RESEARCH ARTICLE

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Fungal isolates of high mountain ethnomedicinal plants in Uttarakhand, India, as a bioresource of industrially pertinent enzymes

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ABSTRACT

Endophytic fungi have been in the spotlight as a reservoir of novel agents with diverse bioactivities. Similarity in chemical diversity with the host plant makes them an amenable target for industrial interventions. A wide range of compounds as secondary metabolites and enzymes are manufactured inside the endophytic fungal factory. However, utilization of endophytic fungi as industrially imperative enzyme producers has been a scarce event. The present study was conducted to bio-prospect the fungal endophytes present in the high mountain medicinal plants of Uttarakhand, as industrially imperative enzyme producers. A total of 58 different endophytic isolates were obtained from *Pinus sabiniana, Cinnamomum tamala, Cinnamomum verum, Ocimum tenuiflorum* and *Rhododendron arboreum*. Endophytic fungal colonization was highest, 31%, in *Pinus sabiniana*. The pure isolates were further explored for the production of amylases, cellulases, proteases and L-asparaginase. Out of 58 isolates, 40 isolates exhibited potent enzyme productivity. #7 PSSTB isolate was considered as a superlative contender on account of its relatively higher production of all the three enzymes viz. amylases, cellulases, proteases. Partial purification of #7 PSSTB extract showed compelling enzymatic activity corroborating the existence of exogenous enzymes in the extract. Interestingly, #9 RASTB, #11 RASTB, and #17 RASTB exhibited the production of therapeutically imperative L-asparaginase enzyme. The present study puts the spotlight on endophytic isolates from five high altitude mountain medicinal plants as a source of enzymes of industrial interest. Production of L-asparaginase paves the way for pharmaceutical intervention to explore anti-oncogenic effects in the endophytic fungal repository of high altitude regions.

Key words: L-asparaginase**,** amylase, proteases, cellulases, endophytic fungi

Introduction

Endophytes are a specific group of micro-organisms that colonize plants internally without apparent adverse effects. Endophytic fungi are a potent source of novel organic compounds with pharmaceutically important biological activities and a high level of biodiversity (Deshmukh et al., 2018; Kapoor et al., 2019). All nonvascular and vascular plants examined until now have been found to harbor endophytic microbes with the potential to produce novel secondary metabolites. Bio-prospecting of endophytes has unraveled new molecules with therapeutic potentials (Strobel and Daisy, 2003). Several endophytic fungi have been reported as potent commercially imperative enzyme producers including *Alternaria tangelonis, Cladosporium cladosporioides, Curvularia akaii, Fusarium subglutinans* (Masumi et al., 2014), *Fusarium solani* (Uzma et al., 2016), and *Aspergillus terreus* (Kalyanasundaram et al., 2015).

Among the repository of fungal enzymes, viz. cellulase, amylase, protease and L-asparaginase, have been investigated for broad-spectrum effects. Cellulolytic enzymes have been a focal point of research as a mediator of baggase degradation and second generation ethanol production. Cellulase enzyme supplementation can improve the enzymatic hydrolysis of lignocellulosic biomass, in terms of speed and hydrolysis yield. Endophytic fungi viz. *Botryosphaeria* sp. and *Saccharicola* sp. have been investigated for the production of cellulase enzyme (Marques et al., 2018). Amylases are one of the prime industrial enzymes that encompass a wide spectrum of functional applications in pharmaceutical, food, textile, and detergent industries. Approx. 30 % of the total enzyme production globally

accounts for amylases (de Souza & Magalhães, 2010). On the other hand, proteases, an enzyme that breaks proteins into smaller constituents, shares two-third of the world enzyme market. Proteases have profound applications in bioremediation, cosmetics, silk degumming, animal cell culture, therapy, diagnosis, pharmaceutical, and food industry (Singh et al., 2016). L-asparaginase (EC 3.5.11. L-asparaginase amidohydrolase) is widely studied in context to its antitumor potential against tumor of lymphoid precursor, acute lymphoblastic leukemia, acute myeloid leukemia, and non-Hodgkin's lymphoma. The regular supply of asparagine, maintained by asparagine synthetase, is requisite for making proteins in the cell. Leukemic cells however are deficient in asparagines synthetase and depend solely on circulating blood for the supply of L-asparagine (Brumano et al., 2019). The immunogenic complications associated with its present microbial sources *Escherichia coli, Erwinia caratovora* limits its medicinal frontier. Administration of bacterial origin L-asparaginase is associated with allergic reactions and anaphylaxis. Thus, exploring the potential of alternate sources for the production of L-asparaginase has put the rich chemical diversity of endophytic fungi under the limelight. L-asparaginase derived from endophytic fungi has many benefits over existing preparations including the non-immunogenicity due to the phylogenetic relatedness and posttranslational modifications (Sarquis et al., 2004). The present study was undertaken to perform systematic screening for the production of enzymes and exploration of fungal endophytes from medicinal plants of high mountain regions of Uttarakhand, India.

