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Molecular Identification, Extracellular Enzyme Production and Antimicrobial Activity of Endophytic Fungi Isolated from *Solanum tuberosum* L. in Egypt

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***Solanum tuberosum* L. possesses economic properties and can host endophytic mycoflora. A total of 19 endophytic fungi were identifiedvia morphological and molecular methods. Amongthem, *Trichoderma harzianum* was the core-group fungus with a relative frequency of 36.7%. In the preliminary antimicrobial assay, all the test pathogens were inhibited by *Alternaria tenuissima, Penicillium pinophilum* and *Penicillium rubens* with a maximum inhibition zone of 26 mm and a minimum zone of 11 mmusing agar-plug method. All the isolated endophytic fungi produced amylase, while cellulase and tyrosinase were recorded for most of the isolated species, whereas laccase and protease and manganese peroxidase were shown by a few taxa. None of the isolated fungi produced chitinase.This study revealed the biodiversity of endophytic fungi isolated from *Solanumtuberosum* that could be a promising source of bioactive compounds applied in many industries.**

Keywords: Biodiversity, Endophytic Fungi, *Solanum tuberosum*,*Trichoderma harzianum.*

Solanum tuberosum L. (potato) belongs to the night shade family (Solanaceae). The global output of potato in 2016 was amounted to be almost 376.83 million tons.The origin of the potato is the Andes of South America [1]. It is common with major producers in China, Russia, Poland, the USA, Ukraine, Germany, and India. In 2017, the total exports of the Egyptian *S.tuberosum* were about 800,000 ton, so it is an important agricultural crop in Egypt [2].

Endophytes are generally all microorganisms living within plants with no symptoms apparent to their hosts [3]. The endophytic fungi hosted approximately one million species of plant[4]. This group of fungi is ubiquitous[5]. Recently, Trabelsi *et al*.[6] isolated endophytic fungi from *Solanumtuberosum* such as *Aspergillus flavus, Aspergillus niger, Penicillium chrysogenum,* and *Penicillium polonicum.* Also, Marakand Kayang[7] isolated a total of 44 endophytic fungi from *Solanumtuberosum* such as *Aspergillus niger, Fusarium oxysporum, Penicillium funiculosum, Trichoderma harzianum.*

Unlike, the utilized chemicals and pesticides for the disease overcoming, the endophytes were a cheap and environmentally safe source of bioactive compounds applied for protection against human pathogens [8]. Nowadays, among the hardest troubles in the world were the developing of the non-susceptible drug in

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pathogenic microbes, such as *Staphylococcus aureus* that was resistant against methicillin[9]. Deshmukh *et al*. [10] have stated that endophytic fungi have antimicrobial compounds extracted from them. Endophytic fungi are a novel hope as various endophytic fungi have antifungal potential [11].

Endophytic fungi were considered as a novel source for acquiring enzymes with unique prospectsas their easy handling and cultivation, fast growth, and high yield[12]. Various extracellular enzymes are secreted by endophytic fungi as cellulase, amylases, laccases, chitinases, and proteinases [13]. Endophytic fungi have been investigated for several applications owing to their extracellular enzymes production[14]. These enzymes operate many functions in an organism extending from obtaining nutrition from their host, food substances hydrolysis and are involved in eliciting defense mechanisms against pathogens. Subsequently, there is an urgent need to discover and use various unique enzymes with great stability for industrial purposes. Few studies have been done on endophytes from *Solanumtuberosum* in Egypt despite their importance. The present study aimed to isolate and identify endophytic fungi from *Solanum tuberosum*. Moreover, the isolated fungi were screened for their antimicrobial activity and extracellular enzymes production.

MATERIALS AND METHODS

Location and sampling

Solanum tuberosum L. (Potato) was collected from Beni-Suef governorate (latitude 29° 42 03 N and longitude 31° 52 03 E), Egypt during March 2015–March 2016. Plants with no visible symptoms of disease were selectedand then transported to the laboratory in sterile bags and handled within 5 hours of sampling.

