

Potential of Endophytic Fungi from *Orobanche* spp. as Novel Sources of L-Asparaginase, an Enzyme with Anticancer Applications

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Abstract

In this study, endophytic fungi present in Orobanche species were evaluated as emerging sources of bioactive compounds, with the aim of identifying microorganisms producing the anticancer enzyme L-asparaginase (L-ASNase). Endophytic fungi were isolated using a standard surface sterilization method. Initial screening for L-ASNase production was performed via a qualitative assay on modified Czapek Dox agar; in this process, the formation of pink halos, resulting from the hydrolysis of asparagine into aspartic acid and ammonia, indicated enzyme production. Enzyme activity was subsequently quantified using the Nessler method. Of the 34 isolated morphotypes, 20 isolates demonstrated L-ASNase production potential, with activities ranging from (0 to 5.79 U/mL). Among these Alternaria longipes, Macrophomina phaseolina, Fusarium verticillioides, and Aspergillus flavus exhibited the highest activity levels. To optimize of enzyme production by Alternaria longipes SH₂₅ isolate, a 2² factorial experimental design was employed. The results demonstrated that L-ASNase production varied between (1.59 to 5.95 U/mL). The maximum enzyme activity (5.95 U/mL) was obtained at pH 5.0 and 1.5% L-asparagine concentration.

Introduction

Endophytic fungi are microorganisms that colonize the intercellular and intracellular tissues of healthy plants without causing visible disease symptoms. They establish mutualistic or neutral associations with their host plants and are widely distributed among diverse plant species. Plant-derived compounds such as flavonoids and phenolic metabolites facilitate the colonization of plant tissues by fungal endophytes originating from the rhizosphere. To maintain long-term symbiotic relationships, endophytic fungi produce a wide range of secondary metabolites and extracellular enzymes that contribute to plant adaptation and defense [2, 34]. Owing to their remarkable biosynthetic potential, endophytic fungi have attracted increasing attention as valuable sources of novel bioactive compounds with pharmaceutical, agricultural, and industrial applications. These metabolites include alkaloids, flavonoids, quinones, steroids, and terpenoids with antimicrobial, antioxidant, antiviral, and anticancer activities [19]. In addition, endophytic fungi produce numerous industrially important enzymes, including amylases, cellulases, lipases, pectinases, proteases, and L-asparaginase, making them promising resources for biotechnology and pharmaceutical industries [3].

L-asparaginase is one of the most important therapeutic enzymes used in the treatment of acute lymphoblastic leukemia (ALL) and certain lymphomas. The enzyme catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia, thereby depriving leukemic cells of an essential amino acid required for their proliferation [24]. Since malignant lymphoblasts express low levels of L-asparagine synthetase, they depend on extracellular L-asparagine for survival. Consequently, depletion of circulating L-asparagine induces apoptosis of leukemic cells while exerting comparatively limited effects on normal cells [5]. Commercial L-asparaginase is mainly produced from *Escherichia coli* and *Dickeya chrysanthemi*. However, bacterial L-asparaginases are frequently associated with hypersensitivity reactions, immunogenicity, hepatotoxicity, pancreatitis, and reduced therapeutic efficacy following repeated administration. Therefore, considerable efforts have been devoted to identifying alternative

microbial sources of this enzyme. Among these, fungal L-asparaginases have attracted increasing attention because they may exhibit lower immunogenicity and improved biocompatibility than their bacterial counterparts, although further investigations are required to confirm these advantages [31].

Among the alternative microbial resources, endophytic fungi are considered particularly promising because of their ability to produce diverse extracellular enzymes and bioactive metabolites. Several studies have demonstrated the production of L-asparaginase by endophytic fungi isolated from medicinal plants. Species belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Cladosporium*, *Curvularia*, and *Phoma* have shown considerable potential for enzyme production. For example, Manasa et al. [23] reported efficient L-asparaginase-producing endophytic fungi isolated from medicinal thyme, whereas Sarquis et al. [32] demonstrated enzyme production in species of *Aspergillus*, *Penicillium*, and *Fusarium*. More recent studies have further highlighted endophytic fungi as valuable sources of pharmaceutically important enzymes and bioactive metabolites [39]. Nevertheless, most previous investigations have focused on endophytes associated with common medicinal plants, whereas microorganisms inhabiting holoparasitic plants remain largely unexplored.