Materials and Methods

Plant Sample Collection and isolation of fungi associated with high mountain medicinal plants

Healthy plant parts (bark, leaf, and stem) of *Rhododendron arboreum* Sm., *Cinnamomum tamala* (Buch.-Ham.) T. Nees & C. H. Eberm., *Cinnamomum verum* J. Presl, *Ocimum tenuiflorum* L. and *Pinus sabiniana* Douglas ex D. Don were collected from Bhowali and Nainital located at 29.3823° N, 79.5196° E (altitude of first collection point); 29.3803° N, 79.4636° E (altitude of second collection point) in Uttarakhand, India. The stems and leaves were surface-sterilized by dipping in 0.1 % sodium hypochlorite for 2-3 minutes followed by 70% ethanol for 1 min and then subsequent washing by dipping in 30% ethanol for 30-45 seconds. Aseptically, the sterilized sample was sectioned into 1-2 mm pieces and they were inoculated onto pre-sterilized Potato Dextrose Agar (PDA) plates. The plates were then incubated at 26±2ºC, 16h/8h light/Dark condition for 8-10 days. The plates were regularly monitored for any fungal growth. The fungal hyphae emerging out of the segment was transferred to a fresh PDA plate aseptically with the help of an inoculation loop to obtain a pure culture (Mitchell et al., 2008; Kapoor et al., 2018).

Production of Secondary metabolites

Each fungal isolate was subjected to culture filtrate production by inoculating 5 mm mycelial plug of 7 days old active culture into 100 ml pre-sterilized PDB medium aseptically and kept on the rotatory shaker at 26±2ºC, 120 rpm for 10 days. After incubation, the culture filtrate rich in bioactive compounds were separated from the fungal mass by filtration through Whatman filter paper no. 4 followed by centrifugation at 12,000 rpm for 15 mins to get cell-free culture filtrate (Raviraja, 2006).

Screening for extracellular enzyme production

Protease assay:

For the protease activity, the skim milk agar plates were prepared to contain 1% skim milk and 1 % agar. 30 µl of each culture filtrate was added into 5 mm wells prepared by sterile cork borer in skim milk agar plates followed by incubation at 37 ˚C for 24 h. Un-inoculated PDB medium served as control. After incubation, a clear zone around the wells indicates the proteolytic activity which was measured in terms of zone diameter and expressed as Mean ± SD. (Kasana et al., 2011; Pant et al., 2015).

Cellulase assay:

The modified agar well diffusion method was employed to assess the cellulase activity of fungal isolates as per the method described by (Marques et al., 2018; Legodi et al., 2019). Briefly, Czapek-Dox agar medium plates supplemented with 1% carboxymethyl cellulose, and 1% Agar was prepared. The plates were allowed to solidify for 30 mins, and 5 mm well were punched out with the help of a sterile cork borer. The culture filtrate of each fungus was loaded into the wells followed by incubation at 37˚C for 18-24 h. After the incubation, the plates were flooded with an aqueous Congo red solution. The appearance of the yellow zone around the fungal colony indicated cellulolytic activity. The zone diameter was measured and represented as Mean ± standard deviation (SD).

Amylase activity assay

Amylase activity was assessed by preparing the 1 % starch agar plate by following the procedure of Hankin & Anagnostakis (1975). The plates were solidified for 30 min and 5 mm well were punched out with help of sterile cork borer. The culture filtrate of each fungus was loaded into the wells followed by incubation at 37 ˚C for 18-24 h. Un-inoculated media served as control. After the incubation, the plates were flooded with the 1 % Iodine solution. Appearance of clear zone around the fungal colony indicated amylolytic activity which was measured in terms of zone diameter and represented as Mean ± SD.

Asparaginase Activity assay

Asparaginase production by the fungal endophytes was assessed by modified Ditch plate assay (Mahajan et al., 2013). L-asparaginase-agar plates were prepared by adding 2% L-asparagine supplemented with 0.009 % phenol red. Each plate was divided into four quadrants followed by preparation of 5 mm wells in each quadrant using pre-sterilized cork borer. Further, 30 µl of culture filtrates of each fungal endophyte were dispensed into the wells followed by incubation at 37°C for 24h. After the incubation period, the plates were observed for the pink halo formation around the wells. The zone diameter was recorded and expressed as Mean ± SD.

