Fungal endophytes of high altitude ethnomedicinal plants as a bioresource of industrially imperative 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 altitude 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. #7PSSTB isolate was considered as superlative contender on account of its relatively higher production of all the three enzymes viz. amylases, cellulases, proteases. Partial purification of #7PSSTB extract showed compelling enzymatic activity corroborating the existence of exogenous enzyme 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 diversity in the high altitude medicinal plants as a source of enzymes of industrial interest. Production of L-asparaginase paves the way of pharmaceutical intervention to explore anti-oncogenic effects in the endophytic fungal repository of high altitude regions.

Introduction

Endophytes are specific group of micro-organisms which colonize plants internally without apparent adverse effects. Endophytic fungi are potent source of novel organic compounds with pharmaceutically important biological activities and a high level of biodiversity (Deshmukh et al. 2018). 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 and 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 enzymes 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 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 and e

Magalhães 2010). On the other hand, proteases, an enzyme which breaks proteins to 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. Regular supply of asparagine, maintained by asparagines 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 rich chemical diversity of endophytic fungi under limelight. L-asparaginase derived from endophytic fungi have many benefits over existing preparations like the non-immunogenic as they are phylogenetically related and posttranslational modifications are present, being the eukaryotic microbes and residing inside the plant body (Sarquis et al. 2004). The present study was undertaken for systematic screening for the production of enzymes and exploration of fungal endophytes from medicinal plants of high altitude regions of Uttarakhand, India.

Methodology

Plant Sample Collection and isolation of endophytic fungi

Healthy plant parts (Bark, leaf and stem) of *Rhododendron arboreum, Cinnamomum tamala, Cinnamomum verum, Ocimum tenuiflorum and Pinus sabiniana* were collected from Bhowali and Nainital located at 29.3823° N, 79.5196° E; 29.3803° N, 79.4636° E in Uttarakhand, India. The stems and leaves were surface sterilized by dipping in 0.1% sodium hypochlorite for 2–3 min followed by 70% ethanol for 1 min and then subsequent washing by dipping in 30% ethanol for 30–45 sec. Aseptically, the sterilized sample was sectioned into 1–2 mm pieces and were inoculated on to pre sterilized Potato Dextrose Agar (PDA) plates. The plates were then incubated at 26 ± 2ºC, 16 h/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 fresh PDA plate aseptically with the help of inoculation loop to obtain pure culture (Hess et al. 2008; Kapoor et al. 2018).

Production Of Secondary Metabolites

Each fungal endophyte 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 fungal mass by filtration through Whatman filter paper no. 4 followed by centrifugation at 12,000 rpm for 15 min to get cell free culture filtrate (Lobo 2006).

Screening For Extracellular Enzyme Production

Protease assay:

For the protease activity, the skim milk agar plates were prepared containing 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 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 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. After the incubation, the plates were flooded with aqueous Congo red solution. The appearance of yellow zone around the fungal colony indicated cellulolytic activity. The zone diameter was measured and represented as Mean ± SD.

Amylase activity assay

Amylase activity was assessed by preparing the 1% starch agar plate by following the procedure of (Hankin and 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). Briefly describing, L- asparaginase-agar (2%) 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 24 h. 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 potential endophytic fungi was subjected to mass fermentation of 1L by inoculating 5 mm active mycelial plug of selected fungal endophyte 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 min 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 20 mM 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 different fungal endophytes were isolated from various medicinal parts (Table 1, Fig. 1). Maximum fungal endophytes were recovered from *Pinus* sp. (31.0%) followed by *C. tamala* (27.5%) and *R. arboreum* (25.8%). However least fungal colonization was observed in *C. verum* (5.1%). The host tissue of each plant sample exhibited a variation in colonization of the endophytic mycoflora. Further, colonization in different parts of plants was estimated. It was observed that maximum fungal colonization was observed in stem (62.1%) followed by leaf (24.1%). However, bark and stem internal tissues were least colonized by endophytes (Fig. 2).