Holoparasitic plants possess unique ecological and physiological characteristics because they rely entirely on their hosts for nutrients and exhibit complex host–parasite interactions. Such distinctive ecological conditions may shape unique endophytic fungal communities with unexplored metabolic capabilities and significant biotechnological potential. Species of *Orobanche* (broomrape) are obligate holoparasitic plants belonging to the family Orobanchaceae, with approximately 130 species distributed mainly throughout the Mediterranean Basin and the Middle East [21]. Although broomrape is considered one of the most destructive parasitic weeds affecting numerous economically important crops, several species have also been used in traditional medicine owing to their content of phenylethanoid glycosides, terpenoids, steroids, and alkaloids [18, 29]. These biological characteristics suggest that *Orobanche* spp. may harbor unique endophytic fungal communities capable of producing valuable bioactive compounds and industrial enzymes.

To the best of our knowledge, no previous study has investigated the diversity of endophytic fungi associated with *Orobanche* spp. for their ability to produce L-asparaginase. Therefore, the objectives of the present study were to isolate and identify endophytic fungi inhabiting *Orobanche* spp., evaluate their ability to produce L-asparaginase using both qualitative and quantitative assays, and identify promising fungal isolates as potential alternative sources of this clinically important enzyme.

Materials and methods

Separation of Endophytic Fungi

In the summer of 2023, a total of 300 healthy *Orobanche* spp. plant samples were collected from agricultural fields in northwestern Iran. The samples were immediately transported to the laboratory and thoroughly washed with tap water to remove adhering soil particles and surface contaminants. The

entire plant stem, including the flowers, was cut into approximately 5 mm long segments. To eliminate surface microorganisms (epiphytes), the segments were first briefly immersed in 70% (v/v) ethanol for 30–60 seconds, followed by immersion in 1% (v/v) sodium hypochlorite (NaOCl) for 3–5 minutes. Subsequently, the segments were rinsed several times with sterile distilled water. To ensure the complete removal of surface contaminants and to verify that any subsequent fungal growth originated solely from endophytic colonization rather than surface contamination, the final rinse water was plated on PDA medium; the absence of microbial growth after the incubation period confirmed the effectiveness of the surface sterilization. Following drying on sterile filter paper, the surface-sterilized segments were transferred onto PDA (Potato Dextrose Agar) medium supplemented with 20 mg/L streptomycin sulfate to prevent bacterial growth. The Petri dishes were incubated at 25°C in complete darkness for 20 days [37].

Identification endophytic species

Genomic DNA extraction was performed using a modified CTAB method based on the protocol of. Fungal mycelia were collected and ground after growing in PDB liquid culture medium for one week (120 rpm). DNA extraction was done using CTAB buffer containing 2-mercaptoethanol. The samples were incubated for 45 minutes at 65°C. To remove proteins, chloroform-isoamyl alcohol mixture (24:1) was used. After centrifugation (12000 rpm for 10 minutes), the aqueous phase was separated and DNA was precipitated with cold isopropanol. After washing with 70% ethanol, the DNA precipitate was dissolved in 70 µL of distilled water. Polymerase chain reaction (PCR) for genomic DNA amplification with a 25 µL reaction mixture containing 1 µL of each primer ITS1 (5'- TCCGTAGGTGAACCTGCGG-3'), ITS4 (3'- TCCTCCGCTTATTGATATGC-5') and TEF1-α (EF1 GAYTTCATCAAGAACATGAT), (EF2 GACGTTGAADCCRACRTTGTC) β-tubulin (Bt2α GGTAACCAAATCGGTGCTGCTTTC), (Bt2b ACCCTCAGTGACCCTTGG), 2 µL of genomic DNA (40 ng), 12.5 µL of Mix Master amplicon Red x2 and 8.5 µL of double distilled water. became. The PCR program consisted of initial denaturation at 95°C for 5 min, 35 cycles with denaturation (95°, 30 s), primer annealing (50–65°, 30 s) and extension (72°, 55 s), and final extension. It was at 72 degrees for 10 minutes. PCR products were electrophoresed on 1% agarose gel, stained with Gel Red and imaged by Gel Documentation system [10]. Sequencing was done by Sanger method in Macrogen (South Korea) and the sequences were edited with ChromasPro software and analyzed by BLAST in NCBI. The final sequences were registered in GenBank and the accession number was obtained (<http://blast.ncbi.nlm.nih.gov/BLAST.cgi>)

