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Hyper-production of taxol from *Aspergillus fumigatus*, an endophytic fungus isolated from *Taxus* sp. of the Northern Himalayan region

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ABSTRACT

Taxol® (generic name Paclitaxel) is a chemotherapeutic drug, effective against head, neck, breast, lung, bladder, ovary, and cervix cancers. Rising demands in chemotherapy and limited supply of natural taxol have ultimately increased the cost of the drug. Semi synthesis using taxol precursors is not able to meet the global supply and has intensified the need to find alternative ways of taxol production. In the present study, 34 different endophytes were isolated from *Taxus* sp. collected from Shimla, Himachal Pradesh (India). Primary screening of taxol-producing fungi was carried out based on the presence of *dbat* gene, essential for the taxol biosynthetic pathway. A fungal isolate TPF-06 was screened to be a taxol-producing strain based on the PCR amplification results. It was characterized and identified as *Aspergillus fumigatus* by *18S rRNA* (Accession No. KU-837249). Multiple sequence alignment (MSA) of nuclear ribosomal internal transcribed spacer (ITS) region and phylogenetic analysis confirmed that strain belonged to *A. fumigatus* clade (Accession No. MF-374798) and is endophytic in nature. Presence of taxol was detected and quantified by High-Performance Liquid Chromatography (HPLC) and characterized by using Thin Layer Chromatography (TLC), Ultraviolet (UV) spectroscopy, Mass spectrometry (MS), Fourier-Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy. Microbial fermentation in the S7 medium yielded 1.60 g/L of taxol, which to the best of our knowledge is the highest taxol production from an endophytic fungus. Findings of the present study suggest that the *A. fumigatus* is an excellent alternate source for taxol supply, and it may become a highly potent strain on a commercial scale. The involvement of *dbat* gene in *A. fumigatus* KU-837249 strain further suggested a way of increasing taxol yield in fungi by medium engineering and recombinant DNA technology in the future.

1. Introduction

Taxol® (generic name Paclitaxel) is a poly-oxygenated cyclic diterpenoid with a characteristic taxane ring system [1]. It is the most effective and widely used chemotherapeutic drug for the treatment of cancers and virus-related sarcoma [2–4]. Each year, approximately 8.1 million new cancer cases are diagnosed worldwide, including India. Around five hundred patients are treated by 1 kg of taxol, which requires 10 tons of bark equivalent to 300 trees [5]. First time, taxol was isolated from the *Taxus brevifolia* (bark, roots, and branches) [1]. So far, the source of taxol is either semi-synthetic precursors like baccatin III and 10-deacetylbaccatin III or natural yew tree. Although the concentration of taxol is very low (0.01 %–0.05 %) from the natural sources, still, the bark of yew (*Taxus*) is the principal source. The high cost of the drug is attributed to the inadequate supply of natural taxol and increasing application in chemotherapy.

Recently, microbial fermentation technology emerged as an alternative approach for cheaper and higher yield of taxol. Particularly, isolation and identification of taxol-producing endophytic fungus is a very prospective and feasible approach for the

Abbreviations: bapt, baccatin III-aminophenylpropanoyl-13-*O*-transferase; *dbat*, 10-deacetylbaccatin III-10-*O*-acetyl transferase; *ITS*, Internal Transcribed Spacer; *ts*, taxadiene synthase; AIDS, Acquired Immuno-Deficiency Syndrome; BLAST, Basic Local Alignment Search Tool; DNA, Deoxyribose Nucleic Acid; HPLC, High Performance Liquid Chromatography; MMA, Modified Mycological Agar; MEGA, Molecular Evolutionary Genetics Analysis 7; PCR, Polymerase Chain Reaction; FTIR, Fourier Transform Infrared Spectroscopy; TLC, Thin Layer Chromatography; UV, Ultra-Violet; MS, Mass Spectroscopy; NMR, Nuclear Magnetic Resonance.