Table 1
Details of fungal endophytes along with assigned culture code, plant name, plant part and sample collection site recovered from different medicinal plants under study

S.NO.	CULTURE CODE	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	*Pinus sabiniana*	Stem	Bhowali
17.	# 2b PSSTB	*Pinus sabiniana*	Stem	Bhowali
18.	# 4 PSSTB	*Pinus sabiniana*	Stem	Bhowali
19.	# 5 PSSTB	*Pinus sabiniana*	Stem	Bhowali
20.	# 7 PSSTB	*Pinus sabiniana*	Stem	Bhowali
21.	# 9 PSSTB	*Pinus sabiniana*	Stem	Bhowali
22.	# 13c PSSTB	*Pinus sabiniana*	Stem	Bhowali
23.	# 14b PSSTB	*Pinus sabiniana*	Stem	Bhowali

Each endophytic fungal isolate was encoded based on the host plant, its part (LF for Leaf, ST for stem, STIT for stem internal tissue and B for Bark) and the place from where it was collected. For instance in #7 RALFB, #7 refers to the segment number, RA refers to the host medicinal plant scientific name, LF refers to leaf as explants for isolation and B refers to Bhowali the place of collection

S.NO.	CULTURE CODE	PLANT NAME	PART	PLACE
24.	# 14c PSSTB	*Pinus sabiniana*	Stem	Bhowali
25.	# 15a PSSTB	*Pinus sabiniana*	Stem	Bhowali
26.	# 15b PSSTB	*Pinus sabiniana*	Stem	Bhowali
27.	# 15c PSSTB	*Pinus sabiniana*	Stem	Bhowali
28.	# 16 PSSTB	*Pinus sabiniana*	Stem	Bhowali
29.	# 16a PSSTB	*Pinus sabiniana*	Stem	Bhowali
30.	# 17c PSSTB	*Pinus sabiniana*	Stem	Bhowali
31.	# 08 PSSTITB	*Pinus sabiniana*	Stem internal tissue	Bhowali
32.	# 10 PSSTITB	*Pinus sabiniana*	Stem internal tissue	Bhowali
33.	# 18a PSSTITB	*Pinus 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

Each endophytic fungal isolate was encoded based on the host plant, its part (LF for Leaf, ST for stem, STIT for stem internal tissue and B for Bark) and the place from where it was collected. For instance in #7 RALFB, #7 refers to the segment number, RA refers to the host medicinal plant scientific name, LF refers to leaf as explants for isolation and B refers to Bhowali the place of collection

S.NO.	CULTURE CODE	PLANT NAME	PART	PLACE
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 was encoded based on the host plant, its part (LF for Leaf, ST for stem, STIT for stem internal tissue and B for Bark) and the place from where it was collected. For instance in #7 RALFB, #7 refers to the segment number, RA refers to the host medicinal plant scientific name, LF refers to leaf as explants for isolation and B refers to Bhowali the place of collection

Screening Of Protease Producing Endophytic Fungal Isolates

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

Table 2

in vitro enzyme production of 40 fungal endophytes (out of total 58 endophytes, 18 endophytes did not exhibited any 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 ^{fgh}	-	-	-
19.	# 15a PSSTB	-	-	-	-
20.	# 15b PSSTB	18.0 ± 0.0 ^{c}	-	-	-
21.	# 15c PSSTB	9.0 ± 1.0 ^{ijk}	-	-	-

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

S.NO.	CULTURE CODE	Average Zone size (in mm)			
		PROTEASE	AMYLASE	CELLULASE	L-ASPARAGINASE
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 of three replicates. Means with different superscript letters are different by Tukey's post-hoc test ($p < 0.05$).

Screening Of Amylase Producing Endophytic Fungal Isolates

In the amylolytic screening assay, only 5 endophytes 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 was found to be potent amylase producer with zone size of 13.0 mm followed by #5 RASTB with zone size of 12.3 mm (Fig. 4a-b). However, least activity was observed in #14b PSSTB.

Screening Of Cellulase Producing Fungal Endophytic Isolates

Cellulose is imperative in leather, detergents and food processing industries. In the cellulolytic activity, 15 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 with a zone size of 17.0 mm followed by #16a PSSTB with zone size of 16.3 mm. However least activity was recorded in #26RASTB and #27RASTB with zone size of 8.0 mm (Fig. 4c-f).