Primary screening for L-asparaginase production

Modified Czapek Dox agar (McDox) was used to investigate fungi for the production of L-asparaginase. For this experiment, a 5-mm mycelial plug of the fungus was inoculated onto the McDox medium containing agar powder (20 g/L), glucose (2 g/L), L-asparagine (10 g/L), KH₂PO₄ (1.52 g/L), KCl (0.52 g/L), MgSO₄·7H₂O (0.52 g/L), Cu(NO₃)₂·3H₂O (0.001 g/L), ZnSO₄·7H₂O (0.001 g/L), and FeSO₄·7H₂O (0.001 g/L). In addition, 0.3 mL of 2.5% phenol red dye was added to the medium as an indicator. Controls were

prepared by inoculating mycelial plugs onto McDox agar without L-asparagine. All plates were incubated at $28 \pm 2^\circ\text{C}$ for 5 days. After the incubation period, the diameter of the pink zone that indicates L-asparaginase production was measured. The above-mentioned method helps in determination and screening of the potential of fungi regarding the production of L-asparaginase, which could be useful in further studies [8]. A negative control consisting of the same culture medium without L-asparagine and a fungal inoculum was included to verify that the fungus alone did not cause a color change. In addition, an uninoculated medium without L-asparagine was used to confirm that the culture medium itself remained unchanged.

L-ASNase production in liquid medium

The L-asparaginase (L-ASNase) production protocol was developed based on the established studies by Loureiro et al. [22] and Silva et al. [33].

In the initial phase, biomass production was initiated using a modified Czapek Dox Medium (CDM) [36], as adapted by Pádua et al. [26]. The medium contained glucose (14.0 g L^{-1}), L-asparagine (10.0 g L^{-1}), KH_2PO_4 (1.52 g L^{-1}), KCl (0.52 g L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.52 g L^{-1}), $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (0.01 g L^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g L^{-1}), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g L^{-1}), and $(\text{NH}_4)_2\text{SO}_4$ (2.0 g L^{-1}), with the pH adjusted to 6.2.

For inoculum preparation, five mycelial discs (5 mm in diameter) were aseptically cut from the actively growing margin of 7-day-old fungal colonies previously cultivated on malt extract agar. The discs were transferred into 250 mL Erlenmeyer flasks containing 100 mL of sterile CDM. The cultures were incubated at 30°C for 96 h under continuous agitation at 120 rpm on a rotary shaker. After incubation, the fungal biomass was separated by filtration using Whatman No. 1 filter paper. The collected biomass was subsequently used for the enzyme production stage.

Fermentative Stage:

For the subsequent fermentative stage, the biomass generated in the pre-fermentative step was utilized as the inoculum. This biomass was introduced into a modified CDM medium, also based on the formulation by Pádua et al. [26]. The key modifications in this medium were a reduced glucose concentration to 2.0 g L^{-1} and the complete exclusion of $(\text{NH}_4)_2\text{SO}_4$. The inoculated media were incubated under identical conditions to the previous stage: 30°C for 96 hours at 120 rpm. Finally, the cultures were filtered once more using Whatman No. 1 filter paper. The enzymatic activity present in the resulting biomass was then quantified according to the methodology detailed below.

Determination of L-ASNase Activity

The enzymatic activity of L-ASNase was quantified following the procedure outlined by Drainas et al. (1977), with modifications adapted from Silva et al. [33] and Pádua et al. [26]. The selected fungal isolates were cultured in 30 ml of McDox broth medium for five consecutive days at $28 \pm 2^\circ\text{C}$ with shaking conditions at a speed of 120 rpm. A volume of 100 μL of the culture medium containing the

enzyme was extracted and transferred to the microcentrifuge tubes. For the preparation of test, 100 µl Tris HCl buffer (pH 7.2), 200 µl asparagine solution (0.04 M) and 100 µl sterile distilled water was added to the sample. The mixture was incubated at $37 \pm 2^{\circ}\text{C}$ for one hour. The reaction was stopped by adding 100 µl of trichloroacetic acid (TCA) at a final concentration of 1.5 M. Thereafter, 100 µl of the mixture was added to new tubes with 750 µl of sterile distilled water and 300 µl of Nessler's reagent. After incubating the tube mixtures for 20 min at $2 \pm 28^{\circ}\text{C}$, absorbance was measured at a wavelength of 450 nm. Liquid culture medium without fungi was used as a blank, while a sample inactivated with TCA prior to the reaction served as the negative control. All experiments were performed in triplicate. A unit of L-asparaginase activity is defined as the amount of enzyme that catalyzes the production of 1 µmol/min of ammonia per minute at $37 \pm 2^{\circ}\text{C}$. After incubation, the absorbance was measured at 450 nm using a spectrophotometer. The amount of ammonia released during the enzymatic reaction was determined from a standard curve prepared using known concentrations of ammonium ions. L-asparaginase activity was calculated according to the following equation:

L-asparaginase activity (U/mL) = ($\mu\text{mol NH}_3$ released / (incubation time (min) $\times$ volume of enzyme extract (mL)). [14].

One unit (U) of L-asparaginase activity was defined as the amount of enzyme required to release 1 µmol of ammonia per minute under the assay conditions.