Mass production and Partial Purification by "salting out"

For the partial purification of desired enzyme, the selected isolate was subjected to mass fermentation of 1L by inoculating 5 mm active mycelial plug of single sample in 10 Erlenmeyer flask containing 100 ml pre-sterilized PDB medium followed by incubation at 28˚C, 120 rpm for 7-10 days. After the incubation period, cell mass and culture filtrate was separated by filtration through Whatman filter paper No. 4 followed by centrifugation at 10,000 rpm for 10 mins at 4^0C in refrigerated centrifuge. The obtained supernatant was then further subjected for precipitation of enzymatic protein by employing ammonium sulphate salting out method. Briefly, ammonium sulphate was slowly added to the culture broth to achieve saturation with slow and continuous stirring at 4°C. The mixture was then incubated overnight at 4°C and the next day protein precipitate was collected by centrifugation at 12,000 rpm for 15 min at 4°C. The obtained precipitate was dissolved in minimum volume of 20mM Tris–HCl buffer (pH 7.8) and analyzed for enzymatic activities (Raul et al., 2014; Pant et al., 2015).

Statistical Analysis

The statistical analysis was done using analysis of variance with GraphPad Prism 5 software followed by Tukey's post-hoc test ($p<0.05$). Data points were obtained from three replicates, and two independent experiments were performed.

Results

Fungal endophytes were isolated from medicinal plants

A total of 58 mycelial colonies of endophytes were isolated from five medicinal plants (Table 1, Figure 1). Maximum fungal isolations were recovered from *Pinus sabiniana* (31 %) followed by *C. tamala* (27.5 %) and *R. arboreum* (25.8 %). Minimal fungal colonization was observed in *C. verum* (5.1 %). The host tissue of each plant sample exhibited a variation in the colonization of the mycobiota. Further, colonization in different parts of plants was estimated. It was observed that maximum fungal colonization was observed in stems (62.1 %), followed by leaves (24.1 %). However, bark and stem internal tissues were least colonized by endophytes (Figure 2).

Screening of protease producing endophytic fungal isolates

In the preliminary screening studies, 31 fungal isolates out of 58 studied exhibited proteolytic activity (Table 2; Figure 3). As per One-way ANOVA analysis [$F_{(38,76)}= 231.4$, <0.001] and Tukey's post hoc analysis, #7 PSSTB isolate exhibited relatively highest protease production with zone size of 24 mm, followed by #13 CTSTN and #6 CVSTN with the zone size of 21.6 mm and 20.6 mm respectively. Further, a moderate level of activity was observed in #15b PSSTB and #18 OTLFN isolates, and the least activity was recorded in #1 RASTB, #4 PSSTB, #8 PSTITB and #5 CTSTN isolates.

Screening of amylase producing endophytic fungal isolates

In the amylolytic screening assay, only 5 endophytic isolates exhibited the amylase-producing potential. As per One-way ANOVA analysis [$F_{(38,76)}= 469.2$, <0.001] and Tukey's post hoc analysis, #1 RASTB isolate was found to be a potent amylase producer with zone size of 13.0 mm followed by #5 RASTB isolate with a zone size of 12.3 mm (Figure 4a-b). However, the least activity was observed in #14b PSSTB isolate.

Screening of cellulase producing fungal endophytic isolates

Cellulose is imperative in the leather, detergent and food processing industries. In the cellulolytic activity, 15 fungal isolates endophytes out of 58 showed positive results. As per One-way ANOVA analysis [$F_{(38,76)}= 1473$, <0.001] and Tukey's post hoc analysis, maximum activity was recorded in #7 PSSTB isolate with a zone size of 17.0 mm followed by #16a PSSTB isolate with a zone size of 16.3 mm. However, the least activity was recorded in #26 RASTB and #27 RASTB isolates with a zone size of 8.0 mm (Figure 4c-f).

Screening of L-asparaginase, produced by the fungal colonies

The isolated mycelia colonies were screened for the production of L-asparaginase enzyme. In the L-asparaginase screening assay, 9 endophytes were found to be potent L-asparaginase producers. One-way ANOVA analysis [$F_{(9,18)}= 130.3$, <0.001] and Tukey's post hoc analysis revealed maximum L-asparaginase production in #9 RASTB isolate with a zone size of 20.3 mm, followed by #11 RASTB and #17 RASTB with zone size of 20 mm [Table 2; Figure 5 (a-c)].

