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Isolation and Characterization of L-Asparaginase Producing Endophytic Fungi from Medicinal Plants of Rutaceae Family

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Endophytic fungi synthesize a diverse set of enzymes and biomolecules with wide array of bioactivities. Thirty-five distinct fungal endophytes have been isolated from medicinal plants of Rutaceae and screened for their ability to produce various industrially important enzymes. Primary enzyme screening revealed that 65% of isolates produced L-Asparaginase, 62% of isolates produced cellulase, 60% of isolates produced amylase, 48% of isolates produced both lipase and pectinase, 45% of isolates produced laccase and 42% of isolates produced protease. Among the different enzymes screened, L-Asparaginase producing isolates were dominant and subjected to quantification using the nesslerization method. The endophytic isolates LCJ315, LCJ324, LCJ326 and LCJ335 were capable of producing high L-Asparaginase activity and their enzyme activity ranged between 7.58 U/ml to 8.84 U/ml. The four isolates were further subjected to molecular identification using 18S rRNA sequencing. The results were then subjected to BLAST analysis. The phylogenetic tree was created by MEGA software. The isolated endophytic fungal strains were identified as LCJ315 (*Aspergillus* sp.), LCJ326 (*Colletotrichum* sp.), LCJ324 and LCJ335 (*Fusarium* sp.). The fungal source of L-Asparaginase is preferred over other microbial sources of L-Asparaginase due to reduced side effects. Given the fact that L-Asparaginase is a crucial anticancer enzyme that is used as the initial therapy for acute lymphoblastic leukemia. Hence, the present study reveals that these endophytic fungal strains isolated from Rutaceae members can be used as an alternate source for L-Asparaginase production.

Keywords: Endophytic fungi; Extracellular enzymes; L-Asparaginase; Medicinal plants; Molecular characterization.

Endophytic fungi are present in the intra and intercellular spaces of the host plants without harming the host plants [1]. Flavonoids, phenols and other molecules produced by the plant attract the fungi present in the rhizosphere to invade the plant. To succeed and maintain the invasion (mutualistic symbiosis) of the plant, endophytic fungi must produce a wide array of secondary metabolites. Endophytic fungi can be considered as luxuriant sources of novel natural biomolecules that have a vast number of applications in the field of healthcare due to their ability to perform biosynthesis and biotransformation [2]. Alkaloids, flavonoids, quinones, steroids and terpenoids are some examples of natural bioactive molecules obtained from endophytic fungi. Recently, endophytic fungi have drawn a lot of attention as the potential candidate for examining sustainable

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and novel antibiotics, antioxidant, antitumour and antiviral agents [2, 3].

Endophytic fungi are also the go to choice for the production of various enzymes that have industrial and healthcare applications. Laccase, lipase, pectinase, amylase, and protease are some of the enzymes obtained from endophytic fungi. Endophytic fungal enzymes are robust and natural products. Hence they are preferred in various industries including pharmaceutical industry.

L-Asparaginase is a therapeutic enzyme that catalyzes the breakdown of L-asparagine into ammonia and L- aspartate [4]. Asparagine is necessary for the proliferation and development of leukemic cells. L-Asparaginase inhibits tumour growth by reducing serum asparagine levels [5]. El-Naggar *et al*., 2020 [6] proposed L-Asparaginase as a possible medication for treating acute lymphoblastic leukemia as an alternative to traditional chemotherapy procedures. L-Asparaginase was discovered first in guinea pig serum [7] and its tumour activity was later documented by detecting the antileukemic activity of the guinea pig serum on treated mice [8].

L-Asparaginase from *E.coli* is currently utilized and it has side effects due to the prokaryotic origin of the enzyme. L-Asparaginase from eukaryotic organisms such as fungi can be considered as an alternative, as they are less likely to cause side effects. Given the importance of this enzyme, it is unavoidable to explore alternative sources for the production of L-Asparaginase. Since fungi meets all the above-said characteristics they can be explored for their ability to act as an alternative source for the enzyme with special attention to endophytic fungi. Many species of Rutaceae are traditionally used as medicines in the treatment of fever, diarrhoea, asthma, cholera, etc. Endophytic fungi from the Rutaceae family are less explored. Hence the present study deals with the isolation of endophytic fungi from the medicinal plants of Rutaceae family and screen their capability to synthesize seven extracellular enzymes. Quantification and molecular identification of L-Asparaginase synthesizing endophytic fungi were studied. The findings of the present study reveal that the endophytic fungi can be used as an alternative source for the production of the therapeutic enzyme L-Asparaginase.

MATERIALS AND METHODS

Isolation of endophytic fungi

Stem and leaf portions of the healthy plants belonging to the Rutaceae family such as *Citrus limon* (L.) Osbeck, *Limonia acidissima* L., *Aegle marmelous* (L.) Correa, *Toddalia asiatica* (L.) Lam., *Glycosmis mauritiana* (Lam.) Tanaka, *Atalantia monophylla* DC. and *Murraya exotica* L. were collected for the endophytic fungi isolation.

Stem and leaf portions were transported to the lab and rinsed with tap water to eliminate surface debris and other contaminants. The isolation of endophytic fungi was done in an aseptic condition. The leaves and stem parts were cut into small pieces (3 cm) and subjected to the process of surface sterilization. Surface sterilization and isolation of endophytic fungi were carried out following the procedures described by Petrini and Dreyfuss [9] with modifications. The cut plant parts were thoroughly washed in running tap water for 10 mins. The cut plant parts were washed using Tween 20 and rinsed with distilled water. Then plant parts were washed with ethanol (70%) for 1 min and then rinsed using distilled water for 30 seconds. Sodium hypochloride solution (1:3) was used to wash the plant parts for 1 min followed by 70% ethanol for 30 seconds. The plant parts were further rinsed four times thoroughly using sterile distilled water. Sterile distilled water used for rinsing the plant part for the fourth time was collected and used as a negative control to confirm the purity of the endophytic fungi. The sterile plant parts were blotted dry to remove the excess moisture. The plant parts that were surface sterilized was cut into small segments (1 cm) using a sterile blade and inoculated onto the petriplates containing Potato Dextrose Agar medium (PDA) with streptomycin supplemented in them to avoid bacterial contamination. All the inoculated petriplates were sealed and incubated at 28±1°C. Fungal growth from the inoculated plant segments was noted periodically for about 2 to 4 weeks. The isolated endophytic fungi were transferred to slants (PDA) and maintained at 4°C as pure cultures for further studies.

