moniliforme* [46].

The ethyl acetate extract of *C. hydrophila* was found to have intermediate antibacterial activity against the tested Gram-positive bacteria, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Micrococcus luteus*, whereas the Gram-negative bacteria, including *Escherichia coli*, and *Proteus* sp. showed resistance to the crude extract. Similar study reported the antibacterial activity of an extract from the mycelia and basidiocarp of some basidiomycetous fungi including *Ganoderma applanatum*, *Trametes corrugata* and *Tricholoma crissum*, which were found to have inhibitory activity against various pathogenic bacteria including, *Micrococcus roseus*, *Mycobacterium phlei* and *Staphylococcus*

aureus [47]. In addition, different Australian basidiomycetous macrofungi, including *Amanita ochrophylla*, *Fomitopsis lilacinogilva* as well as some *Agaricus* sp. showed excellent inhibition against *S. aureus* and *E. coli* [48].

NMR spectroscopy is a crucial analytical tool in the field of metabolomic research due to its capacity to determine metabolite quantities, experimental reproducibility, nondestructive nature, and exceptional structure elucidation capabilities [49]. Using both ^{1}H and ^{1}H-^{13}C HSQC NMR data to study the metabolic profile of biological samples is a solid and dependable technique [50]. In the present study, the metabolic profiling of the *C. hydrophila* polar extract was performed using the NMR spectroscopy. The data showed a presence of twenty-three metabolites. Six aliphatic amino acids (e.g. alanine, betaine, isoleucine) have been identified in the aliphatic region δ 0.5–3.0 ppm; the identification of these metabolites was confirmed by comparing our 1 D and 2 D data with HMDB and data published in the literature [51–54].
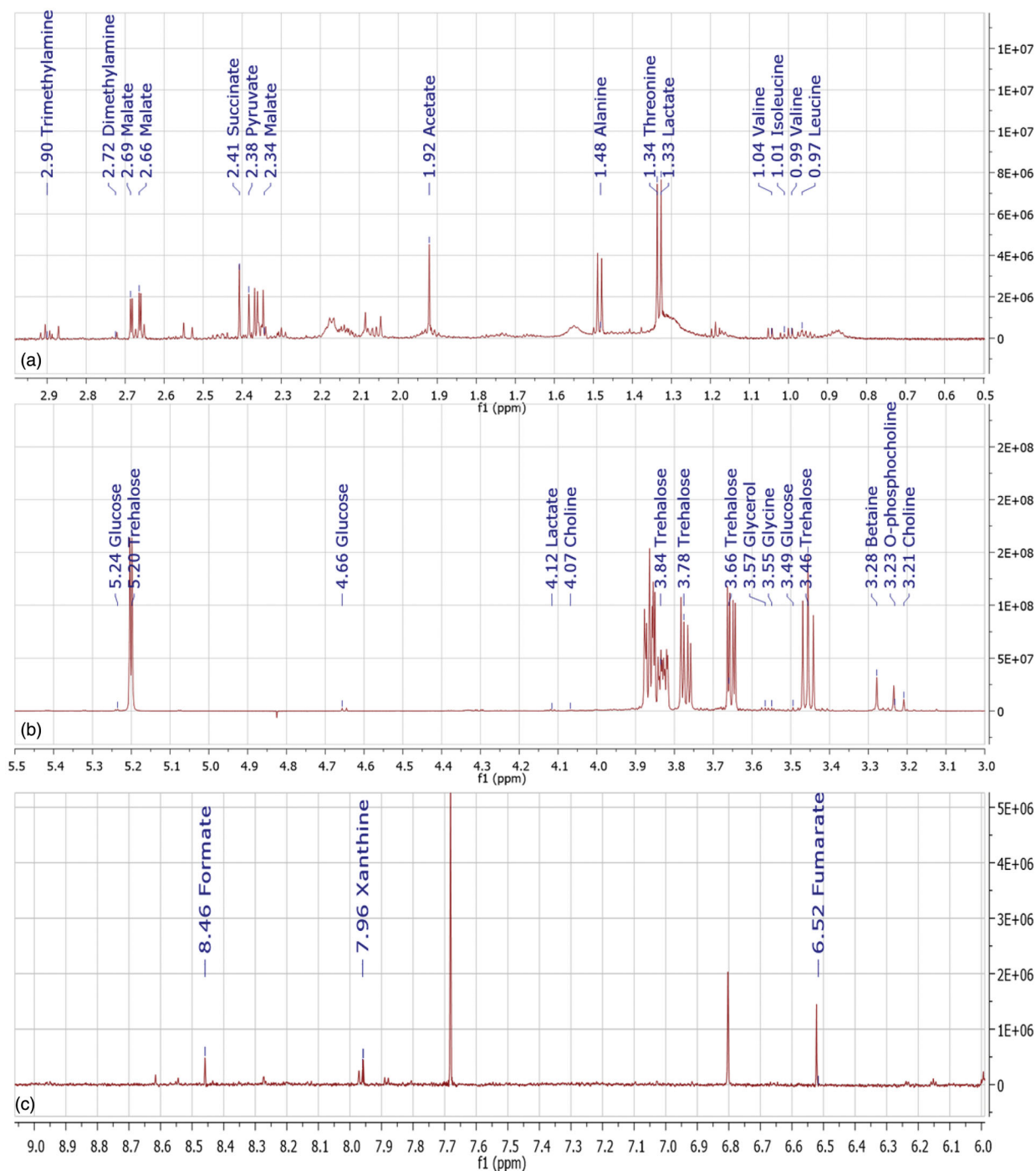
Figure 4. ^{1}H NMR spectra of the polar extract of *Ceratorhiza hydrophila*. (a) Aliphatic region, (b) sugars abundance signals region, (c) aromatic region.