Bioactivity screening of partially purified protein

Further, the protein purification was done from the endophytic fungal isolates using the salting-out method and

Table 1. *Details on fungal isolations with culture codes, plant names, plant parts and sample collection sites obtained five medicinal plants.*

S.NO.	CULTURE COD	PLANT NAME	PART	PLACE
1.	# 7 RALFB	*R. arboberum*	Leaf	Bhowali
2.	# 18 RALFB	*R. arboberum*	Leaf	Bhowali
3.	# 21 RALFB	*R. arboberum*	Leaf	Bhowali
4.	# 25 RALFB	*R. arboberum*	Leaf	Bhowali
5.	# 1 RASTB	*R. arboberum*	Stem	Bhowali
6.	# 4 RASTB	*R. arboberum*	Stem	Bhowali
7.	# 5 RASTB	*R. arboberum*	Stem	Bhowali
8.	# 11 RASTB	*R. arboberum*	Stem	Bhowali
9.	# 15 RASTB	*R. arboberum*	Stem	Bhowali
10.	# 17 RASTB	*R. arboberum*	Stem	Bhowali
11.	# 26 RASTB	*R. arboberum*	Stem	Bhowali
12.	# 27 RASTB	*R. arboberum*	Stem	Bhowali
13.	# 28 RASTB	*R. arboberum*	Stem	Bhowali
14.	# 29 RASTB	*R. arboberum*	Stem	Bhowali
15.	# 9 RASTITB	*R. arboberum*	Stem internal tissue	Bhowali
16.	# 2a PSSTB	*P. sabiniana*	Stem	Bhowali
17.	# 2b PSSTB	*P. sabiniana*	Stem	Bhowali
18.	# 4 PSSTB	*P. sabiniana*	Stem	Bhowali
19.	# 5 PSSTB	*P. sabiniana*	Stem	Bhowali
20.	# 7 PSSTB	*P. sabiniana*	Stem	Bhowali
21.	# 9 PSSTB	*P. sabiniana*	Stem	Bhowali
22.	# 13c PSSTB	*P. sabiniana*	Stem	Bhowali
23.	# 14b PSSTB	*P. sabiniana*	Stem	Bhowali
24.	# 14c PSSTB	*P. sabiniana*	Stem	Bhowali
25.	# 15a PSSTB	*P. sabiniana*	Stem	Bhowali
26.	# 15b PSSTB	*P. sabiniana*	Stem	Bhowali
27.	# 15c PSSTB	*P. sabiniana*	Stem	Bhowali
28.	# 16 PSSTB	*P. sabiniana*	Stem	Bhowali
29.	# 16a PSSTB	*P. sabiniana*	Stem	Bhowali
30.	# 17c PSSTB	*P. sabiniana*	Stem	Bhowali
31.	# 08 PSSTITB	*P. sabiniana*	Stem internal tissue	Bhowali
32.	# 10 PSSTITB	*P. sabiniana*	Stem internal tissue	Bhowali
33.	# 18a PSSTITB	*P. sabiniana*	Stem internal tissue	Bhowali
34.	# 1 OTLFN	*O. tenuiflorum*	Leaf	Nainital
35.	# 14 OTLFN	*O. tenuiflorum*	Leaf	Nainital
36.	# 16 OTLFN	*O. tenuiflorum*	Leaf	Nainital
37.	# 18 OTLFN	*O. tenuiflorum*	Leaf	Nainital
38.	# 10 OTSTN	*O. tenuiflorum*	Stem	Nainital
39.	# 15 OTSTN	*O. tenuiflorum*	Stem	Nainital
40.	# 1 CTLFN	*C. tamala*	Leaf	Nainital
41.	# 15 CTLFN	*C. tamala*	Leaf	Nainital
42.	# 18 CTLFN	*C. tamala*	Leaf	Nainital
43.	# 21 CTLFN	*C. tamala*	Leaf	Nainital
44.	# 27 CTLFN	*C. tamala*	Leaf	Nainital
45.	# 32 CTLFN	*C. tamala*	Leaf	Nainital
46.	# 2 CTSTN	*C. tamala*	Stem	Nainital
47.	# 4 CTSTN	*C. tamala*	Stem	Nainital
48.	# 5 CTSTN	*C. tamala*	Stem	Nainital
49.	# 6 CTSTN	*C. tamala*	Stem	Nainital
50.	# 8 CTSTN	*C. tamala*	Stem	Nainital
51.	# 10 CTSTN	*C. tamala*	Stem	Nainital
52.	# 11 CTSTN	*C. tamala*	Stem	Nainital
53.	# 13 CTSTN	*C. tamala*	Stem	Nainital
54.	# 14 CTSTN	*C. tamala*	Stem	Nainital
55.	# 11 CTSTITN	*C. tamala*	Stem internal tissue	Nainital
56.	# 5 CVBN	*C. verum*	Bark	Nainital
57.	# 6a CVBN	*C. verum*	Bark	Nainital
58.	# 18 CVBN	*C. verum*	Bark	Nainital

Each endophytic fungal isolate is encoded on the plant part (LF - Leaf, ST - stem, STIT - stem internal tissue, B - Bark), and collection sites (Place). For example, in #7 RALFB, #7 - refers to the segment number, RA - plant scientific name, LF – leaf explants used for isolation and B – Bhowali, place of collection.