Screening for extracellular enzymes

The isolated fungal endophytes were screened to identify their capability to synthesize

extracellular enzymes (amylase, lipase, pectinase, cellulase, protease and laccase) using the Sunitha *et al*., 2013 [10] methods and L-Asparaginase activity was calculated based on Imada *et al*., 1973 [11].

Amylase

Amylase activity was confirmed by inoculating the endophytic fungi in Glucose Yeast extract Peptone Agar Medium (GYP) (pH 6.0) with soluble starch as substrate (0.2%). After an incubation period of 5 days, the petriplates were flooded with a solution containing iodine (1%) in potassium iodide (2%). The formation of a clear white zone around the fungus colony indicates positive amylase activity.

Lipase

Fungal endophytes were inoculated on Peptone Agar Medium with sterile Tween 20 (1%) revealing lipase activity. Formation of the halo zone around the fungal culture after an incubation period of 5 days represents positive activity.

Pectinase

The fungal isolates were cultured in Pectin Agar Medium with a pH of 5.0 and incubated. At the end of the incubation time, 1% hexadecyltrimethylammonoium bromide was added to the petriplates and the formation of a hydrolytic zone around the fungal culture confirmed pectinase activity.

Cellulase

The cellulase activity of each fungal isolate was determined by growing it on GYP agar (pH 6.0) containing 0.5% carboxy-methylcellulose and incubated. Following fungal development, the petriplates were brimmed with Congo red solution (0.2%) and washed with NaCl (1 M) for 15 minutes. The presence of red or yellow zones around fungal endophyte colonies confirmed the existence of cellulolytic activity.

Protease

Endophytic fungal proteolytic activity was evaluated on the GYP medium containing 0.4% gelatin with pH adjusted to 6.5 and maintained at room temperature for 3 to 5 days. Ammonium sulphate (saturated aqueous solution) was used to brim the incubated petriplates and the formation of a halo zone surrounding the fungus confirmed the existence of proteolytic activity.

Laccase

Laccase assay of the fungal endophytes was studied by culturing it on GYP agar supplemented with 1-naphthol (0.005%). Since laccase oxidizes 1-naphthol, the colourless medium turns blue as the fungus grows indicating a positive reaction.

L-Asparaginase

L-Asparaginase activity of the isolated fungal endophytes was investigated by using Czapek Dox medium with 2.5% of phenol red. After the incubation period of 5 days, a change in medium colour from yellow to pink indicated a positive reaction.

L-Asparaginase assay

The endophytic fungal cultures that showed a positive reaction for L-Asparaginase activity were cultivated in an Erlenmeyer flask (500 ml) containing 200 ml of Modified Czapek Dox medium. The flasks were incubated in an orbital shaker (120 rpm) at room temperature. Imada *et al*., 1973 [11] described Nesslerization as a method for estimating L-Asparaginase. Samples were drawn out daily to make a reaction solution (2.5 ml) that contained 500 µl of each of the following crude enzyme (broth), 0.5M Tris buffer, 0.04 L-Asparagine and distilled water. The reaction solution was incubated for about 30 mins. 500 µl of Trichloroacetic acid was mixed to the reaction solution to halt the enzymatic reaction. Followed by centrifugation at 10,000 rpm for 5 mins. 100 µl was pipetted out from the supernatant of the reaction solution to which distilled water (3700 µl) and Nessler's reagent (200 µl) was added and incubated for 20 mins. The brownish yellow colour developed was read at 450 nm using an UV visible spectrophotometer (Elico). At 37± 2 °C, 1 unit of asparaginase is defined as the enzyme quantity required to catalyze the creation of 1 µmol of NH_4/ min [12].

$$\text{Units/mL enzyme} = \frac{(\mu\text{M of ammonia liberated})\ (2.5)}{(0.1)\ (30)\ (0.5)}$$

Volume of reaction solution - 2.5 (mL)
Volume of supernatant - 0.1 (mL)
Incubation Time - 30 (min)
Volume of crude enzyme - 0.5 (mL)

Molecular characterization

Cultures of promising fungal isolates were cultivated on PDA media for seven days in test tubes for DNA extraction and sequencing. 18S rRNA (ITS sequencing) sequencing was used for

molecular identification. NucleoSpin® Tissue Kit was employed to extract genomic DNA.

Genomic DNA, Universal primers such as ITS1 (5'TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3'), DNA polymerase, Taq Buffer, and dNTP Mix were used to amplify the gene using PCR. PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) was used for PCR amplification. The amplification process consists of the following parameters on a thermal cycler: the reaction mixture is preheated for 5 min at 95° C; denaturation for 30 sec at 95° C; annealing for 40 sec at 58° C; extension for 60 sec at 72° C and final extension for 5 min at 72° C. The products of PCR were examined using a UV transilluminator with 1.2% agarose gel electrophoresis. BLAST search was used to compare the results to the National Center for Biotechnology Information (NCBI). MUSCLE was used to align the sequences with similar ones. MEGA ver. 6 was used to construct Phylogenetic tree.

Statistical analysis

The studies were carried out in triplicates and the results were statistically analyzed. ANOVA rules were followed for the analysis of variance. Duncan's multiple range test (DMRT) was employed to detect significant discrepancies between the means.

