Journal of Nepal Biotechnology Association, 6 (1), 2025: 9-14 ISSN: 1728-1628
DOI: https://doi.org/10.3126/jnba.v6i1.76913

Research Article

Isolation and Purification of L-Asparaginase Producing Endophytic Fungi from *Ocimum tenuiflorum* L.

Saru Kayastha, Ganga Bir Rai, Shiva Dahal, Bimala Subba

Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal

ARTICLE INFO

Submission: 25/02/2025
Acceptance: 14/03/2025

CORRESPONDENCE

Bimala Subba

Central Department of Chemistry
Tribhuvan University
Kirtipur, Kathmandu
Nepal

Email: bimala.subba@cdc.tu.edu.np

0000-0001-9211-5072

Abstract

Endophytic fungi are essential sources of bioactive chemicals and enzymes. L-asparaginase is a key enzyme used in the treatment of acute lymphoblastic leukemia and lymphoblastic lymphoma. However, the usage of commercial L-asparaginase generated from prokaryotes is limited due to severe clinical hypersensitivity. Studies showed that L-asparaginase from eukaryotic sources can significantly reduce allergy reactions. Thus, nowadays, researchers are looking for eukaryotic sources to manufacture L-asparaginase. This work isolated and purified four endophytic fungi known as (Tulsi 'a', 'b', 'c', and 'd') from the leaf tissue of *Ocimum tenuiflorum* (Holy Basil). Morphological analysis revealed Tulsi 'b' corresponds to *Microsporum* sp., Tulsi 'c' corresponds to *Penicillium* sp., while Tulsi 'd' corresponds to *Trichoderma* sp. Tulsi 'a' did not produce spores so morphological identification of Tulsi 'a' was done. Screening for L-asparaginase biosynthesis on modified Czapek Dox (MCDox) medium showed varying activity: Tulsi 'b' and 'c' exhibited a faint pink hue (pH-dependent), but Tulsi 'a' and 'd' caused the medium to turn yellow. Submerged fermentation followed by nesslerization method was used to measure the concentration of ammonia formed. One unit of asparaginase catalyzes the formation of 1 μM of ammonia per minute. So, amount of ammonia in the sample gives enzyme activity. Tulsi 'a' demonstrated the maximum activity (31.24 U/ml) on day 5, while Tulsi 'd' reached its peak on day 16 (77.43 U/ml). Temporal variations in activity indicate phase-dependent enzyme synthesis. These findings highlighted the potential of endophytic fungi from *O. tenuiflorum* as a source of L-asparaginase could serve as an alternative to bacterial-derived enzymes in medical and industrial applications.

Keywords: Endophyte, L-asparaginase, Nesslerization, *Ocimum tenuiflorum*

Introduction

Endophytes are microorganisms that live in the internal tissues of living plants without harming them. They are found in all plants and are highly valuable since they are known to produce a variety of beneficial bioactive chemicals that play a role in phytopathogen defense mechanisms. Endophytic bioactive chemicals have been studied in recent years and found to have a wide range of antibacterial, anticancer, antioxidant, and anti-inflammatory properties (Chow & Ting, 2015).

L-Asparaginase (E.C. 3.5.1.1) is one of the enzymes being researched for its production from endophytes. It catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Yap et al., 2021). This enzyme is used to treat disorders such as acute lymphoblastic leukemia in conjunction with vincristine and a glucocorticoid (such as dezamethasone) (Vimal & Kumar, 2017). It is also used to treat autoimmune illnesses, canine and feline cancer, and has antibacterial properties (Vimal & Kumar, 2017). In the food business, it is used to make acrylamide-free food. Commercial L-asparaginase for clinical use is derived from the bacterium *E. chrysanthemi* and *E. coli* (Vimal & Kumar, 2017). However, clinical hypersensitivity in individuals receiving L-asparaginase has resulted in substantial drawbacks (Narta et al., 2007). Prokaryotic bacterial enzymes cause a modest to severe immunological response (Manasa & Nalini, 2014). Eukaryotic fungal L-asparaginase has been examined since it has fewer adverse effects than prokaryotic L-asparaginase (Kumar et al., 2016).