The presence of amino acids alanine, glutamate, glutamine and arginine has been reported in the wild edible *Boletus* mushroom [55]. Valine and glycine were identified in other members of the family Basidiomycota (*Pleurotus tuoliensis*) [56]. Betaine is used as a source of carbon and energy source by fungi as reported by [57]. Furthermore, the antibacterial activity of betaine has been reported [58] as well as its activity against skin-associated fungus [59]. In addition, betaine has an important medicinal value in several human diseases, such as obesity, diabetes, cancer, Alzheimer's and gastrointestinal diseases [60]. Moreover, alkyl betaines are used as

antistatic and viscosity-increasing agents in cosmetic products such as shampoo [61]. Betaine is used as a surfactant to remove various environmental pollutants such as toluene [62]. In addition, betaine can improve the PCR reaction during DNA amplification by reducing the formation of secondary structure in GC-rich regions [63].

In the present work, trehalose and glucose signals were clearly distinguished in the spectral region δ 3.0–5.5 ppm [64]. Trehalose is a non-reducing disaccharide produced by many organisms, including bacteria, fungi, and plants [65]. Trehalose was reported as an essential energy source in

filamentous fungi and yeast [66], and it has a key role during fermentation in *Saccharomyces cerevisiae* [67]. The metabolic profiling of three strains of *Grifola frondosa* showed that trehalose, glycerol, and glucose were the most abundant metabolites [68].

Moreover, the organic acids like acetic acid, formate, fumarate, lactate, malate, malonate, succinate and pyruvate have been identified. In other studies, acetic acid has been reported in the Antarctic yeasts metabolic profiling [69]. Fumarate, lactate, malate, succinate and pyruvate have been identified in *Grifola frondosa* [70]. The detection of malonate in the culture medium of the ectomycorhhizal fungus *Suillus luteus* was also reported [71]. Moreover, through organic transformations, malonates can be used to produce fragrances, vitamins, agrochemicals, and pharmaceutical compounds [71].

Choline was also identified in the *C. hydrophila* extract. It acts as a synthetic precursor for various important compounds including, the amino acid betaine, the neurotransmitter acetylcholine, and membrane phospholipids [72].

Xanthine was identified in the fungal polar extract. It is an alkaloid with well-known pharmacological properties mainly in treatment of respiratory disease [73], it also possesses anti-inflammatory, and anti-cancer activities. It is a precursor of several alkaloids and used as drug manufactory [74]. Xanthine tablets dispensed in the market for treatment of pulmonary disorder (e.g. Xanthin Plus Tablets 20's® manufactured by Vega Pharmaceuticals (Pvt) Ltd).

In conclusion, this is the first report for isolation of *C. hydrophila* a basidiomycetous fungus from *Myriophyllum spicatum* plant in Egypt. All the results obtained from this study were reported for the first time. *C. hydrophila* showed the ability to produce cellulase and protease enzymes. The ethyl acetate extract of *C. hydrophila* showed intermediate antibacterial activity against all tested Gram-positive bacteria. Metabolic profiling using NMR spectroscopy showed that the fungal metabolite contents include carbohydrates, organic acids, amino acid, amines, and alkaloids. Some metabolites as choline and xanthine are used as nutritional supplement and in drug industry. It is expected that the results obtained in this study will pave the way to other perspective in enormous applications using *C. hydrophila* as a new promising source of antimicrobial agents and essential metabolites. Also, our findings about *C. hydrophila* can be valuable in classification and chemotaxonomy of the species.

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Disclosure statement

The authors declared that there is no conflict of interest.

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