RESULTS AND DISCUSSION

Isolation of endophytic fungi

Thirty five different endophytic fungal strains were explored from the medicinal plants belonging to the Rutaceae family (*Citrus limon* (L.) Osbeck, *Limonia acidissima* L., *Aegle marmelous* (L.) Correa, *Todallia asiatica* (L.)Lam., *Glycosmis mauritiana* (Lam.) Tanaka, *Atalantia monophylla* DC. and *Murraya exotica* L.). The endophytic fungal isolates were obtained from the fresh stem and leaf portions of the plants (Fig. 1). Table 1 describes the list of endophytic fungal isolates obtained from different parts of the Rutaceae family members.

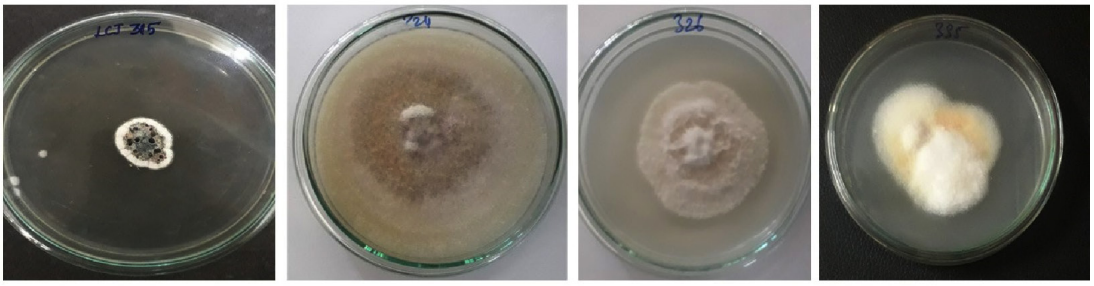
Screening for extracellular enzymes

Endophytic fungi as a part of their strategy to invade the host plant and gather nutrients for their development produce enzymes [13]. Thirty five isolated endophytic fungi from the medicinal plants of Rutaceae family members were subjected to the production of extracellular enzymes and all were able to produce extracellular enzymes (Fig. 2). The above mentioned enzyme activity was tested using the suitable media and their results were calculated by the diameter of enzyme hydrolytic zone formation (Table 2).

Amylase

Thirty five isolated endophytic fungi were subjected to amylase enzyme screening and among them, 60% of the isolates showed positive reaction. The enzyme activity of amylase enzyme ranges from 0.8 cm to 3.36 cm. LCJ327 exhibited the highest amylase activity. Isolates LCJ303, LCJ308, LCJ309, LCJ311, LCJ314 and LCJ328 are some of the isolated endophytic fungal cultures that showed negative activity.

Amylases are one of the key enzymes with uses ranging from the food industry to pharmaceutical industry. In industrial production, fungal amylases are widely used owing to their beneficial properties like simple production process (cost-effective, reduced time and space), consistency and optimization [14]. Sunitha *et al*., 2012 [15] isolated thirty endophytic fungi among them 37% of isolates showed positive amylase activity.



LCJ315 LCJ324 LCJ326 LCJ335

Fig. 1. Isolated endophytic fungal strains

They were the first to report amylase activity from endophytic fungi isolated from *Alpinia calcarata.* Reyes *et al*., 2021[16] isolated 11 endophytic fungi from the plant *Citrofortunella microcarpa* (Bunge) Wijnands belonging to the Rutaceae family and all the isolates showed positive reaction for amylase activity.

Protease

42.5% of the isolated endophytic fungi showed positive reaction to protease activity. The enzyme activity varied from 0.74 cm to 3.78 cm. LCJ301, LCJ304, LCJ305, LCJ306, LCJ311, LCJ317, LCJ318 and LCJ319 are some of the isolates that showed negative reaction.

Proteases are utilized in a variety of industries, including the pharmaceutical (treatment of cancer), leather, detergent and food industries (bakery and meat tenderization) [17]. Proteases are catalytic enzymes that hydrolyze peptide bonds in proteins to produce amino acids and peptides. They belong to the hydrolase enzyme family, which includes a vast number of enzymes [18]. Elgammal *et al*., 2020 [19] reported alkaline thermophilic protease produced from the fungal endophyte *Aspergillus*

Table 1. List of isolated endophytic fungi from medicinal plants of Rutaceae family

Isolate no	Medicinal Plant	Plant part
LCJ301	*Citrus limon* (L.) Osbeck	Stem
LCJ302	*Limonia acidissima* L.	Stem
LCJ303	*Limonia acidissima* L.	Leaf
LCJ304	*Limonia acidissima* L.	Petiole
LCJ305	*Limonia acidissima* L.	Stem
LCJ306	*Citrus limon* (L.) Osbeck	Stem
LCJ307	*Citrus limon* (L.) Osbeck	Stem
LCJ308	*Citrus limon* (L.) Osbeck	Leaf
LCJ309	*Citrus limon* (L.) Osbeck	Leaf
LCJ310	*Citrus limon* (L.) Osbeck	Leaf
LCJ311	*Citrus limon* (L.) Osbeck	Stem
LCJ312	*Limonia acidissima* L.	Stem
LCJ313	*Aegle marmelous* (L.) Correa	Stem
LCJ314	*Aegle marmelous* (L.) Correa	Stem
LCJ315	*Murraya exotica L.*	Leaf
LCJ316	*Aegle marmelous* (L.) Correa	Stem
LCJ317	*Aegle marmelous* (L.) Correa	Leaf
LCJ318	*Aegle marmelous* (L.) Correa	Leaf
LCJ319	*Toddalia asiatica* (L.) Lam.	Stem
LCJ320	*Toddalia asiatica* (L.) Lam.	Stem
LCJ321	*Toddalia asiatica* (L.) Lam.	Leaf
LCJ322	*Glycosmis mauritiana* (Lam.) Tanaka	Leaf
LCJ323	*Glycosmis mauritiana* (Lam.) Tanaka	Stem
LCJ324	*Glycosmis mauritiana* (Lam.) *Tanaka*.	Stem
LCJ325	*Atalantia monophylla* DC.	Stem
LCJ326	*Atalantia monophylla* DC.	Leaf
LCJ327	*Atalantia monophylla* DC.	Stem
LCJ328	*Atalantia monophylla* DC.	Stem
LCJ329	*Atalantia monophylla* DC.	Leaf
LCJ330	*Murraya exotica* L.	Leaf
LCJ331	*Murraya exotica* L.	Stem
LCJ332	*Murraya exotica* L.	Stem
LCJ333	*Murraya exotica* L.	Stem
LCJ334	*Murraya exotica* L.	Stem
LCJ335	*Murraya exotica* L.	Leaf