Partial optimisation of L-ASNase production

To partially optimise L-ASNase production, a statistical experimental design (2^2 full factorial design with four central points) was employed [8]. The experimental design comprised eight trials, involving two independent variables (L-asparagine concentration and pH), with four replicates at the central point. The pre-fermentation conditions were maintained as previously described in the 'L-ASNase production in liquid medium' section. The modified CDM was utilized for fermentation, with variables adjusted according to the experimental design matrix. The flasks were incubated at 120 rpm and 30°C for 120 h. Following the incubation period, the fungal biomass was harvested via filtration and used to determine enzyme activity. Based on the results obtained from the factorial design, further experiments were conducted to enhance enzymatic production.

Statistical evaluation

The experiments were conducted as a completely randomized design (CRD) with three replications for each investigated parameter. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0. Prior to analysis of variance, the normality of data distribution and homogeneity of variances were evaluated using the Shapiro–Wilk test and Levene's test, respectively. The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's HSD test for mean comparisons at a significance level of $P < 0.05$.

Results

Isolation of endophytes

In this study, 300 samples of *Orobanch* spp were transferred to the laboratory and the stem and flower parts were separated from each other and each was cultivated separately. 83 isolates were isolated from the stem and 42 isolates were isolated from the flower. The isolates were divided into 34 morphotypes, each morphotype having similar morphological characteristics. For further experiments, representative of the morphotypes were used.

Molecular identification

The phylogenetic tree showed that the *Alternaria* isolates clustered with their corresponding reference species with high bootstrap support, confirming their taxonomic identity (Fig. 2). The obtained isolates were assigned to species including *Alternaria longipes*, *A. alternata*, *A. tenuissima*, *A. solani*, *A. brassicicola*, and other closely related taxa. The isolate SH₂₅ clustered with reference strains of *A. longipes* with 100% bootstrap support, while SH₂₃, SH₇₄, SH_{20A}, and SH_{AA} grouped with *A. alternata*, *A. tenuissima*, *A. solani*, and *A. brassicicola*, respectively. The overall topology of the tree was consistent with the sequence similarity analysis and clearly resolved the *Alternaria* species into distinct, well-supported clades.

Fig .1 Phylogenetic relationships of *Alternaria* isolates based on TEF1- α gene sequences. The phylogenetic trees were constructed using the Maximum Likelihood and Neighbor-Joining methods with 1000 bootstrap replicates.

Identification of L-asparaginase producing endophytes and their activities

Qualitative assay

For initial screening, endophyte isolates were cultured on Modified Czapek Dox agar (McDox). In this medium, L-asparagine served as the sole nitrogen source, and its hydrolysis by the enzyme released ammonia. This reaction increased the pH of the medium, causing the phenol red reagent from yellow to pink and forming pink halos formed around the colonies (Fig. 2). The diameter of the halos ranged from 0.3 to 3.50 cm, with a median of 1.4 cm. The largest pink zone diameter (3.5 cm) was observed for *Fusarium clavum* (SH₄), followed by *Alternaria longipes* (2.9 cm). This qualitative measurement served as an initial screening tool, and isolates that produced visible pink halos were selected for subsequent quantitative analysis.

Quantitative assay

Qualitatively positive isolates were cultured in McDox liquid medium (Falcon tubes) to determine their actual L-asparaginase activity. After the incubation period, samples were collected and reacted with Nessler reagent to quantify the released ammonia. Enzyme activity was expressed in units per milliliter (U/mL), and results indicated that the L-asparaginase ranged from 0.701 to 5.79 U/mL (Table 1, Fig. 3).

Four isolates (*Alternaria longipes*, *Macrophomina phaseolina*, *Aspergillus flavus*, *Fusarium verticillioides*) exhibited the highest enzymatic activity, whereas the remaining isolates showed low to moderate activity (0–2.17 U/mL). Notably, some isolates that formed large halos in the qualitative assay, such as *Fusarium proliferatum* (3.50 cm), exhibited little or no L-asparaginase activity. These results indicate that halo size on solid media does not always accurately reflect the actual enzymatic activity. To examine the relationship between halo size in the qualitative assay and quantitative L-asparaginase activity, simple regression analysis was performed.

The results revealed a weak and non-significant correlation between halo diameter and enzymatic activity. Although the quantitative analysis of halo zone diameter did not reveal significant differences among fungal isolates based on one-way ANOVA, qualitative assessment showed significant variation in the distribution of L-asparaginase-producing and non-producing isolates. These findings indicate that while the extent of halo formation among positive isolates was statistically similar, the occurrence of L-asparaginase production differed among fungal isolates (Table 1).