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Table 2. *In vitro enzyme production of 40 fungal isolates (of 58 tested, 18 isolates showed no activity)*

S.NO.	CULTURE CODE	Average Zone size (in mm)			
		PROTEASE	AMYLASE	CELLULASE	L-ASPARAGINASE
1.	# 21 RALFB	-	-	12.3 ± 0.58 ^{d}	17.6 ± 0.58 ^{b}
2.	# 25 RALFB	8.3 ± 0.58 ^{jk}	-	-	20.0 ± 0.0 ^{a}
3.	# 1 RASTB	7.0 ± 0.0 ^{jk}	13.0 ± 1.0 ^{a}	8.0 ± 0.0 ^{h}	-
4.	# 4 RASTB	14.3 ± 0.58 ^{defg}	-	-	19.6 ± 0.58 ^{a}
5.	# 5 RASTB	9.0 ± 0.0 ^{ijk}	12.3 ± 0.58 ^{ab}	10.0 ± 0.0 ^{f}	-
6.	# 9 RASTB	-	-	-	20.5 ± 1.52 ^{a}
7.	# 11 RASTB	-	-	-	20.0 ± 0.0 ^{a}
8.	# 17 RASTB	12.0 ± 0.0 ^{gh}	-	10.0 ± 0.0 ^{f}	20.0 ± 0.0 ^{a}
9.	# 26 RASTB	13.3 ± 0.58 ^{efgh}	-	8.0 ± 0.0 ^{h}	15.3 ± 0.58 ^{c}
10.	# 27 RASTB	11.3 ± 0.58 ^{hi}	-	8.0± 0.0 ^{h}	16.0 ± 0.0 ^{bc}
11.	# 28 RASTB	9.3 ± 0.58 ^{ij}	-	9.0 ± 0.0 ^{g}	-
12.	# 29 RASTB	-	-	9.0 ± 0.0 ^{g}	-
13.	# 2b PSSTB	12.0 ± 0.0 ^{gh}	-	-	-
14.	# 4 PSSTB	6.67 ± 0.58 ^{k}	-	-	-
15.	# 7 PSSTB	24.0 ± 1.0 ^{a}	11.0 ± 1.0 ^{c}	17.0 ± 0.0 ^{a}	-
16.	# 9 PSSTB	8.67 ± 0.58 ^{jk}	-	-	-
17.	# 14b PSSTB	15.67 ± 1.52 ^{cde}	7.3 ± 1.1 ^{d}	-	-
18.	# 14c PSSTB	12.6 ± 1.15 f^{gh}	-	-	-
19.	# 15a PSSTB	-	-	-	-
20.	# 15b PSSTB	18.0 ± 0.0 ^{c}	-	-	-
21.	# 15c PSSTB	9.0 ± 1.0 ^{ijk}	-	-	-
22.	# 16 PSSTB	-	12.0 ± 0.0 ^{b}	11.6 ± 0.58 ^{de}	-
23.	# 16a PSSTB	-	-	15.6 ± 0.58 ^{b}	-
24.	# 8 PSSTITB	7.0 ± 0.0 ^{jk}	-	-	-
25.	# 10 PSSTITB	8.0 ± 0.0 ^{jk}	-	9.0 ± 0.0 ^{g}	-
26.	# 18a PSSTITB	15.0 ± 2.0 ^{def}	-	-	-
27.	# 14 OTLFN	12.0 ± 0.0 ^{gh}	-	-	-
28.	# 18 OTLFN	18.0 ± 1.0 ^{c}	-	-	-
29.	# 10 OTSTN	-	-	-	-
30.	# 15 OTSTN	7.3 ± 0.58 ^{jk}	-	-	-
31.	# 1 CTLFN	15.0 ± 1.0 ^{def}	-	-	8.0 ± 0.0 ^{d}
32.	# 32 CTLFN	16.0 ± 1.0 ^{cd}	-	-	-
33.	# 5 CTSTN	7.0 ± 0.0 ^{jk}	-	-	-
34.	# 6 CTSTN	20.6 ± 0.58 ^{b}	-	14.6 ± 0.58 ^{c}	-
35.	# 8 CTSTN	14.0 ± 0.0 ^{defg}	-	-	-
36.	# 10 CTSTN	-	-	10.0 ± 0.0 ^{f}	-
37.	# 13 CTSTN	21.6 ± 0.58 ^{ab}	-	14.6 ± 0.58 ^{c}	-
38.	# 14 CTSTN	15.6 ± 0.58 ^{cde}	-	-	-
39.	# 5 CVBN	12.3 ± 2.08 ^{gh}	-	-	-
40.	# 6a CVBN	12.0 ± 0.0 ^{gh}	-	11.0 ± 1.0 ^{e}	

*Data presented as mean ± standard deviation (SD) of three replicates. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$).