Surface sterilization of *Solanum tuberosum* and isolation of endophytic fungi

According to the method of [15], plants were washed in running tap water to eliminate soil debris. Leaf, stem and root samples were cut into small segments of 1 cm long and 3 mm broad. Those segments were sunken in 70% ethanol for 1-3 minutes, 4% NaOCl for 1.5 minutes, 70% ethanol for 1minute and finally rinsed 3 times with sterile distilled water, then they were dried by sterile filter paper. Five segments were placed on potato dextrose agar medium with 250mg/l chloramphenicol. The plates were incubated at 25°C in a dark condition. The emerged fungi from segments were observed every 2 days for at least 3 weeks. The mycelia emerged from the segments were regularly isolated and the hyphal tips were transferred to the PDA plates free of antibiotics. Single spore cultures were prepared for each strain to ensure purity of strains.

Data analysis

The colonization and relative frequency were calculatedaccording to Petrini et al. [16]:

$$\text{Colonization Frequency(CF\%)} = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segments observed}(or\ used)} \times 100$$

$$\text{Relative Frequency (RF \%)} = \frac{\text{Number of isolates of species}}{\text{Total number of isolates}} \times 100$$

Morphological identification of endophytic fungi

For morphological identification of endophytic fungi, hyphae and conidia were taken from purified colonies and examined by optical microscope according to [17].

Molecular identification of endophytic fungi

Genomic DNA was extracted from mycelia grown on 3% MEA (Malt Extract Agar) incubated at 28°C harvested after 2 days with the Plant DNeasy Minikit (QIAgen GmbH, Hilden, Germany) according to the manufacturer's instructions. A region of nuclear DNA containing the ITS1 and two regions of the rRNA gene cluster was amplified by PCR using the primer combinations SR6R and LR1 (21) as described by Kullnig-Gradinger *et al*. [18]. PCR products were sequenced. The sequences were dropped in NCBI GenBank and compared with those available in the data base using a sequence similarity search tool (blastn) [19].

Screening of endophytic fungi for antimicrobial activity

The preliminary screening of antimicrobial activity was carried out against *Candida albicans*, *Salmonella typhi, Sarcinaventriculi* and *Staphylococcus aureus* obtained from (Microbiology Laboratory, Faculty of Science, Ain Shams University, Egypt) using the agar plug method [20].The plates were poured with nutrient agar (NA) medium and inoculated with 100 µl of the pathogen suspension (1.5x108 CFU/ ml) then regularly spread by a sterile cotton swab on the medium. The grown mycelial discs (6 mm) of all

isolates (15 day-olds) grown on potato dextrose agar (PDA) medium (potato infusion 200 g, dextrose 20 g, agar 20 g, distilled water 1 L and pH 6) were taken from actively growing edges of isolates using a sterile cork borer and put on the surface of nutrient agar medium (NA) (peptone 5g, yeast extract 5g, beef extract 3g, agar 20g, distilled water 1 L and pH 7) previously inoculated with test organisms. The plates were wrapped by parafilm and incubated at 37° C for 24 h. Then, the antibacterial activity was documented by the visualization and estimation of inhibition zones.

Extracellular Enzyme production

Amylase production

The amylase production was screened using glucose yeast extract-peptone (GYP) agar medium (glucose 1g, yeast extract 0.1g, agar 15 g, distilled water 1L and pH 6) which included 1% soluble starch. After incubation, the plates were flooded with 1% iodine and 2% potassium iodide. The clear zone surrounding the colony indicated amylase production [21].

Cellulase production

Cellulase production was estimated by GYP agar medium supplemented with 0.5% Na-carboxy-methylcellulose. Plates were incubated for 5 days at 28°C then immersed with a solution of 1% Congo red dye for 20 minutes and destained with 1N NaCl solution for 15 minutes. The cellulase enzyme was evaluated by the presence of a light yellow area around the colony of the fungus [21].

Tyrosinase production

Tyrosinase production was assessed using tyrosine agar medium (peptone 5g, beef extract 3g, agar 20g, L-tyrosine 5g and pH 7). The brown color around the colony indicated tyrosinase enzyme [9].

Protease production

Protease production was screened by GYP agar medium amended with 1% casein and pH 6.5and incubated for 5 days. The clear zone around the colony indicated protease enzyme[21].

Chitinase production

Chitinase production was estimated by chitin agar medium (yeast extract 1.5 g, chitin 2.0 g, agar 20 g and distilled water1 L). Plates were inoculated with test cultures and then incubated at 26°C up to 72 h. The appearance of a clear zone around the culture indicated the chitinase enzyme.