Screening Of L-asparaginase Producing Fungal Endophytes

The isolated endophytic fungal isolates were screened for the production of L-asparaginase enzyme. In the L-asparaginase screening assay, 9 endophytes were found to be potent L-asparaginase producer. 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 with a zone size of 20.3 mm followed by #11 RASTB and #17 RASTB with zone size of 20 mm (Table 2; Fig. 5).

Bioactivity Screening Of Partially Purified Protein

Further the protein purification was done from the endophytic fungal isolates using salting out method and evaluated for enzyme activities. The partially purified protein of #7PSSTB, 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 size of 16 mm, 12 mm and 16 mm respectively was obtained (Fig. 6).

Discussion

Endophytic fungi have been investigated as repository of bioactive metabolites for industrial and therapeutic applications. Being in symbiotic association with the host plant endophytic fungi shares the similar chemical diversity as that of the plant (Jia et al. 2016; Khare et al. 2018). 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. A study of endophytic fungi from tropical and temperate forests supports the high estimates of species diversity (Strobel and Daisy 2003). Over two decades endophytic fungi have been investigated for the novel chemistries targeting a spectrum of

bioactivities. They have been recognized as 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, cellulase and proteases by endophytic fungi is done to combat the host plant defence 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 was done to investigate the potential of endophytic fungi isolated from high altitude 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 different fungal endophytes were isolated (Table 1). Maximum colonization of endophytic fungi was observed in *Pinus sabiniana* and stem as an explant source (Fig. 2). Further, the isolates were screened for production of industrially and medicinally useful enzymes such as amylases, cellulases, proteases and L-asparaginase. Out of the 58 isolated endophytic fungi 40 isolates were found to be enzyme producers with varying degree of productivity whereas 18 isolates did no showed any enzymatic activity. #7PSSTB isolate was selected as the best candidate for further screening owing to its potential of relatively higher production of all the 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 exogenous enzyme in the isolated endophytic fungi. L-asparaginase has been shown to exhibit a profound tumor suppressive activity (Mohamed Shebany et al. 2016). Interestingly, L-asparaginase production was also observed in the endophytic isolates mainly #9 RASTB, #11 RASTB and #17 RASTB. Production of L-asparaginase further substantiated the imperative reserve of endophytic fungi as 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 plant. 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*. The present study paves the way for exploration of endophytic fungi as industrially viable enzyme producers. More studies are warranted to optimize the enzyme production from isolated fungal endophytes and characterization of its physiochemical properties.

Abbreviations

PDA
Potato Dextrose Agar
Rpm

Rotation per minute
SD
Standard Deviation
ANOVA
Analysis of Variance
LF
Leaf
ST
stem
STIT
stem internal tissue
B
Bark
RA
Rhododendron arboreum
CT
Cinnamomum tamala
CV
Cinnamomum verum
OT
Ocimum tenuiflorum
PS
Pinus sabiniana

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All the authors have read and are in consent to submit it to *Bioresources and Bioprocessing*.

Availability of data and materials

The data pertaining to this article are incorporated in the manuscript.

Competing interest

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Author's 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 in writing the manuscript.