Table 1

GenBank accession numbers for partial ITS and TEF1- α sequences of reference species used in phylogenetic analyses: marked taxa(∞) were utilized for partial sequencing of β -tubulin

Isolate	GenBank Accession No. (TEF1- α / β -tubulin, ITS)	Closest related species	Similarity (%)
SH ₄	PX050527 / PV926797	<i>Fusarium clavum</i>	100
SH ₂₃	PX050528 / PV927073	<i>Alternaria alternata</i>	100
SH ₇₄	PX050529 / PV929796	<i>Alternaria tenuissima</i>	100
SH _{51K}	PX050526 / PV929794	<i>Fusarium nygamai</i>	99
SH _{672A}	PX050543 / PV927074	<i>Aspergillus flavus</i> ∞	100
SH _{20A}	PX050530 / PV927075	<i>Alternaria solani</i>	100
SH ₂₅	PX050531 / PV927076	<i>Alternaria longipes</i>	100
SH _{A03}	PX050544 / PV927077	<i>Penicillium simplicissimum</i> ∞	100
SH ₉₉	PX050532 / PV931816	<i>Fusarium poae</i>	99
SH _{GG}	PX050533 / PV927079	<i>Fusarium graminearum</i>	100
SH ₇₂	PX050545 / PV927078	<i>Penicillium chrysogenum</i> ∞	100
SH ₇₈	PX050534 / PV929795	<i>Fusarium acuminatum</i>	100
SH ₆₇₂	PX050539 / PV929762	<i>Trichoderma harzianum</i>	100
SH ₁₆₆	PX050535 / PV927080	<i>Fusarium solani</i>	98
SH ₄₉	PX050536 / PV927081	<i>Fusarium verticillioides</i>	100
SH _{4A}	PX050537 / PV927082	<i>Macrophomina phaseolina</i>	100
SH _{AAA}	PX050538 / PV927084	<i>Alternaria brassicicola</i>	100
SH _{20B}	PX050542 / PV927104	<i>Trichoderma viride</i>	100
SH _{AA}	PX050540 / PV927106	<i>Fusarium proliferatum</i>	98
SH _{49B}	PX050541 / PV927107	<i>Fusarium oxysporum</i>	100

Table 2

L-asparaginase activity of endophytic fungal isolates from *Orobanch* spp.. Mean and standard deviation are provided. Means with different letters indicate statistically significant differences as determined by Tukey's Studentized Range Test (HSD0.05).

Endophytic isolate	L-asparaginase activity (U/mL)	SD	Tukey group
SH4	1.08	0.570	c
SH23	1.31	0.042	c
SH74	0.00	0.000	e
SH51K	0.00	0.000	e
SH672A	2.04	0.438	b
SH20A	1.06	0.127	c
SH25	5.79	0.332	a
SHA03	0.00	0.000	e
SH99	1.11	0.120	c
SHGG	1.09	0.048	c
SH72	0.00	0.000	e
SH78	1.33	0.184	c
SH672	0.00	0.000	e
SH166	1.08	0.113	c
SH49	2.24	0.368	b
SH4A	3.30	0.566	a
SHAAA	0.83	0.113	d
SH20B	0.00	0.000	e
SHAA	0.00	0.000	e
SH49B	2.17	0.148	b

Table 3
Analysis of variance (ANOVA) of L-asparaginase production by endophytic fungal isolates in liquid culture, based on quantitative enzyme activity assay ($P < 0.05$).

p-value	F-value	MS	df	Source of variation
0.025	44.61	3.087	19	Isolates
		0.0692	40	Error
			59	Total

L-ASNase production using the 2^2 factorial design varied from 1.590 to 5.95 U/mL (Table 4). The highest L-ASNase production (5.95 U/mL) was obtained at pH 5.0 with 1.5% L-asparagine (Assay 2, Table 4). Analysis of variance (ANOVA) showed that L-asparagine concentration had a statistically significant effect on enzyme production ($p = 0.011$), whereas pH alone did not show a significant effect ($p = 0.291$). Notably, the interaction between L-asparagine and pH was statistically significant ($p = 0.029$), indicating that the effect of L-asparagine on enzyme production depends on the pH level. The lack of fit was not statistically significant, and the coefficients of determination (R^2) and adjusted R^2 were 0.949 and 0.880, respectively, indicating a strong model fit.

Table 4
Production of L-asparaginase by *Alternaria longipes* SH25 in the 2^2 factorial experimental design

Assay	L-asparagine (%)	pH	L-Asparaginase activity (U/mL)
1	0.5	5.0	1.590
2	1.5	5.0	5.95
3	0.5	7.0	2/79
4	1.5	7.0	3/59
5	1.0	6.0	4/43
6	1.0	6.0	4/82
7	1.0	6.0	3/80
8	1.0	6.0	4.01

Thermal stability of L-asparaginase

The thermal stability of partially purified L-asparaginase was evaluated by incubating the enzyme at 35°C for different time intervals. The enzyme retained a high proportion of its initial activity throughout the incubation period (Table 5). After 30 and 60 min, the residual activities were 99.36% and 68.98%,

respectively. Even after 120 min, the enzyme maintained approximately 69.22% of its original activity, indicating relatively good thermal stability at moderate temperatures.