Table 1. List of endophytic fungi isolated from different parts of potato and their GenBank accession numbers and relative frequency

TUCIM No.	Most similar GenBank entry	Species identification	% similarity / % query coverage	Number of isolated colonies				% Relative frequency
				Root	Stem	Leaf	Total	
6640	KX664322	*Alternaria tenuissima*	99/99	12	69	8	89	7.9
6676	JF951750	*Aspergillus flavus*	99/90	27	63	10	100	8.9
6670	KF154412	*Aspergillus niger*	100/96	62	69	119	250	22.2
6644	KC859010	*Aspergillus ochraceus*	100/93	_	4	9	13	1.15
6671	KP307914	*Aspergillus oryzae*	99/99	13	2	_	15	1.2
6637	NR_144848	*Chaetomium cervicicola*	98/100	_	4	_	4	0.35
6645	KF753941	*Curvularia lunata*	100/96	23	_	10	33	2.9
6636	KC311517	*Fusarium equiseti*	99/99	12	5	_	17	1.5
6634	HF546381	*Fusarium nygamai*	99/99	_	2	39	41	3.6
6635	LT746253	*Fusarium oxysporum*	99/99	_	_	3	3	0.27
6649	KY655194	*Lasiodiplodiatheobrome*	100/96	_	_	4	4	0.4
6641	GU183120	*Penicillium funiculosum*	99/98	_	14	22	36	3.2
6642	JN620402	*Penicillium minioluteum*	99/94	_	_	40	40	3.5
6668	AB606412	*Penicillium pinophilum*	99/98	_	_	2	2	0.12
6669	KX958077	*Penicillium polonicum*	99/99	_	2	_	2	0.12
6673	LC105692	*Penicillium rubens*	100/97	_	_	18	18	1.6
6648	MG065799	*Stemphylium vesicarium*	99/100	14	_	_	14	1.24
6638	KM491893	*Trichoderma harzianum*	99/99	175	102	137	414	36.7
6647	KU945936	*Ulocladium sp.*	100/96	10	16	7	33	2
	_	Total	_	348	352	428	1128	_

Estimation of Lignin-degrading enzymes

Manganese peroxidase production

Manganese peroxidase production was estimated using discs cut from the edge of 6 days old fungal cultures. The fungal discs were inoculated on Boyd and Kohlmeyer (B&K) medium (glucose 10 g, peptone 2 g, yeast extract 1 g, agar 18 g, distilled water 1 L and pH 6.0) amended with 4 m Mguaiacol[22]. The plates were incubated at 25±2°C for 2 weeks after enclosing them with black polythene bags. The formation of reddish brown color under and around the fungal colony was owing to guaiacoloxidation.

Laccase production

Laccase production was performed using GYP agar medium supplemented with 0.005% 1-naphthol. The blue color indicated laccase enzyme due to oxidation of 1-naphthol [23].

Statistical analysis

The tests were performed in triplicate and the results were analyzed statistically by the SPSS program version 20. The analyses of variance were according to the rules of the ANOVA. The significance of differences between the means as determined through Duncan's multiple range Test.

RESULTS

Isolation and identification of endophytic fungi

In the present study, 2520 potato segments analyzed (840 of leaves, 840 of stems and 840 of roots segments), 1128 isolates were taxonomically identified. Colonization frequencies of endophytic fungi from leaves, stems, and roots were 50.9, 41.9 and 41.4%, respectively. The percentages of endophytic fungi isolated from leaves, stems, and roots were 37.9, 31.2, and 30.9%, respectively. Morphological and molecular identification using IT Srevealed nineteen taxa of endophytic fungi (Figure1 &Table1). All the identified endophytic fungi belong to Ascomycota.The isolates belonging to three classes, Sordariomycetes, Dothideomycetes, and Eurotiomycetes. *Trichoderma harzianum*

Table 2. Preliminary screening of antimicrobial activity of cultures of endophytic fungi isolated from different parts of the potato plant. Values are means of three independent replicates. ± indicate standard error. Means followed by the same letter within the same column are not significantly different according to Duncan test ($P \leq 0.05$)