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Figures

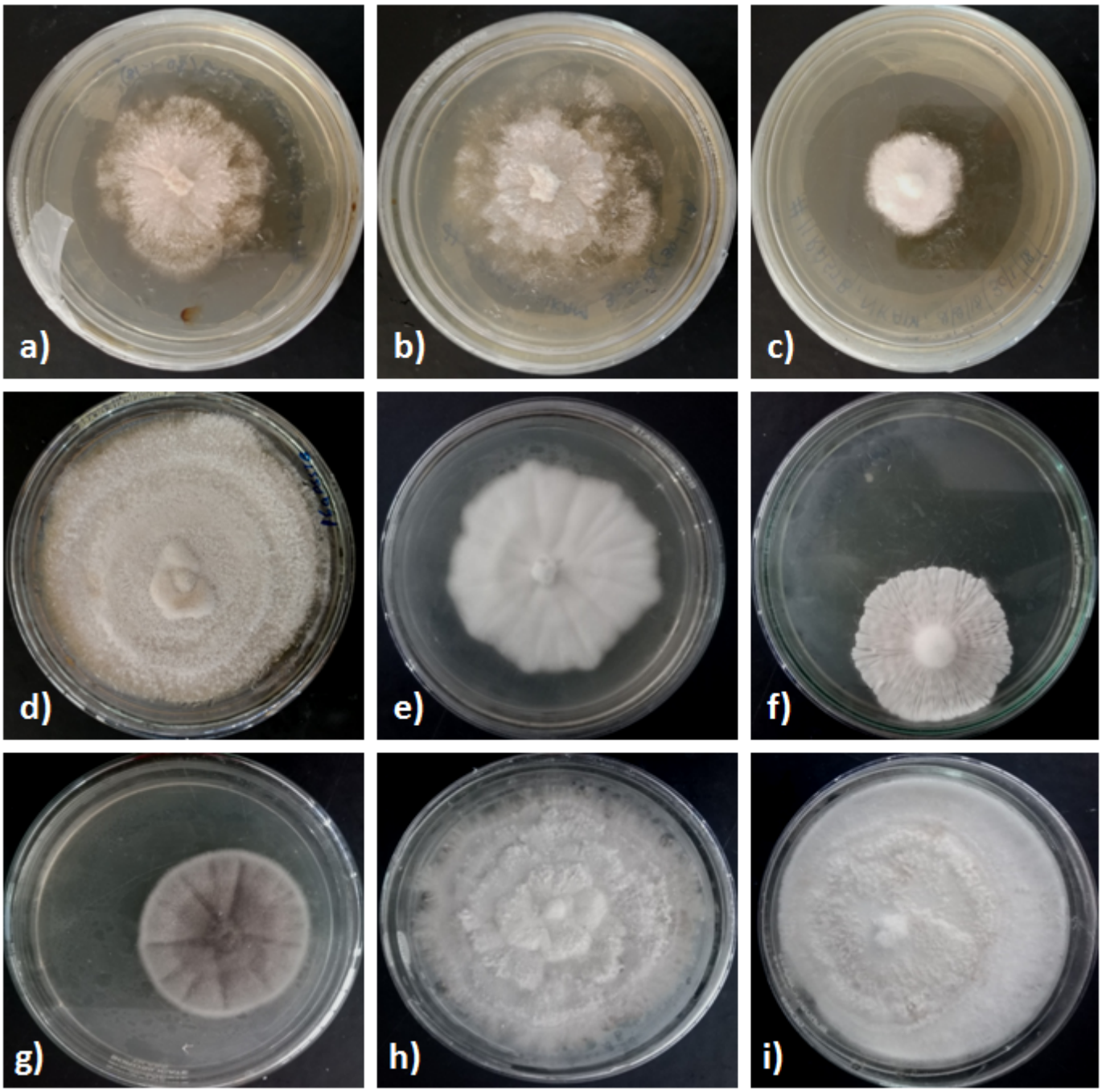

Figure 1

Fungal endophytes obtained from high altitude ethnomedicinal plants of Uttarakhand viz. Pinus sabiniana, Cinnamomum tamala, Cinnamomum verum, Ocimum tenuiflorum and Rhododendron arboreum used in the study: (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.