Table 5
Effect of temperature on L-asparaginase activity of the selected fungal isolate (SH₂₅)

Temperature (°C)	L-asparaginase activity (μmol mL ⁻¹ min ⁻¹)
25	1.53 ± 0.25
30	2.18 ± 0.36
35	3.16 ± 0.21
40	1.45 ± 0.49
45	1.27 ± 0.55
Note: Values are presented as mean ± standard deviation (SD) of three measurements.	

Table 6
Thermal stability of L-asparaginase produced by the selected fungal isolate SH25 at 35°C

Incubation time (min)	Residual L-asparaginase activity (%)
0	100
30	99.36
60	68.98
120	69.22

These results suggest that the enzyme possesses sufficient stability for potential industrial and biotechnological applications requiring prolonged incubation under mild thermal conditions.

Discussion

The qualitative plate assay revealed that 20 out of 34 fungal isolates obtained from Orobanche species produced distinct pink zones, indicating their potential ability to produce L-asparaginase. However, the diameter of the pink zones did not show a strong correlation with the enzyme activity quantified by the Nesslerization method. For example, *Fusarium clavum* (SH₄) exhibited the largest pink zone (3.50 cm), while its quantitative enzyme activity was relatively low (1.08 U/mL). In contrast, *Alternaria longipes* (SH₂₅) showed a high enzyme activity (5.79 U/mL) with a pink zone diameter of 2.90 cm. Similar inconsistencies between qualitative screening and quantitative enzyme assays have been reported in previous studies [37, 20]. Therefore, plate-based assays are considered useful for rapid screening of potential isolates, whereas quantitative assays are necessary for accurate evaluation of enzyme production.

In this study, endophytic fungi associated with *Orobanche* sp. collected from agricultural fields of Iran were investigated as potential sources of L-asparaginase. Among all evaluated isolates, *Alternaria longipes* (SH₂₅) exhibited the highest enzyme activity (5.79 U/mL), representing the most promising candidate for further investigation. To our knowledge, this is the first report of L-asparaginase production by an endophytic *A. longipes* isolate obtained from *Orobanche* sp. Previous studies have reported variable L-asparaginase production among *Alternaria* isolates. Araújo-Magalhães et al. [4] and De Andrade et al. [12] reported low or undetectable enzyme activity in some *Alternaria* isolates obtained from different hosts. These differences may be associated with fungal species identity, host association, environmental adaptation, and genetic variation among isolates. Therefore, the high activity observed in *A. longipes* SH₂₅ highlights the importance of exploring native endophytic fungi as novel sources of industrially valuable enzymes.

The remarkable L-asparaginase production by *A. longipes* SH₂₅ may be related to specific physiological and metabolic adaptations of this fungus. L-asparaginase production is often influenced by fungal nitrogen metabolism, enzyme secretion capacity, and regulatory mechanisms controlling nitrogen acquisition. Since L-asparagine can serve as an alternative nitrogen source, efficient utilization of this compound may provide a selective advantage for fungi inhabiting nutrient-limited environments. Endophytic fungi colonizing internal plant tissues frequently experience variable nutrient availability and therefore may develop specialized mechanisms for nutrient acquisition, including production of extracellular hydrolytic enzymes [9]. In this context, the high activity of *A. longipes* SH₂₅ may reflect its enhanced ability to mobilize nitrogen sources through L-asparagine degradation.

Furthermore, members of the genus *Alternaria* possess considerable metabolic flexibility and are known to produce diverse extracellular enzymes and secondary metabolites. Such metabolic versatility may contribute to differences in enzyme production among closely related fungal species or even among individual isolates of the same species. Variations in genes involved in nitrogen regulation, enzyme biosynthesis, secretion pathways, and adaptation to host-associated environments could explain the superior L-asparaginase production observed in *A. longipes* SH₂₅ compared with other isolates. Although the exact molecular mechanisms responsible for this high activity require further investigation, the interaction between *A. longipes* SH₂₅ and its *Orobanche* host may have promoted metabolic traits associated with efficient nutrient acquisition and environmental adaptation.