ochraceus. Two endophytic fungi (*Curvularia australiensis and Alternaria citrimacularis*) from *Aegle marmelos* showed intermediate protease activity [20].

Lipase

48.5% of the endophytic fungal isolates exhibited positive lipase activity. The highest activity of 3.48 cm was exhibited by LCJ323

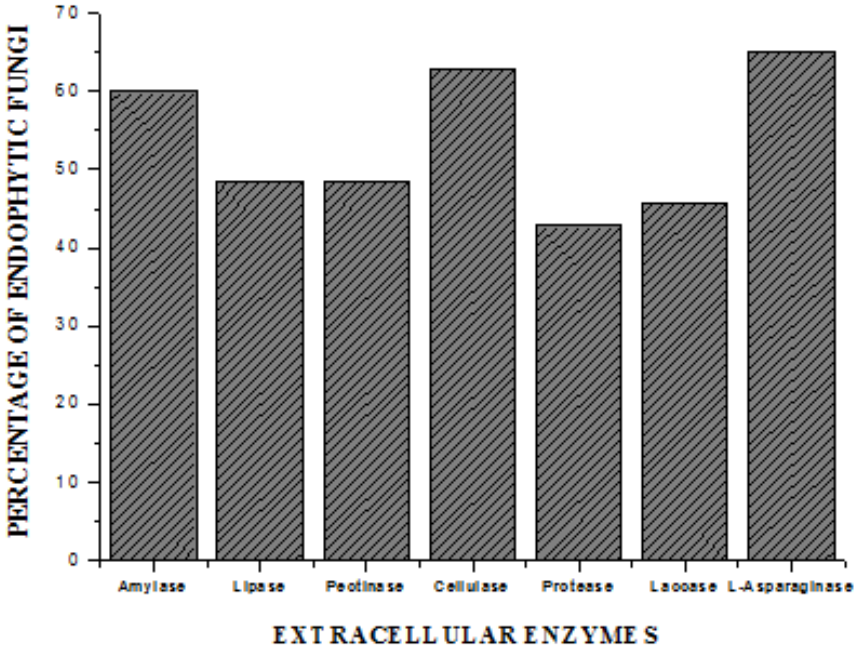


Fig. 2. Percentage of positive activity of the extracellular enzymes shown by the endophytic fungal cultures isolated from Rutaceae family members

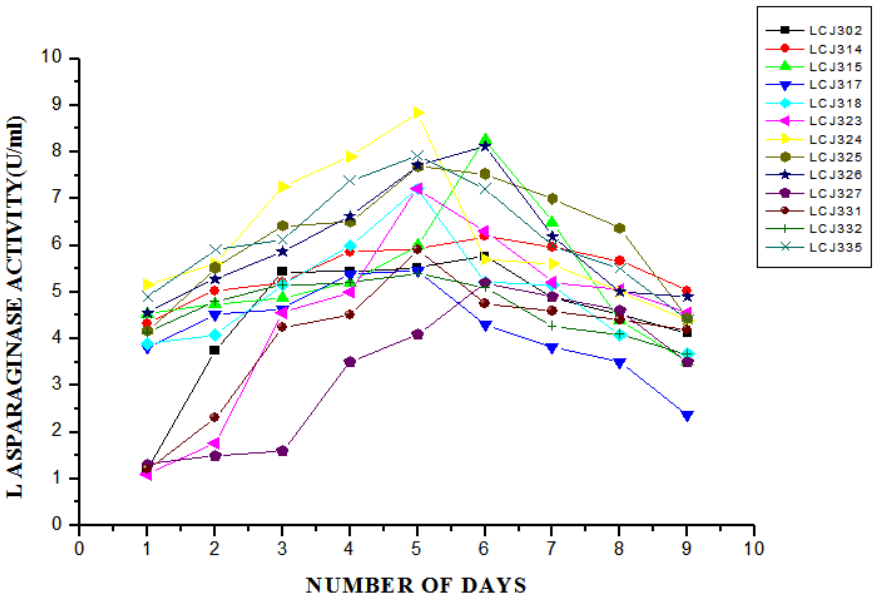


Fig. 3. Secondary screening of L-Asparaginase enzyme activity by potential endophytic fungal cultures

and the lowest activity of 0.62 cm was exhibited by LCJ335. LCJ303, LCJ305, LCJ311, LCJ316, LCJ317, LCJ319, LCJ324 and LCJ327 all produced lipase activity in the range of 2 to 3 cm.

Jenila and Gnanadoss, 2018[21] reported that 73% of the endophytic fungi showed positive reaction for lipase activity. Mani *et al*., 2018 [20] described moderate lipase activity for endophytic fungi isolated from *Aegle marmelos*. In the study conducted by Toghueo *et al*., 2017 [22] among the 87 isolated endophytic fungi 59.8% of isolates exhibited lipase activity. Lipases catalyze the entire or partial hydrolysis of glycerols into free fatty acids. Lipases have a broad array of applications, ranging from the food industry to the chemical industry [23].