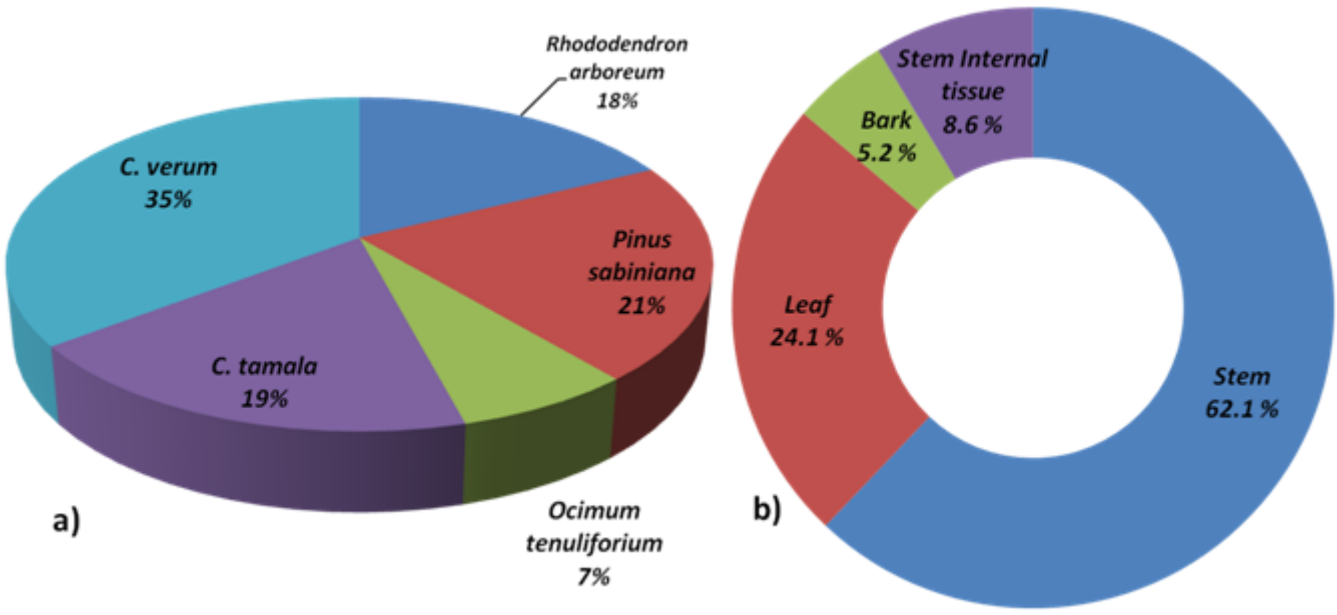

Figure 2

Colonization frequency of fungal endophytes was graphed as percentage distributions of fungal endophytes in (a) host plant, maximum colonization of 31% was observed in Pinus sabiniana. (b) host tissue, maximum colonization was observed in stem compared with leaf, stem internal tissue and bark.

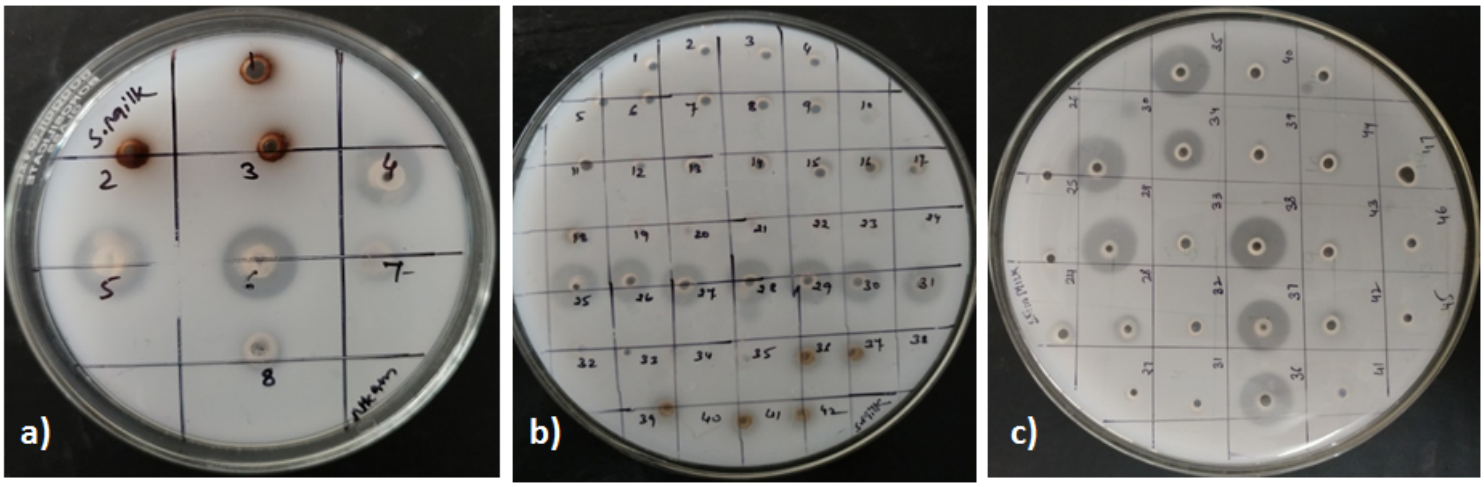

Figure 3

(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.