Molecular identification and phylogenetic analysis further confirmed the identity of the fungal isolates. The phylogenetic placement of *A. longipes* SH₂₅ within the cluster of closely related reference sequences supported its species-level identification. Molecular markers such as ITS and protein-coding genes provide reliable tools for accurate identification of fungal species, which is particularly important because enzyme-producing ability may vary significantly among closely related taxa. The confirmed identity of the high-producing isolate strengthens the potential value of *A. longipes* SH₂₅ as a novel source of L-asparaginase.

The high enzyme activity and favorable thermal stability observed for *A. longipes* SH₂₅ indicate its potential for further optimization and evaluation for biotechnological applications. Enzymes with suitable stability characteristics are preferred in biotechnological processes because they can maintain activity during processing and storage conditions. Previous studies have shown that optimization of culture conditions and fermentation parameters can significantly enhance fungal L-asparaginase production [40, 28]. Future studies focusing on optimization of culture conditions, genetic improvement, mutagenesis, and protein engineering approaches may further enhance L-asparaginase production. Overall, this study demonstrates that *Orobanche* sp., despite being an agriculturally important parasitic plant, harbors diverse endophytic fungi with valuable biotechnological potential. The identification of *A. longipes* SH₂₅ as a high-producing L-asparaginase producer highlights the importance of exploring unexplored fungal communities as promising resources for enzyme discovery and sustainable biotechnology development.

Declarations

The authors have no relevant financial or non-financial interests to disclose.

Author Contribution

R.G has conducted tests as PhD student. She also has written the initial manuscript. R.H. is the supervisor who has reviewed the M.S.