O. tenuiflorum known as Holy Basil in English and Tulsi in Nepali, is a member of the *Ocimum* genus in the Lamiaceae family of plants. It is a perennial plant which is about 30-60 cm tall. It is an erect and branched herb and have hair. The flower may be white or purple in a green or purplish stem. It is a traditional plant used for healing properties in Ayurveda. Different parts of plant like leaf, stem, root, flower and seed are used for the treatment of diseases such as bronchitis, arthritis, malaria diarrhea, dysentry, skin diseases, insect bites and many more (Pathak & Niraula, 2019). *O. tenuiflorum* contains a variety of therapeutic qualities, including antioxidant, anti-diabetic, anti-inflammatory, anti-cancer, anti-fertility, anti-helmintic, antibacterial, and cardio protective effects (Singh & Chaudhuri, 2018; Bhattarai et al., 2024). *O. tenuiflorum* contains phytochemicals including euginol, euginal, ursolic acid, carvacrol, linalool, limatrol, caryophyllene, methyl carvicol, rosmarinic acid, luteolin, ursollic acid, and limonene (Pattanayak et al., 2010).

Bacillus stratosphericus endophytic bacteria were isolated from *O. tenuiflorum* and showed considerable L-asparaginase production (Pola et al., 2018). Endophytic fungi identified from *O. tenuiflorum* include *Collectrichum* sp., *Pleosporales* sp., *Phomopsis* sp., *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., *Diaporthe* sp., *Rhizoctonia* sp., and *Chaetomium* sp. (Manikandan & Ramanathan, 2023). Different enzymes, such as protease, amylase, cellulase, and laccase have been reported from endophytic fungi isolated from *O.tenuiflorum*. One of the enzymes produced by *O. tenuiflorum* is L-asparaginase. *O. tenuiflorum*, a medicinal herb utilized in Ayurveda, possesses several endophytes, although study on its fungus population is limited. Thus, this study aimed to isolate endophytic fungi from *O. tenuiflorum* and evaluate their L-asparaginase production, with a focus on their potential as biocatalysts for medical and industrial applications.

Materials and Methods

Collection of plant sample and isolation of fungi

Healthy *O. tenuiflorum* leaves was collected from the local garden cultivation in Bagdol, Lalitpur district [27.6681° N; 85.3039° E]. Isolation of fungi was done by the method given by (Kumar et al., 2016). The plant sample was thoroughly washed with distilled water and then soaked in 4% sodium hypochlorite solution for one minute. Then it was surface sterilized with 90% ethanol.

After complete sterilization, the plant parts were cut into 2 mm x 2 mm pieces and seeded into PDA plate equidistantly at six points. The seeded petri plates were then incubated at 28 ± 2 °C for 1 to 2 weeks. The mycelial growth in the petri plate where the plant parts were seeded indicated the production of endophytic fungi.

Morphological characteristics of fungi

Morphological characteristics were observed macroscopically and microscopically. Macroscopic observation included the texture, colour of fungi at center and margin, colour of fungi in the reverse side, shape, presence or absence of exudates and sulcation, initial colour of colonies and colour of colonies after maturation. Similarly, microscopic observation was done under 4x, 10x, 40x and 100x magnification on the optical microscope (Alsohaili & Bani-Hasan, 2018).

Screening of L-asparaginase producing fungi

The screening of L-asparaginase producing fungi was done using the method given by (Chow and

Ting, 2015). The mycelial plug of the fungi was transferred to the modified Czapek Dox (MCDox) media [contains agar 20.0 g/l; glucose 2.0 g/l; L-asparagine 1.0 g/l; KH_2PO_4 1.52 g/l; KCl 0.052 g/l; $MgSO_4$.7H2O 0.052 g/l and trace amount of $CuNO_3.3H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$] at 6.8 pH and incubated at the temperature of 28 ± 2 °C. Phenol red indicator was used to indicate whether the given fungi produced L-asparaginase or not. For control, the MCDox agar medium did not contain L-asparagine but $NaNO_3$ as nitrogen source. Activity was indicated by pink (alkaline pH) or yellow (acidic metabolites) zones.

Enzyme activity assay

L-asparaginase converts L-asparagine into aspartic acid and ammonia. The amount of ammonia produced during the reaction is determined by Nesslerization method as it is highly specific for ammonia. The amount of ammonia produced during the reaction is equal to the enzyme activity of L-asparaginase enzyme (Zhao et al., 2019). In this study, the method given by (Hatamzadeh et al., 2020) is followed for the determination of L-asparaginase enzyme activity. The five days old mycelial plug was placed in the MCDox broth and incubated in a shaker at 150 rpm for five days at the temperature of 28 ± 2 °C. On the 5th day, 100 µl of broth, 100 µL of 0.1 M Tris-HCl (pH 7.2), 200 µl of 0.04 M L-asparagine and 100 µl of sterile distill water was incubated at 37 ± 2 °C. After 1 hour, 100 µl of TAA was added to the mixture to stop the enzymatic reaction.