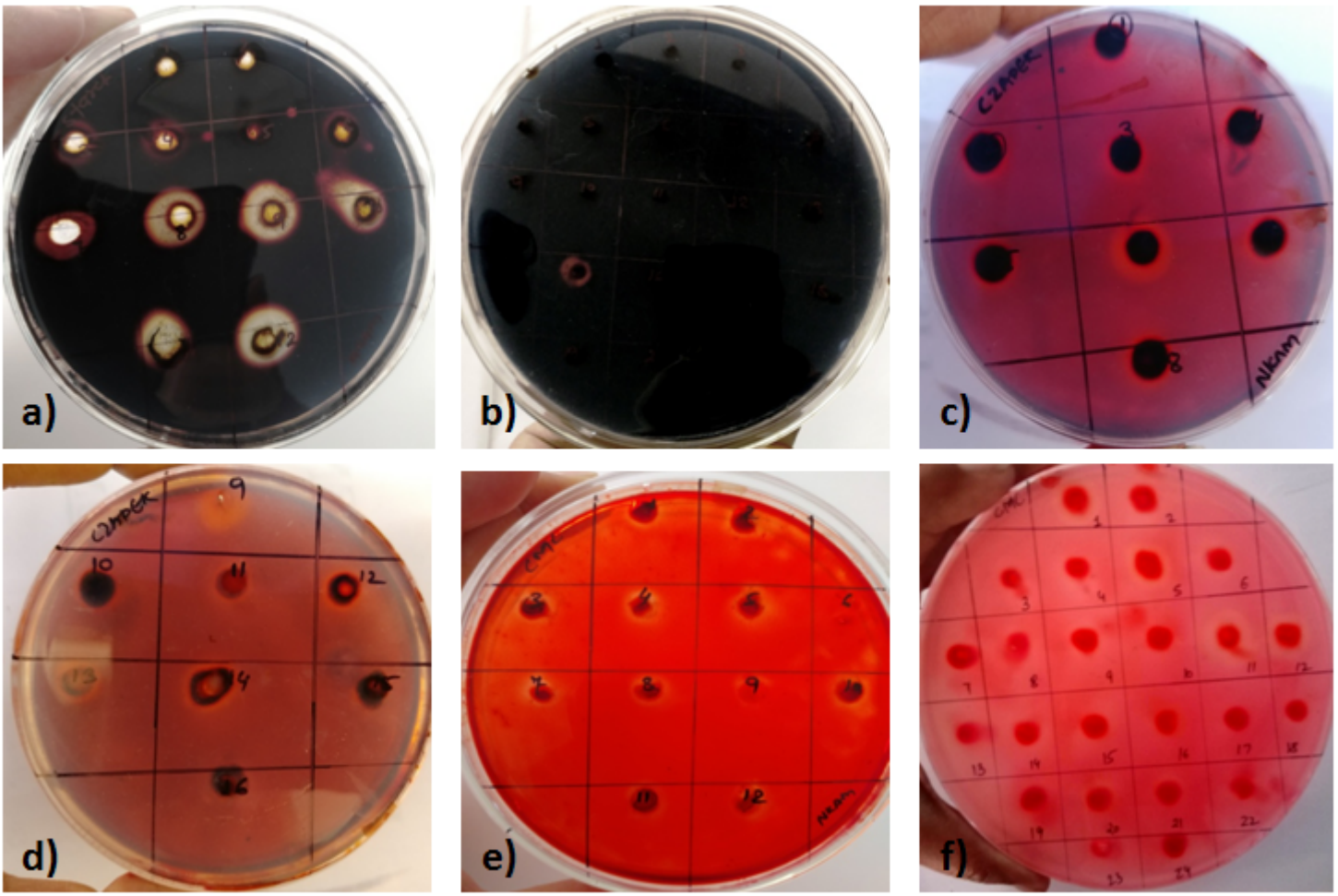

Figure 4

Amylase and cellulase production exhibited by culture filtrates of different fungal endophytes. (a-b) amylolytic activity as indicated by clear zone around the well over starch agar plate stained by Iodine solution (c-f) cellulolytic activity as indicated by pale yellow halo around well over carboxymethyl cellulose agar plate stained with congo red dye.