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Figures

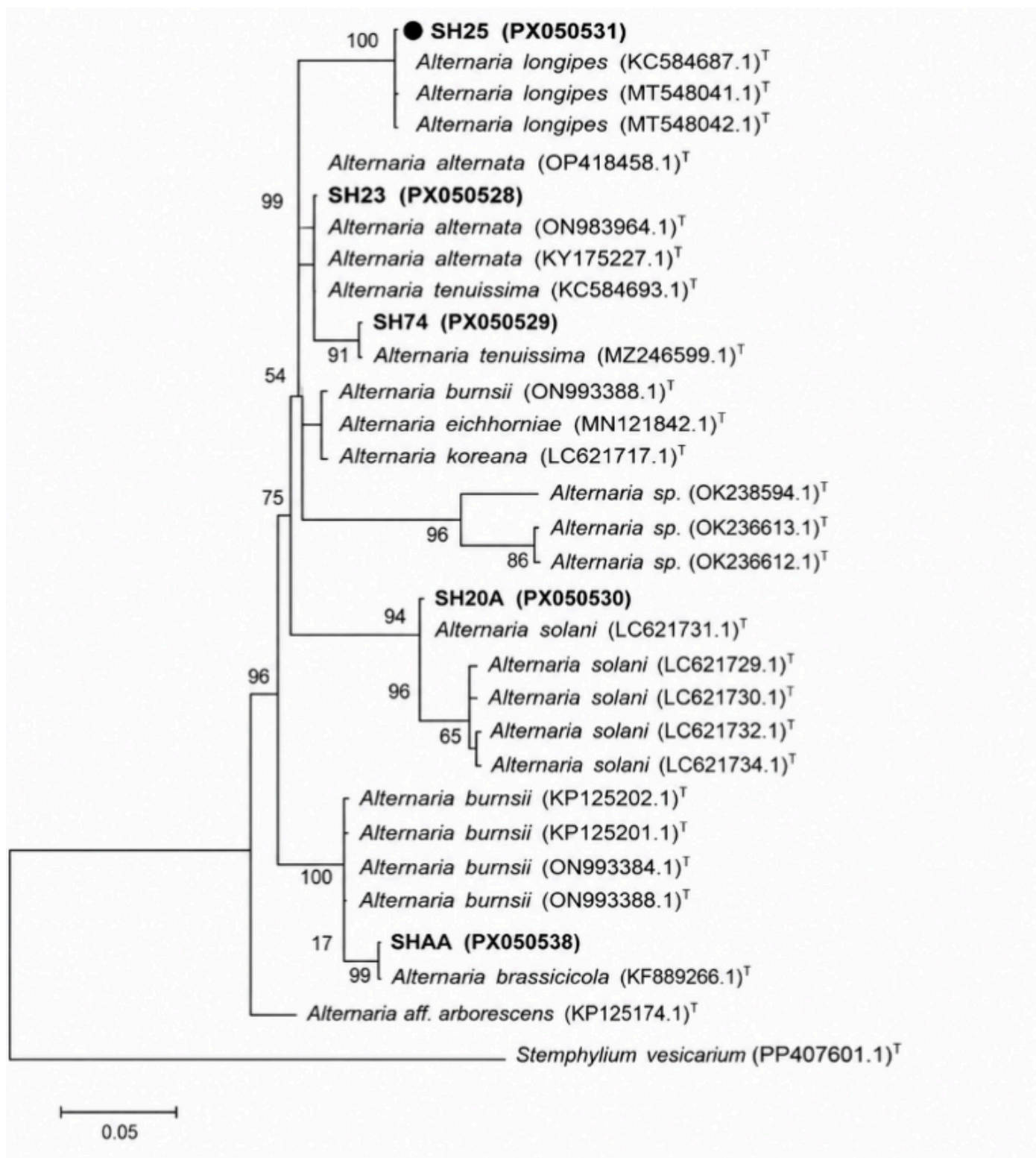


Figure 1

Phylogenetic relationships of *Alternaria* isolates based on TEF1-α gene sequences. The phylogenetic trees were constructed using the Maximum Likelihood and Neighbor-Joining methods with 1000 bootstrap replicates.

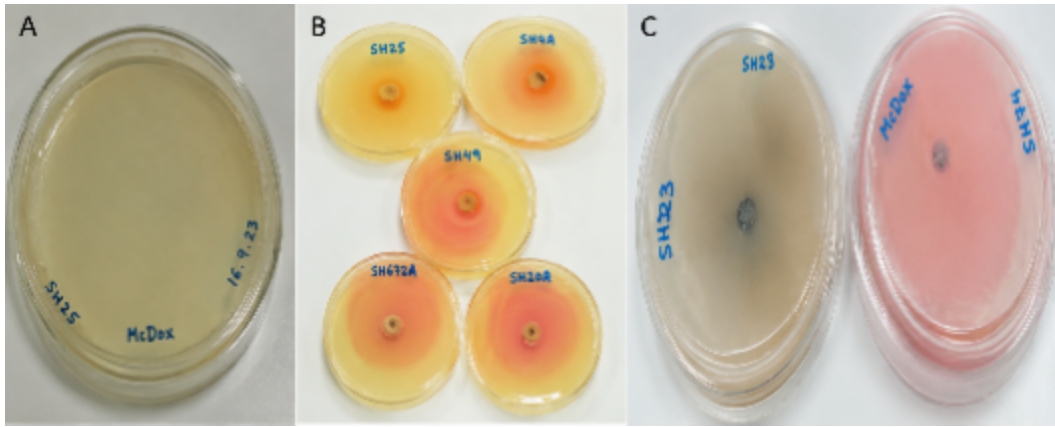


Figure 2

Fungal endophytes producing L-asparaginase were identified based on the formation of pink area (A) and the endophyte with the highest enzyme activity along with L-asparaginase control (B) (*Alternaria longipes*). The cultures were 5 days old. Negative Control: uninoculated culture medium without L-asparagine (c).

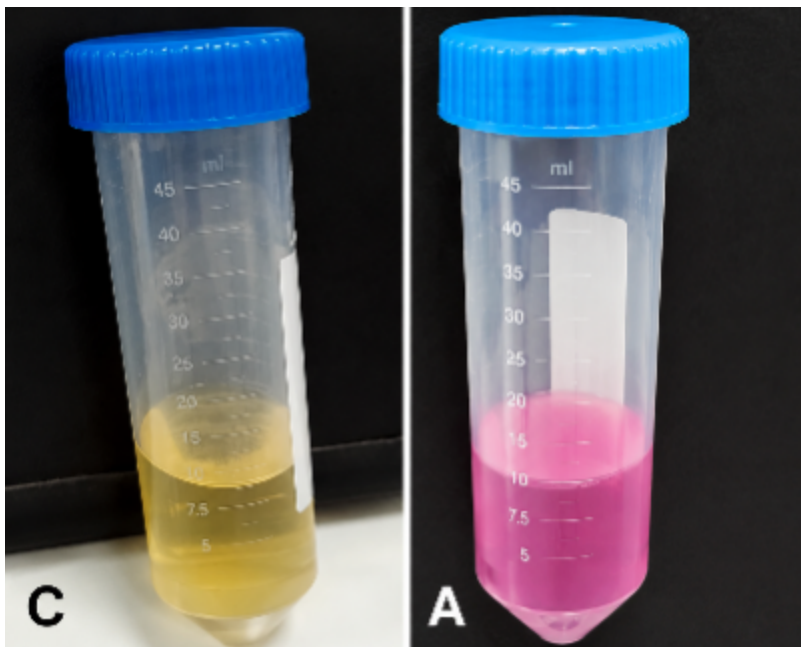


Figure 3

the endophyte with the highest enzyme activity along with L-asparaginase control of McDox broth medium. Negative Control(C). endophyte *Alternaria longipes* with the highest enzyme activity(A)