Endophytic fungi	Zone of inhibition (mm)			
	Candida albicans	*Salmonella typhimurium*	*Sarcinavent riculi*	*Staphylococcus aureus*
Alternaria tenuissima	26.0±0.35^{g}	21.0±0.3^{e}	20.0±0.47^{d}	11.0±0.25^{b}
Aspergillus flavus	19.0±0.35^{d}	11.0±0.3^{a}	15.0±0.47^{b}	-
Aspergillus niger	-	-	-	-
Aspergillus ochraceus	-	-	-	-
Aspergillus oryzae	-	-	-	-
Chaetomium cervicicola	-	-	-	-
Curvularia lunata	-	-	-	-
Fusarium equiseti	20.0±0.35^{e}	23.0±0.3^{f}	21.0±0.47^{e}	-
Fusarium nygamai	-	-	-	-
Fusarium oxysporum	-	13.0±0.3^{b}	23.0±0.47^{g}	-
Lasiodiplodiatheobromae	-	-	-	10.0±0.25^{a}
Penicillium funiculosum	16.0±0.35^{c}	19.0±0.3^{d}	11.0±0.47^{a}	-
Penicillium minioluteum	-	-	-	-
Penicillium pinophilum	25.0±0.35^{f}	25.0±0.3^{g}	22±0.47^{f}	18.0±0.25^{d}
Penicillium polonicum	-	-	-	-
Penicillium rubens	11.0±0.35^{a}	15.0±.3^{c}	17.0±0.47^{c}	13.0±0.25^{c}
Stemphylium vesicarium	-	-	20.0±0.47^{d}	-
Trichoderma harzianum	-	-	-	-
Ulocladium sp.	13.0±0.35^{b}	_-	_-	-_

was the most dominant endophytic fungus with a relative frequency (RF) of 36.7%, *Aspergillus niger* was the second most one with RF=22.2% while *Penicillium pinophilum* and *Penicillium polonicum* showed theminimum percentage (RF=0.12%) for each (Table1).On the other hand, the genus *Penicillium* was the most diverse (5 different species) among other taxa. All isolates showed a good similarity(98 - 100 %) to the GenBank sequences (Table1).

Preliminary screening of antimicrobial activity

Out of the 19 isolates, three isolates were able to inhibit all the test organisms. The results revealed that all the endophytic fungi exhibited a varied degree of inhibition against the test pathogens (Figure 2). Out of the 19 isolates only 10, endophytic fungi revealed inhibition of at least one of the test pathogens. *Candidaalbicans* and *Salmonella typhi* were inhibited by seven strains while *Sarcinaventriculi* and *Staphylococcus aureus* were inhibited by eight and four, respectively (Figure 2 & Table 2). Among the tested endophytic fungi, *Alternaria tenuissima Penicillium pinophilum and Penicillium rubens* inhibited all the test pathogens with a maximum zone of inhibition of 26 mm and a minimum of 11 against test pathogens, while all the other endophytic fungi except *Alternaria tenuissima*, *Aspergillus flavus*, *Fusarium equiseti, Fusarium oxysporum*, *Lasiodiplodia theobromae, Penicillium funiculosum*, *Penicillium pinophilum Penicillium rubens*, *Stemphylium vesicarium*, and *Ulocladium* sp. showed no inhibition to test pathogens (Table 3).

Enzyme production by endophytic fungi

All the 19 fungal isolates secreted amylase. Cellulase was secreted by twelve while tyrosinase, protease, manganese peroxidase, and laccase were produced by six, three, eleven and two, respectively. None of the isolated fungi produced chitinase (Table 3 and Figure 3).

DISCUSSION

The results showed that higher number endophytic fungi were isolated from leaves than stems and roots tissues. This may be attributed to the large surface area of leaves exposed to the surroundings and the presence of stomata that aid fungal mycelium entrance[24]. The morphological and molecular identification of isolated endophytic fungi revealed 19 species. It was believed that one

Table 3. Extracellular enzyme production of endophytic fungi isolated from different parts of the potato plant. Where- = No production; + = Weak production; ++ = Medium production; +++ = High production