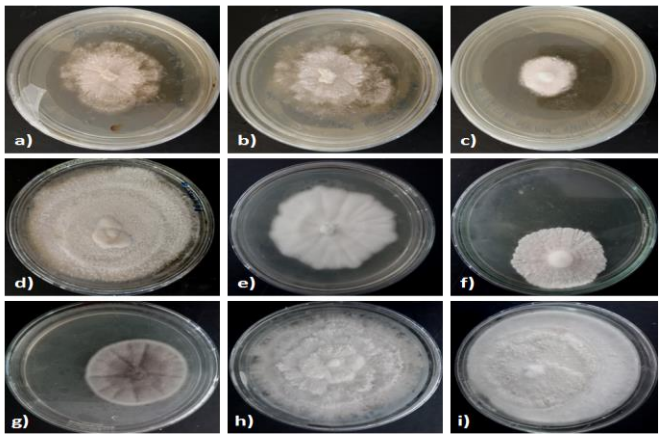

Figure 2. *Colonies of fungal endophytes (7 days old culture) obtained from high mountain ethnomedicinal plants in Uttarakhand (Pinus sabiniana, Cinnamomum tamala, Cinnamomum verum, Ocimum tenuiflorum, Rhododendron arboretum): (a) #12 RASTB, (b) #5 RASTITB, (c) #11 RASTB, (d) # 16a PSSTB, (e) #15a PSSTB, (f) #5 CTSTN, (g) #9 CTSTN, (h) #18 RALB, (i) #12 RALB.*

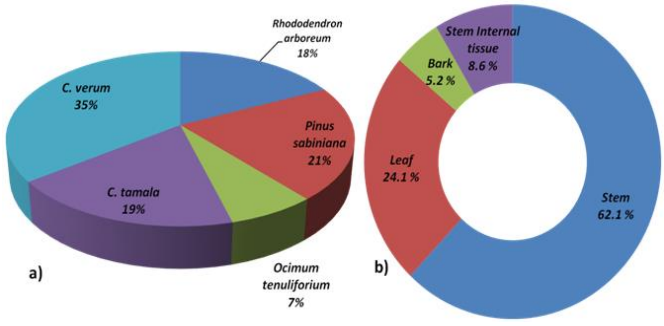

Figure 1. *Colonization frequency of fungal isolations shown by distribution in:* ***(a)*** *host plants, maximum (31% of isolates) was observed in Pinus sabiniana;* ***(b)*** *host tissues, maximum fungal isolations observed in stems, compared to leaves, internal stem tissues and bark.*

evaluated for enzyme activities. The partially purified protein of #7 PSSTB, opted due to consistent best results in all the three screenings, was again subjected for bioactivity profiling. In protease, amylase and cellulase activity assay, zone sizes of 16 mm, 12 mm, and 16 mm respectively was

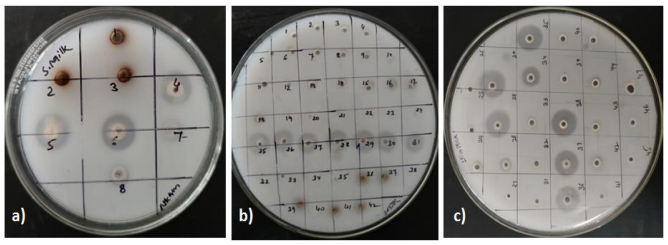

Figure 3. ***(a-c)*** *Proteolytic activity of culture filtrates obtained from 10 days old endophytic fungal batch fermentation. Zone of clearance against white background is observed among positive isolates over skim milk agar plate*