Cellulase

62.8% of the endophytic fungal isolates were capable of producing cellulase enzymes. The highest cellulase activity of 3.65 cm was exhibited by LCJ317 and the lowest cellulase activity of 0.72 cm was exhibited by LCJ313. Most of the isolates showed positive reaction for cellulase enzyme indicating that the endophytic fungal isolates derived energy from cellulose.

64.4% of isolated endophytic fungi from three tropical plants with medicinal properties exhibited cellulase activity [22]. 30% of endophytic

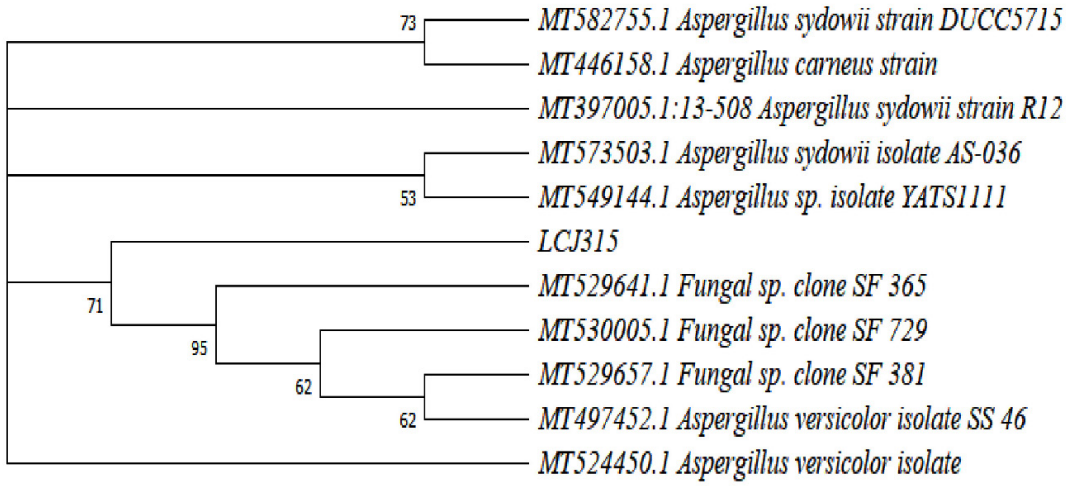


Fig. 4. Phylogenetic tree for the strain LCJ315 *Aspergillus* sp.

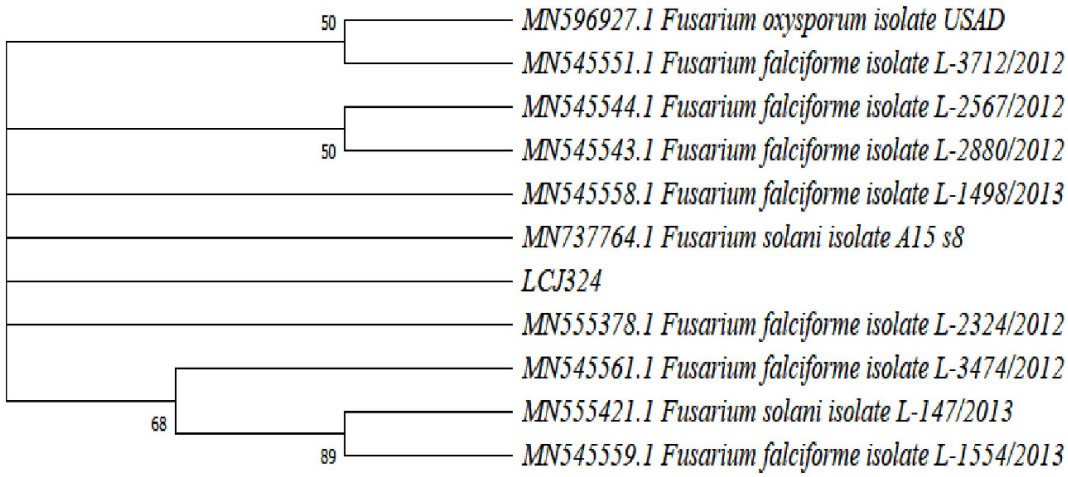


Fig. 5. Phylogenetic tree for the strain LCJ324 *Fusarium* sp.

fungi isolated from Thai mangrove plants exhibited cellulase activity [24]. Cellulases produced by endophytic fungi not only help them in gaining nutrients (by hydrolyzing cellulosic substrates to glucose) but also assist the host in defending against pathogen attacks [25]. Cellulase enzymes are widely used in paper, food industry and biofuel production. Bioethanol production from cellulosic materials using cellulase is currently popular [26].

Pectinase

Among the thirty five isolates, 48.5% of isolates can synthesize pectinase enzyme. The enzyme activity ranged from the lowest of 0.5 cm (LCJ306 and LCJ316) to the highest of 3.49 cm (LCJ305). When compared with other extracellular enzymes tested, pectinase showed less activity.

Fungal endophytes were obtained from the Thai orchids and screened for extracellular activity. Among the 52 isolates screened for pectinase activity, 49% showed positive pectinase activity [27]. Jenila and Gnanadoss, 2018[21] reported that most of their endophytic fungi isolated from medicinal plants exhibited positive pectinase activity. Only 24% of the 25 isolates obtained from *Tinospora cordifolia* (Willd.) Hook. f. & Thomson exhibited pectinase activity [28]. Pectinases are enzymes that degrade the lengthy and complicated molecules of pectic compounds [29]. They degrade the polysaccharide complex to monomers such as D-galacturonic acid. Pectinase enzymes are used in the food industry, especially for clearing condensed fruit juices [30].