Endophytic fungi	Amylase	Cellulase	Tyrosinase	Protease	Chitinase	Manganese peroxidase	Laccase
Alternaria tenuissima	+++	-	++	-	-	-	+++
Aspergillus flavus	+++	+++	+++	-	-	-	-
Aspergillus niger	+	+++	-	-	-	-	-
Aspergillus ochraceus	+	++	-	+	-	-	-
Aspergillus oryzae	+	+	+++	+++	-	-	-
Chaetomium cervicicola	+	++	+++	-	-	+++	-
Curvularia lunata	++	+	+++	-	-	+	+
Fusarium equiseti	++	-	-	-	-	-	-
Fusarium nygamai	++	++	+++	-	-	-	-
Fusarium oxysporum	++	-	+++	-	-	-	-
Lasiodiplodiatheobromae	+++	+++	-	-	-	-	-
Penicillium funiculosum	+	+	++	-	-	-	-
Penicillium minioluteum	+	+	-	-	-	-	-
Penicillium pinophilum	+	-	-	-	-	+	-
Penicillium polonicum	+	+++	+++	-	-	-	-
Penicillium rubens	+	+	-	-	-	+++	-
Stemphylium vesicarium	++	-	++	-	-	-	-
Trichoderma harzianum	+++	-	-	-	-	+++	-
Ulocladium sp.	++	-	++	++	-	+	-

plant can be a habitat of six fungi, but after adding fungal endophytes, the ratio of fungal: plant species has now been altered to 33:1 [25]. The low rate of colonization (19 species) may be ascribed to the secretion of the phytochemicals[26].

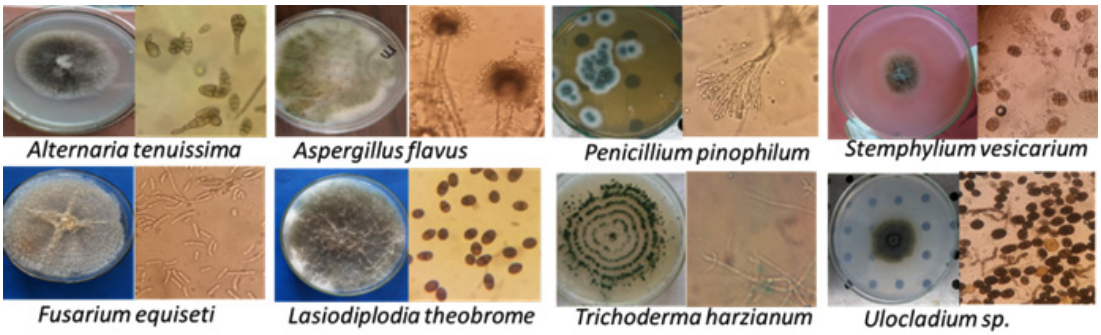

Fig. 1. Pure cultures of some endophytic fungi isolated from different parts of the potato plant

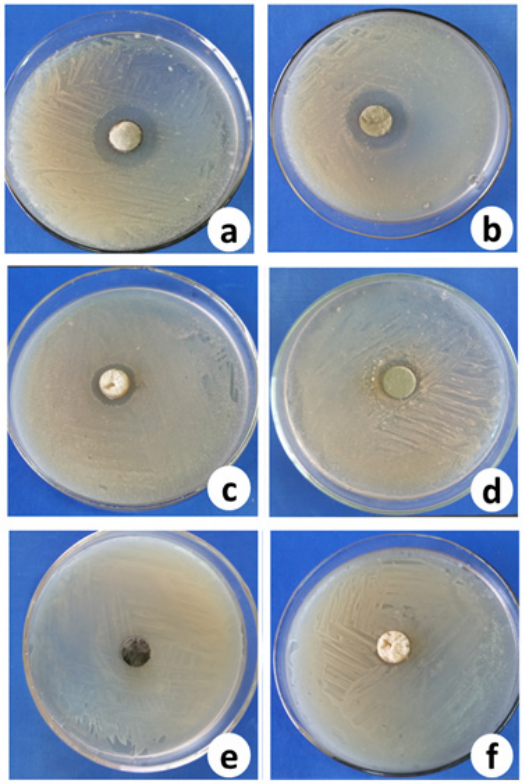

Fig. 2. Antimicrobial activity of six endophytic fungi against human pathogens (a, b, c and d) positive results while (e and f) negative results, a- *Alternaria tenuissima* against *Candida albicans*, b- *Penicillium pinophilum* against *Salmonella typhimurium*, c- *Fusarium oxysporum* against *Sarcinaventriculi*, d- *Penicillium pinophilum* against *Staphylococcus aureus*, e- *Curvularia lunata* against *Sarcinaventriculi* and f- *Fusarium nygamai* against *Candida albicans*

Aspergillus, Fusarium, and *Penicillium* were the predominant genera. Their prevalence may owe to the high spore production that facilitates their widespread and getting established as endophytes [27].