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production of a large amount of taxol [6,7]. Endophytes are designated as a promising source of novel natural metabolites exhibiting a variety of biological activities, including anti-cancer properties [8–11]. Recently, several endophytes from different genera such as *T. Anderanae*, *Alternaria alternate, Fusarium* sp. are reported to produce taxol [12]. Production of secondary metabolites is significantly affected by genetic, developmental, and environmental factors. Recombination DNA technologies like gene manipulation and metabolic pathway alterations may improve the endophytic strains and enhance taxol production [13].

In the past decades, numerous taxol-producing endophytic fungi have been isolated. However, none of them achieved an industrial production platform because of the low amount of taxol production. Therefore, researchers are looking for newer approaches using recombinant technology to improve the yield from isolated taxol-producing fungi, in addition to search novel high taxol-producing stable microbial isolates from nature. However, due to the rapidly growing market, low availability, and the fact that *Taxus* spp. are rare, endangered [14,15] and grow very slowly, an alternative source is needed to produce taxol at large commercial scale. Considering these factors, the current study was carried out on isolation, identification and extracellular production of taxol from *Aspergillus fumigatus* isolated from *Taxus* sp. collected from the Northern Himalayan region of India.

2. Materials and methods

2.1. Chemicals and molecular reagents

All the reagents and chemicals were of high purity and analytical grade. Standard paclitaxel was procured from MP Biomedicals (USA). Media components used for the growth and maintenance of taxol-producing endophytes were purchased from Hi-Media (Mumbai). The electrophoresis reagents, molecular grade chemicals used for DNA isolation were purchased from SDFCL (India) and Sigma-Aldrich (USA), respectively. Pre-coated Silica gel 60, F_{254} TLC plates, and HPLC solvents were of HPLC grade and procured from Merck (Germany). Universal primers for *ITS* and *dbat* genes were procured from Sigma (USA) and Bioservice, respectively.

2.2. Collection of plant samples and isolation of taxol-producing endophytes

Taxol-producing endophytes were isolated from different plant tissues (bark, stem, and needle) of *Taxus* sp. collected from Shimla, Himachal Pradesh (India). The location map of the plant tissue collection sites is shown in Fig. 1. The bark, stem, and needle samples were surface sterilized under a laminar airflow chamber with ethanol (70 %; v/v) for 30 s and sodium hypochlorite (3.5 %; v/v) for 2 min, followed by washing with sterilized water. Subsequently, the outer surface was peeled off using a sterilized surgical blade. The bark, stem, and needles were chopped into small pieces of $\sim 0.5 \times 0.5 \times 0.5$ cm and were aseptically placed on the surface of modified mycological agar (MMA) medium [16], composed of glucose 40 g/L, bacto-soytone 10 g/L, sodium acetate 1 g/L, sodium benzoate 50 mg/L, bacto-agar 15 g/L, pH 6.0–6.5 and were incubated at 25 ± 1 °C for 72 h. Morphologically different colonies were picked up depending upon shape, size, and color. Pure line cultures were established by repeatedly streaking single colonies on the MMA medium, and pure cultures were maintained on slants at 4 °C. It was decided to further restrict the studies to endophytic fungi in a quest for taxol-producing microorganisms.