From this, 100 µl of solution was taken and 750 µl of sterile distill water and 300 µl of Nessler's reagent was added. It was allowed to incubate at 28 ± 2 °C for 20 mins. The UV absorbance of the enzyme was measured at λ_{max} which was found to be 450 nm. The same procedure was done for the MCDox broth on 16th day. One unit of asparaginase is expressed as the amount of enzyme that catalyzes the formation of 1 µmol of ammonia per minute.

The concentration of ammonia in the broth was measured by using the formula adopted by Aisha et al. (2022).

$$\text{Concentration of ammonia in sample (µg/ml)} = \text{Abs. of sample at 450 nm / slope}$$

Similarly, enzyme activity (EA) was measured following Hatamzadeh et al. (2020).

$$EA = (\text{Conc. of ammonia} \times VTS) / VS \times T \times AA$$

Volume of Total Solution (VTS) = 0.6 ml
Volume of Sample (VS) = 0.1 ml
Time in Minutes (T) = 60 minutes
Amount of Asparagine (AA)= 0.2 ml

Results and Discussion

Isolation and morphology of Fungi

Four endophytic fungi (Tulsi 'a', Tulsi 'b', Tulsi 'c', and Tulsi 'd') were isolated from *O. tenuiflorum* leaves. Tulsi 'b', Tulsi 'c' and Tulsi 'd' exhibited sporulation, with morphological features resembling *Microsporum*, *Penicillium* and *Trichoderma* species respectively (Kidd et al., 2022; Tobeigei et al., 2023). Tulsi 'a' did not produce spores. All the isolated fungi were obtained from the leaf tissue of the sample plant as shown in Figure 1.

Screening of L-asparaginase producing fungi

During screening of L-asparaginase production, the isolated fungi Tulsi 'a' and Tulsi'd' did not produce pink colour but there was slight change in colour of the media to yellow in Tulsi 'c'. Tulsi 'b' showed slight pink colour in the culture media. The change in colour was due to the catalytic hydrolysis of L-asparagine into aspartic acid and ammonia.

Enzyme activity

The enzyme activities of the isolated fungi were studied for the 5th and 16th day. On the fifth day, the highest enzyme activity was observed for Tulsi 'a' which was 31.24 U/ml and the least was observed for Tulsi 'd' with 21.98 U/ml. Similarly, for the 16th day, the highest L-asparaginase activity was shown by Tulsi 'd' with 77.43 U/ml and the least was for Tulsi 'c' 21.07 U/ml. The activity for all the isolates were shown in Figure 2. This result suggested that the enzyme activity of the isolated fungi varied with time. Tulsi 'd' showed higher enzyme activity after some time than other isolates. In isolates Tulsi 'a', Tulsi 'b' and Tulsi 'd', enzyme activity increased from day 5 to day 16. Tulsi 'c' showed a decline in activity from 25.43 U/ml (day 5) to 21.07 U/ml (day 16).