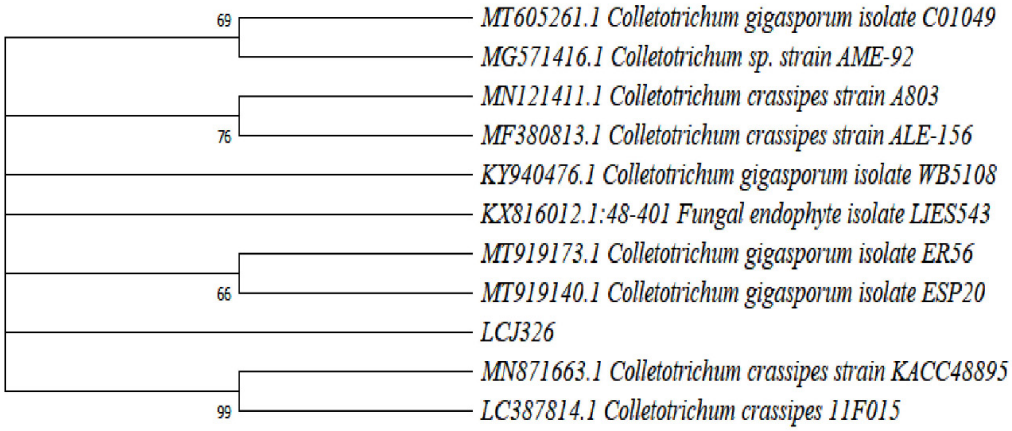


Fig. 6. Phylogenetic tree for the strain LCJ326 *Colletotrichum* sp.

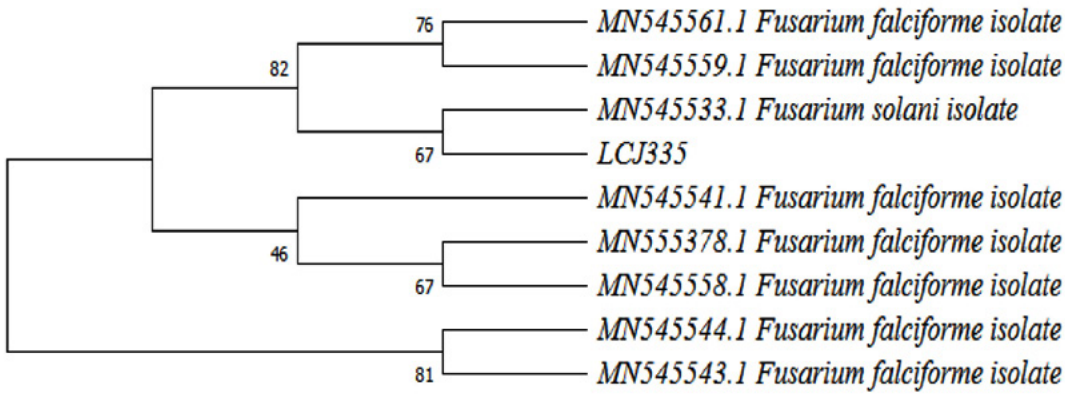


Fig. 7. Phylogenetic tree for the strain LCJ335 *Fusarium* sp.