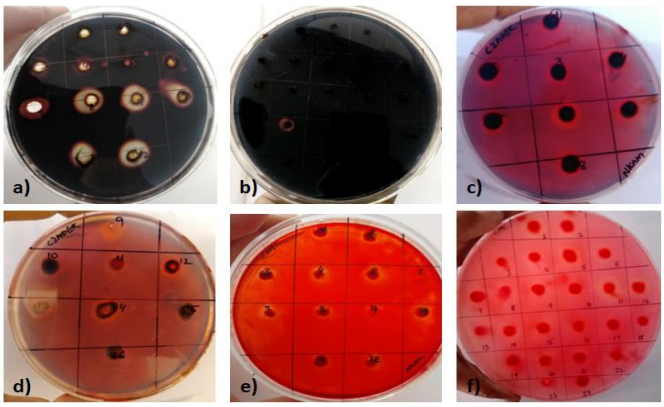

Figure 4. *Amylase and cellulase production exhibited by culture filtrates of fungal endophytic isolates.* ***(a-b)*** *amylolytic activity, indicated by clear zone around the well over starch agar plate stained by Iodine solution* ***(c-f)*** *cellulolytic activity, indicated by pale yellow halo around well on carboxymethyl cellulose agar, stained in aqueous Congo red.*

Discussion

Endophytic fungi have been investigated as a repository of bioactive metabolites for industrial and therapeutic applications. Being in the symbiotic association with the host plant, endophytic fungi share a similar chemical diversity as that of the plant (Jia et al., 2016; Khare et al., 2018; Gambhir et al., 2020). Therefore, the endophytic fungi, ubiquitous in medicinal plants, have been investigated for the production of novel metabolites with cost-effective industrial applications. Studies have revealed that around 1 million species of endophytic fungi are residing in plants (Xiang & Guo, 2012).

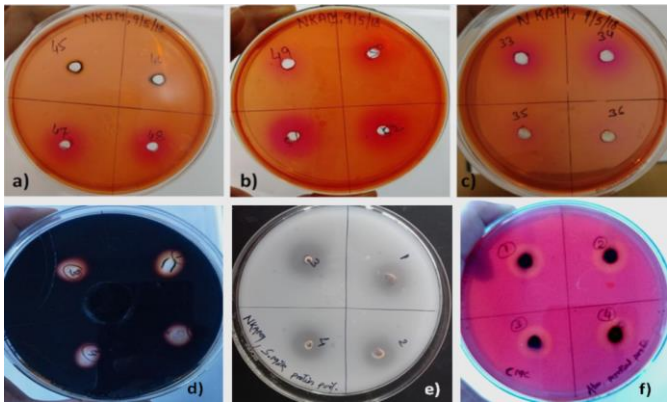

Figure 5. ***(a-c)*** *L-asparaginase enzyme production of culture filtrates, obtained from 10 days old fungal endophytic isolates, indicated by dark pink-coloured halo around the well on Asparagine-phenol red agar. Maximum L-asparaginase production observed in #9 RASTB isolate (zone size 20.5mm).* ***(d-f)*** *Bioactivity profiling of partially purified protein of #7 PSSTB isolate. The cultural filtrate was processed for partial purification of protein by salting out. The bioactivity profiling exhibited:* ***(a)*** *Amylolytic activity;* ***(b)*** *Protease activity;* ***(c)*** *Cellulase activity with a zone size of 16 mm, 12 mm and 16 mm respectively.*

A study of endophytic fungi from tropical and temperate forests supports the high estimates of species diversity (Strobel & Daisy, 2003). Over two decades endophytic fungi have been investigated for novel chemistries targeting a spectrum of bioactivities. They have been recognized as a primary resource for harnessing antibiotics, anticancer, immunomodulatory, antiparasitic, etc. compounds. Apart from these therapeutic compounds, endophytic fungi have long been known as potent hydrolytic and oxidative enzyme producers (Sahoo et al., 2018). Production of enzymes such as amylases, cellulases, and proteases by endophytic fungi is done to combat the host plant defense and to attain the desired nutrients for their sustenance. However, less attention has been given to the use of endophytic fungi as potent industrial enzyme producers.

The present study investigates the potential of endophytic fungal isolates from high mountain medicinal plants of Uttarakhand, as commercially imperative enzyme producers. Medicinal plants viz *Rhododendron arboreum, Cinnamomum tamala, Cinnamomum verum, Ocimum tenuiflorum,* and *Pinus sabiniana* were selected for bioprospecting of endophytic fungi. The explants were subjected to endophytic isolation and 58 fungal endophytic isolates were studied (Table 1). Maximum colonization of endophytic fungi was observed in *Pinus sabiniana* and stem as an explant source (Figure 2). Further, the isolates were screened for the production of industrially and medicinally useful enzymes such as amylases, cellulases, proteases, and L-asparaginase.