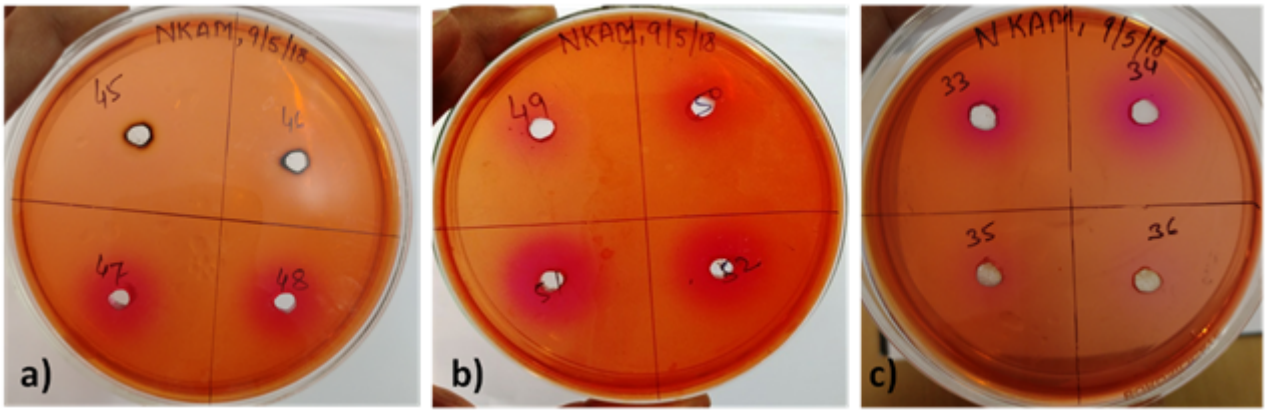

Figure 5

(a-c) L-asparaginase enzyme production by culture filtrates obtained from 10 days old of different fungal endophytes as indicated by dark pink color halo around the well over Asparagine-phenol red agar plate.

Maximum L-asparaginase production was observed in #9 RASTB with a zone size of 20.3 mm.

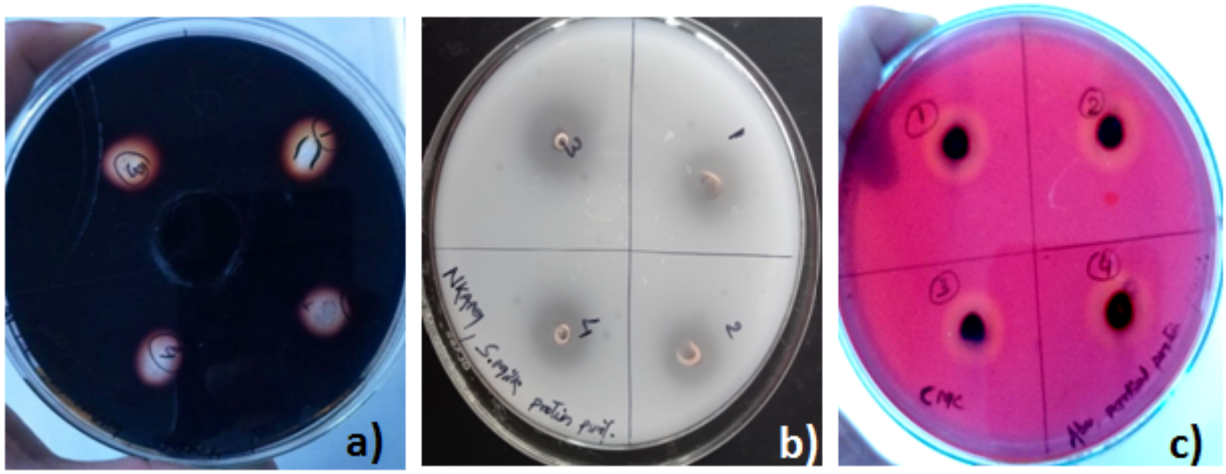

Figure 6

Bioactivity profiling of partially purified protein of #7PSSTB. The cultural filtrate of most promising candidate #7PSSTB 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

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