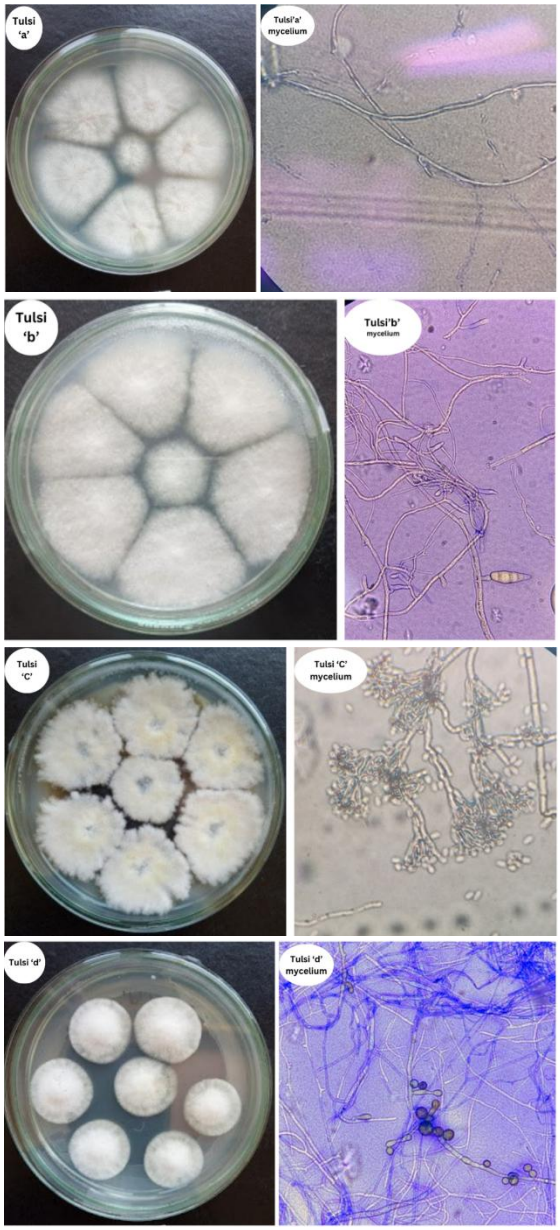

Figure 1: Morphological view of endophytic fungi isolated from *O. tenuiflorum* and microscopic observation of hyphae and sporulation in isolated fungi.

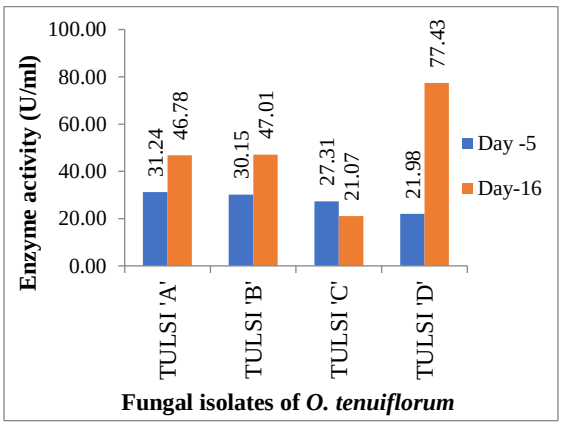

Figure 2: Enzyme Activity of isolated fungi of *O. tenuiflorum* on day 5 and day 16.

The study found that endophytic fungi from *O. tenuiflorum* can produce L-asparaginase, with activity levels changing between isolates and incubation periods. Tulsi 'a', Tulsi 'b', and Tulsi 'd' enzyme activity appears to be increasing over time, possibly because they are entering a secondary metabolic phase that enhances enzyme production. Tulsi 'c', on the other hand, has decreased activity over time, which can be attributed to nutrient depletion or autolysis.

The results of this investigation are highly aligned with previously reported literature (Cheruiyot et al., 2024), who isolated a *Penicillium* sp. with peak L-asparaginase activity on day 6, followed by a gradual decrease (Cheruiyot et al., 2024). Ahiravan & Gnanadoss (2022) had reported isolation of 10 endophytic fungi from *O. tenuiflorum*, among which five isolates showed positive L-asparaginase activity. The highest activity was shown by *Fusarium* sp. which was 9.36 ± 0.52 U/ml (Kathiravan & Gnanadoss, 2022). Notably, Tulsi 'd', tentatively identified as *Trichoderma* sp. based on conidial morphology, showed a significant increase in enzyme activity over time (21.98 U/ml on day 5 to 77.43 U/ml on day 16). To our knowledge, this is the first documentation of L-asparaginase production by *Trichoderma* sp., therefore augmenting the repertoire of fungal genera with biotechnological potential.

Comparably, Tulsi 'b,' displaying morphological traits like *Microsporum* sp., showed higher activity from 27.15 U/ml (day 5) to 45.67 U/ml (day 16) (Tobeigei et al., 2023). *Microsporum* is usually known as a dermatophyte, but its enzymatic properties are yet unknown, so this finding is unique. But, the molecular identification of these isolates, specifically *Trichoderma* and *Microsporum* is essential to verify their taxonomy and associate genetic characteristics with enzymatic activity.

The limits of phenotypic assays are shown by the difference between colorimetric screening (pink zone) and quantitative enzyme activity. For example, Tulsi 'a' lacked a pink zone but showed great activity (31.24 U/ml on day 5), most likely from melanin or another pigment interfering. The findings indicate the potential of endophytic fungi as a safer alternative to bacterial enzymes and a sustainable source of L-asparaginase. Future studies should look into the genetic underpinnings of enzyme synthesis and improve cultural settings including pH levels, carbon sources, temperature etc.

Conclusion

This study demonstrated that the amount of L-asparaginase generated varied even across endophytes isolated from the same plant. Furthermore, this work supports the hypothesis that endophytes derived from medicinal plants have therapeutic capabilities. Because endophytes have varied growth rates and enzyme activity, growing strategies must be tuned to get larger yields and increased L-asparaginase activity.

Acknowledgements

We thank Research Directorate, Tribhuvan University, and University Grants Commission, Nepal for financial support.

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