Table 2. Extracellular enzyme activity of isolated endophytic fungi

Isolate no	Diameter of enzymatic hydrolysis zone (cm)						
	Amylase	Lipase	Pectinase	Cellulase	Protease	Laccase	L.Asparaginase
LCJ301	2.9 ± 0.144^{a}	0 ± 0^{d}	1.8 ± 0.289^{b}	2.1 ± 0.277^{b}	0 ± 0^{cd}	0.5 ± 0.0577^{c}	0 ± 0^{cd}
LCJ302	1.75 ± 0.139^{b}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}	1.8 ± 0.121^{b}	0 ± 0^{c}	3.41 ± 0.393^{a}
LCJ303	0 ± 0^{e}	2.62 ± 0.133^{b}	0 ± 0^{e}	1.98 ± 0.312^{c}	3.15 ± 0.173^{a}	0 ± 0^{e}	0.84 ± 0.0866^{d}
LCJ304	1.64 ± 0.289^{b}	0.95 ± 0.127^{c}	0 ± 0^{d}	3.52 ± 0.346^{a}	0 ± 0^{d}	1.64 ± 0.115^{b}	0 ± 0^{d}
LCJ305	2.86 ± 0.144^{ab}	1.9 ± 0.121^{c}	3.49 ± 0.525^{a}	0 ± 0^{d}	0 ± 0^{d}	2.18 ±0.231^{bc}	0 ± 0^{d}
LCJ306	1.2 ± 0.115^{c}	0 ± 0^{e}	0.5 ± 0.115^{de}	0.96 ±0.0577^{cd}	0 ± 0^{e}	3.38 ± 0.346^{a}	1.74 ± 0.237^{b}
LCJ307	1.14 ± 0.121^{c}	0 ± 0^{d}	0 ± 0^{d}	1.56 ± 0.162^{bc}	2.39 ± 0.52^{ab}	2.72 ± 0.52^{a}	1.97 ± 0.277^{ac}
LCJ308	0 ± 0^{d}	0 ± 0^{d}	1.42 ± 0.121^{c}	3.26 ± 0.346^{a}	1.86 ± 0.121^{bc}	0 ± 0^{d}	2.28 ± 0.358^{b}
LCJ309	0 ± 0^{d}	3.36 ± 0.167^{a}	0 ± 0^{d}	2.63 ± 0.173^{b}	1.14 ± 0.133^{c}	2.6 ± 0.294^{b}	2.6 ± 0.358^{b}
LCJ310	3.19 ± 0.144^{a}	0 ± 0^{c}	1.98 ± 0.052^{b}	0 ± 0^{c}	1.88 ± 0.375^{b}	0 ± 0^{c}	0 ± 0^{c}
LCJ311	0 ± 0^{c}	2.29 ± 0.208^{a}	0 ± 0^{c}	2.43 ± 0.115^{a}	0 ± 0^{c}	1.44 ± 0.202^{b}	0 ± 0^{c}
LCJ312	0 ± 0^{d}	0.78 ±0.0577^{bc}	0.82 ± 0.3^{b}	0 ± 0^{cd}	1.98 ± 0.502^{a}	0 ± 0^{cd}	2.34 ± 0.289^{a}
LCJ313	2.79 ± 0.144^{a}	0 ± 0^{d}	0 ± 0^{d}	0.72±0.00577^{c}	1.56 ± 0.0924^{b}	2.64 ± 0.318^{a}	1.81 ± 0.266^{b}
LCJ314	0 ± 0^{e}	1.06 ± 0.0866^{d}	1.67±0.0346^{d}	1.78 ± 0.144^{cd}	3.28 ± 0.144^{a}	2.38 ± 0.41^{bc}	3 ± 0.398^{ab}
LCJ315	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}	1.35 ± 0.0693^{b}	0.74 ±0.0577^{bc}	3.1 ± 0.473^{a}	3.92 ± 0.531^{a}
LCJ316	0 ± 0^{c}	2.32 ± 0.548^{b}	0.5 ± 0.0693^{c}	0 ± 0^{c}	3.66 ± 0.693^{a}	0 ± 0^{c}	0.8 ± 0.121^{c}
LCJ317	0 ± 0^{c}	1.93 ± 0.104^{b}	0 ± 0^{c}	3.65 ± 0.572^{a}	0 ± 0^{c}	0 ± 0^{c}	3.33 ± 0.572^{a}
LCJ318	1.72 ± 0.323^{b}	0 ± 0^{d}	0.8 ± 0.0924^{c}	2.86 ± 0.202^{a}	0 ± 0^{d}	0 ± 0^{d}	2.68 ± 0.289^{a}
LCJ319	0.91 ± 0.115^{b}	2.89 ± 0.346^{a}	0 ± 0^{c}	2.42 ± 0.346^{a}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}
LCJ320	0 ± 0^{c}	0 ± 0^{c}	0.79 ± 0.312^{c}	3.74 ± 0.323^{a}	2.73 ± 0.572^{b}	0 ± 0^{c}	2.41 ± 0.381^{b}
LCJ321	2.93 ± 0.237^{a}	0 ± 0^{c}	1.14 ± 0.306^{b}	0 ± 0^{c}	0 ± 0^{c}	3.1 ± 0.0808^{a}	0 ± 0^{c}
LCJ322	2.18 ± 0.15^{b}	0 ± 0^{c}	2.38 ± 0.196^{b}	0 ± 0^{c}	0 ± 0^{c}	2.9 ± 0.185^{a}	2.14 ± 0.139^{b}
LCJ323	0 ± 0^{c}	3.48 ± 0.271^{a}	3.23±0.485^{ab}	0 ± 0^{c}	0 ± 0^{c}	2.7 ± 0.312^{b}	0 ± 0^{c}
LCJ324	0 ± 0^{d}	2.74 ± 0.15^{b}	1.18 ± 0.202^{c}	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}	4.28 ± 0.208^{a}
LCJ325	3.17 ± 0.202^{a}	3 ± 0.566^{ab}	0 ± 0^{c}	1.93 ± 0.133^{b}	3.78 ± 0.566^{a}	0 ± 0^{c}	3.54 ± 0.485^{a}
LCJ326	2.74 ± 0.0866^{b}	0 ± 0^{c}	0 ± 0^{c}	3.29 ± 0.502^{ab}	0 ± 0^{c}	0 ± 0^{c}	3.88 ± 0.699^{a}
LCJ327	3.36 ± 0.589^{a}	1.99 ± 0.191^{b}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}	3.4 ± 0.0635^{a}
LCJ328	0 ± 0^{c}	0 ± 0^{c}	2.54 ± 0.294^{a}	1.8 ± 0.127^{b}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}
LCJ329	0.64 ± 0.0635^{b}	2.22 ± 0.468^{a}	0 ± 0^{b}	0 ± 0^{b}	1.89 ± 0.358^{a}	2.4 ± 0.26^{a}	0 ± 0^{b}
LCJ330	1.92 ± 0.0924^{b}	0 ± 0^{c}	0 ± 0^{c}	1.98 ± 0.144^{b}	2.12 ± 0.323^{b}	0 ± 0^{c}	2.9 ± 0.144^{a}
LCJ331	0 ± 0^{b}	0 ± 0^{b}	2.73 ± 0.277^{a}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}
LCJ332	1.74 ± 0.11^{b}	0 ± 0^{d}	2.42 ± 0.289^{a}	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}	0.94 ± 0.121^{c}
LCJ333	1.86 ± 0.121^{b}	0.76 ± 0.104^{d}	0 ± 0^{e}	2.81 ± 0.121^{a}	0 ± 0^{e}	0 ± 0^{e}	1.16 ± 0.11^{c}
LCJ334	0.81 ± 0.0924^{b}	0 ± 0^{c}	0 ± 0^{c}	0.66 ± 0.231^{b}	0 ± 0^{c}	1.94 ± 0.462^{a}	0 ± 0^{c}
LCJ335	2.1 ± 0.312^{b}	0.62 ± 0.0635^{c}	0 ± 0^{c}	2.68 ± 0.115^{b}	0 ± 0^{c}	0.52 ±0.0577^{c}	3.79 ± 0.693^{a}

Table 3. Identified endophytic fungi with their accession number obtained from NCBI

Isolate no	Fungal sp.	Accession number
LCJ315	*Aspergillus* sp.	MZ646107
LCJ324	*Fusarium* sp.	MZ646108
LCJ326	*Colletotrichum* sp.	MZ646112
LCJ335	*Fusarium* sp.	MZ646131

Laccase

Only 45% of the isolated endophytic fungi were capable of synthesizing laccase enzyme. LCJ306 and LCJ321 exhibited high laccase activity followed by LCJ322 and LCJ313.