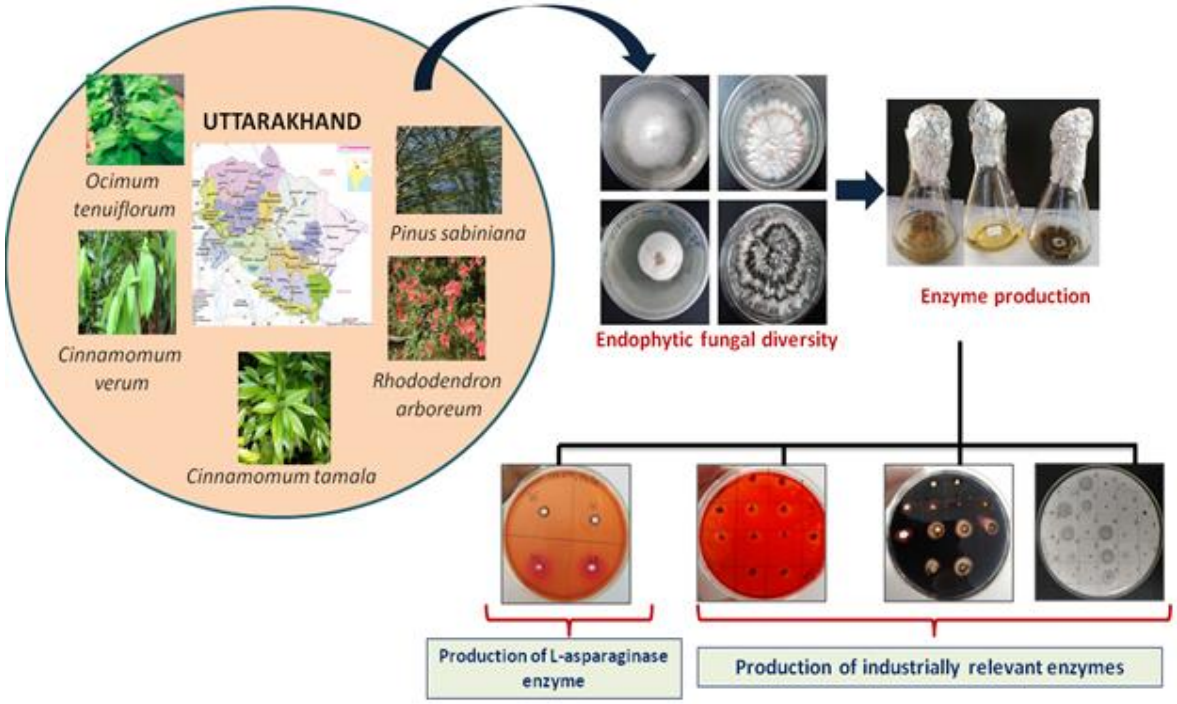

Figure 6. *Scheme of production of industrially relevant enzymes by endophytic fungal isolates from medicinal plants in Uttarakhand.*

Out of the 58 isolated endophytic fungal colonies, 40 isolates were found to be enzyme producers with varying degrees of productivity, whereas 18 isolates showed no enzymatic activity. #7PSSTB isolate was selected as the best candidate for further screening, due to its potential of relatively high production of three enzymes, viz. amylases, cellulases, proteases (Table 2). Further, crude protein mixture obtained after partial purification of extracts exhibited potent enzymatic activity contemplating the presence of the exogenous enzyme in the endophytic fungal isolates. L-asparaginase has been shown to exhibit a profound tumor-suppressive activity (El-Said et al., 2016). Interestingly, L-asparaginase production was also observed in the endophytic isolates mainly #9 RASTB, #11 RASTB, and #17 RASTB. The production of L-asparaginase further substantiated the imperative reserve of endophytic fungal isolates as a pharmaceutically important repository.

Conclusion

Medicinal plants of high altitude Himalayan regions of Uttarakhand have been used for potent medicinal properties. Endophytic fungi present in medicinal plants provide an alternate itinerary to exploit the bioactive chemistries analogous to host plants. Very few reports have demonstrated the bioprospecting endophytic fungi from high-altitude medicinal plants. The present study demonstrates the colonization of endophytic fungi in *Rhododendron arboreum*, *Cinnamomum tamala*, *Cinnamomum verum*, *Ocimum tenuiflorum*, and *Pinus sabiniana* (Figure 6). The present study suggests the possible exploration of endophytic fungal isolates as industrially viable enzyme producers. More studies are warranted to optimize the enzyme production from isolated fungal colonies and characterization of their physiochemical properties.

Conflict of interest

There is no actual or potential competing interest.

Authors` contributions

NK and AM performed the experiments. NK and LG conceived the idea and designed the research. LG and NK analyzed the data and all the authors contributed to writing the manuscript.

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