In the preliminary antimicrobial assay, three strains showed inhibition to all the test pathogens (*Candida albicans*, *Salmonella typhi, Sarcinaventriculi* and *Staphylococcus aureus*). *Fusariumequiseti* and *Penicillium pinophilum* revealed significant inhibition of *Salmonella typhimurium*. This may drive to find a new antibiotic against typhoid caused by this bacteria.

Fungal amylase has enormous uses in the food and pharmaceutical industries [14]. In this study, all endophytes degrade starch by amylase enzyme. Out of the 19 endophytic isolates only 12, endophytic fungi produced cellulase enzyme and this enzyme involved in biofuels production [28]. It was established that *Ulocladium* sp., *Aspergillus oryzae,* and *Aspergillus ochraceus* produced protease enzyme, almost this enzyme is applied in treatments of diabetes [29]. Tyrosinase was produced by eleven strains and it is known for lignin degradation and synthesis of melanin [14].*Alternaria tenuissima* and *Curvularia lunata* showed laccase production. Laccase plays a role in the degradation of lignin, and can, therefore, be classed as lignin-modifying enzymes [30]. Lately, the effectiveness of laccases has also been applied to nanobiotechnology [31]. In similar studies, Rajagopal *et al*. [26] reported that *Curvularia lunata* produced laccase. Among all isolates, only six isolates produced manganese peroxidase and this enzyme

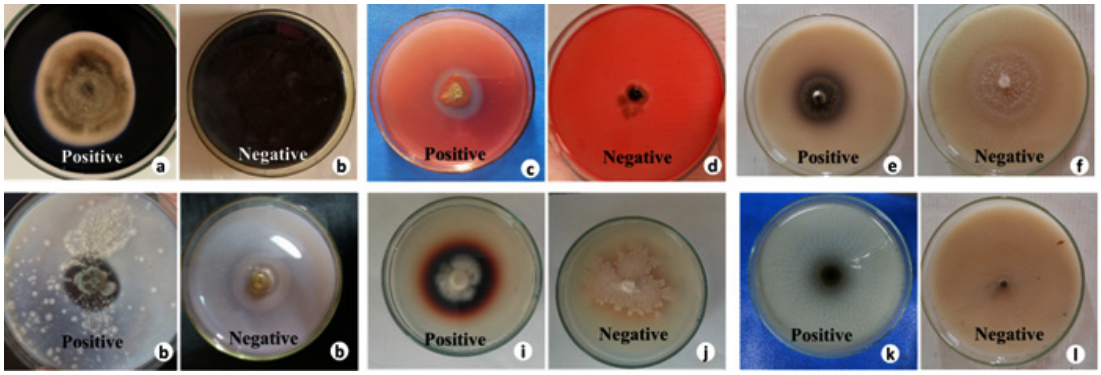

Fig. 3. Enzyme production by endophytic fungi isolated from different parts of potato plant, (a and b) amylase production, (c and d) cellulase production, (e and f) tyrosinase production, (g and h) protease production, (i and j) manganese peroxidase production, (k and l) laccase production

used for the degradation of lignin that plays a major role in biomass biodegradation[32]. Endophytic fungi produced a variety of enzymes in this study that may work as a unique source for applications in industry.

CONCLUSION

In the present study, 19 endophytic fungi were isolated and identified from leaves, stems, and roots of the potato plant. These fungi were able to produce extracellular enzymes such as amylase, cellulase, tyrosinase, protease, manganese peroxidase and laccase. Also, Fouda *et al*.[13] were able to produce enzymes such as cellulase, amylases, laccases, chitinases, and proteinases from endophytic fungi. The results demonstrated that all the test pathogens were inhibited by *Alternaria tenuissima, Penicillium pinophilum* and *Penicillium rubens*. This evidenced that endophytic fungi have antimicrobial compounds extracted from them Deshmukh *et al*.[10]. According to the obtainable results, it can be obviously realized that endophytes isolated from potato plants could be valuable and applicable in many industries, mainly the pharmaceutical industries.

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