Only 10.5% of the 19 isolated endophytic fungi from the plant *Solanum tuberosum* showed positive reaction for laccase [31]. Sunitha *et al*., 2013 [10] and Reyes *et al*., 2021 [16] have all reported that only a few of their endophytic isolates showed positive laccase activity. Fungal laccases are multicopper oxidases with immense biotechnological potential since they can oxidise a broad spectrum of organic pollutants utilizing oxygen from the air. The fact that endophytic fungi are low producers of laccase enzyme may be due to the capability of laccase to breakdown lignin and can potentially lead to the mutilation of the host [10].

L-Asparaginase

65% of the isolated endophytic fungi produced L-Asparaginase enzyme. The highest enzyme activity of 4.28 cm was exhibited by LCJ324 followed by LCJ315, LCJ326 and LCJ335 exhibited enzyme activity of 3.92 cm, 3.88 cm and 3.79 cm respectively.

Arumugam *et al*., 2021 [32] have reported that 36% of 27 isolated endophytic fungal isolates exhibited positive reactions for L-Asparaginase enzyme activity. Asparaginase (EC 3.5.1.1) is an amidohydrolase that hydrolyzes aspartic acid (aspartate) and ammonium (NH_4^+) from asparagine [33]. Purified L- Asparaginase from *Erwinia chrysanthemi* and *Escherichia coli* was initially utilized in clinical trials for the treatment of acute lymphoblastic leukaemia in humans but it lead to allergic reactions that caused death [34]. As a result, researchers began to focus on fungi as they are eukaryotic members thus they can act as a safe alternative eukaryotic source for producing asparaginase to reduce allergic reactions [35]. 45% of eighty four fungal endophytes isolated from Asteraceae family members exhibited positive L-Asparaginase enzyme activity [3]. Four endophytic fungal isolates showing L-Asparaginase activity were isolated from *Ocimum sanctum* with activity ranging from moderate to high [36]. Sixty six endophytic fungal strains were obtained from the leaves of *Mandevilla catimbauensi.* Among them, 20 isolates were screened for their ability to produce L-Asparaginase. 70% of isolates exhibited positive L-Asparaginase activity [37].

L- Asparaginase assay

Among the thirty five endophytic fungi, thirteen isolates were good producers of L-Asparaginase (Fig. 3). So the thirteen isolates were subjected to quantification of L-Asparaginase enzyme using the nesselarization method. The isolates LCJ315, LCJ324, LCJ326 and LCJ335 showed the best L-Asparaginase activity. LCJ315 and LCJ324 are the two isolates that showed atmost L-Asparaginase activity of 8.26 ±0.462 U/ml on the 6th day and 8.84 ±0.346 U/ml on the 5th day respectively in Modified Czapek's Dox Medium. Singh and Sao, 2021[36] isolated four endophytic fungi from *Ocimum sanctum* and among them two isolates i.e. TSF2 and TSF1 showed L-Asparaginase activity of 6.054 U/ml and 5.282 U/ml respectively.

Thirty eight endophytic fungi isolated from seven plants belonging to the Asteraceae family with medicinal values were quantified for L-Asparaginase activity. The activity ranges from 0.019 to 0.492 U/ml [3]. Fifteen endophytic fungi were isolated from *Tabernaemontana heyneana* Wall., among them, eleven isolates were quantified for L-Asparaginase activity ranging from 1.44 to 3.42 U/ml [38]. Yap *et al*., 2021 [39] isolated the endophytic fungus *Fusarium proliferatum* from the medicinal plant *Cymbopogon citatus* produced 16.75 U/ml of L-Asparaginase. L-Asparaginase activity of different *Fusarium* sp. reported by Manasa and Nalini, 2014 [40], Jenila and Gnanadoss, 2018[21] had considerably low L-Asparaginase activity when compared to the present study.

Molecular Characterization

The endophytic fungal isolates LCJ315, LCJ324, LCJ326 and LCJ335 showed the best L-Asparaginase activity. Hence these isolates were subjected to molecular characterization to identify them. DNA extraction and amplification were done

using PCR with the help of ITS primer. The DNA sequencing was done using Sanger's dideoxy technique. Using BLAST, the acquired sequences were matched to the nucleotide sequence database of the NCBI. BLAST analysis revealed the identity of the isolated endophytic fungal strains and the obtained sequences were submitted to Genbank as well as accession numbers were acquired (Table 3). MEGA software was employed to construct the phylogenetic tree (Fig. 4-7).

Fusarium sp. and *Aspergillus* sp. were considered as the most frequently colonizing endophytic fungi in different parts of the host plant *Aegle marmelos* [41]. *Aspergillus* sp. of endophytic fungus was isolated by El-Said *et al*., 2016 [42] from *Datura innoxia* and *Hyoscyamus muticus* was the most frequent colonizing fungal genera. Hemalata and Amani, 2019 [43] reported *Aspergillus* sp. and *Fusarium* sp. were isolated from *Murraya koenigii* belonging to Rutaceae family. Kathiravan and Gnanadoss, 2023[44] reported *Fusarium* sp. as L-Asparaginase producing endophytic fungi isolated from Lamiaceae medicinal plants.

CONCLUSION

From this study, we can conclude that endophytic fungi *Aspergillus* sp. and *Fusarium* sp. may be used as potential candidates for the production of L-Asparaginase. Our findings support the concept that endophytes linked with medicinal plants have therapeutic properties. Fungal sources of enzymes are robust and stable when compared to other microbial sources of enzymes. The capacity of endophytic fungi to produce extracellular enzymes opens up new possibilities for the synthesis of industrial-based biocatalysts. The enzymatic biotransformed compounds derived from endophytic fungus could be employed as targets for drug discovery with therapeutic uses. However, the selection of host plants for the isolation of endophytic fungi as well as optimization of cultivation techniques are needed to obtain higher yield along with increased enzyme activity.

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Conflict of Interest

The authors have no conflict of interest.

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