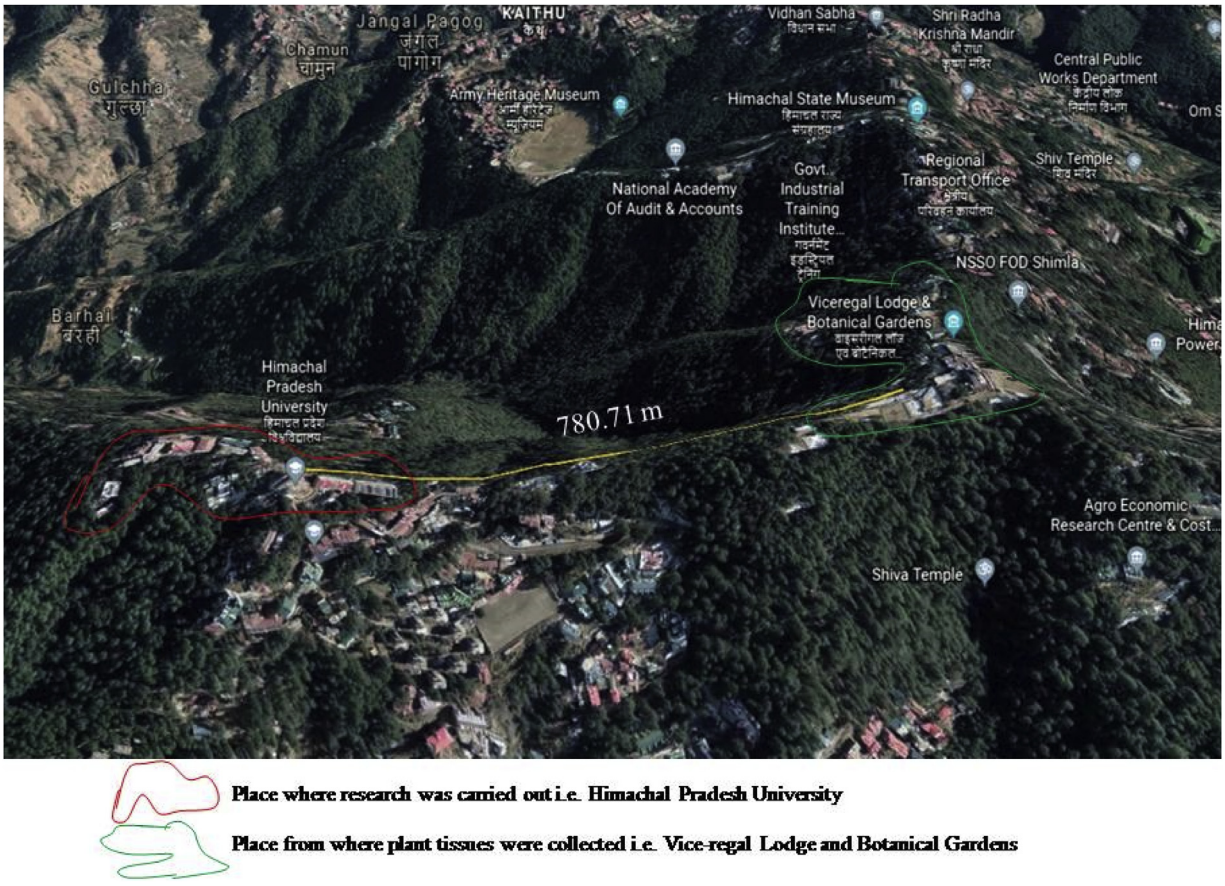

Fig. 1. Location map of the plant tissues collection sites.

2.3. Molecular screening of taxol-producing endophytic Fungi

All the isolated fungal cultures were grown individually in Erlenmeyer flasks containing 25 mL modified S7 liquid broth [16] consisted of glucose 3 g/L, sodium acetate 1 g/L, sucrose 18 g/L, beef-extract 5 g/L, fructose 9 g/L, soytone 1 g/L, thiamine 1 mg/L, biotin 1 mg/L, pyridoxal 1 mg/L, calcium pantothenate 1 mg/L, magnesium sulphate 3.6 mg/L, calcium nitrate 6.5 mg/L, copper nitrate 1 mg/L, zinc sulphate 2.5 mg/L, manganese chloride 5 mg/L, iron (III) chloride 2 mg/L, phenylalanine 5 mg/L, sodium benzoate 100 mg/L, pH 6.0–6.5 and 1 mL of 1 M sodium phosphate buffer, pH 6.8 and incubated at 25 ± 1 °C for 5–7 days at 150 rpm in an incubator shaker. Genomic DNA extraction was carried out from the harvested mycelia.

2.3.1. Fungal DNA extraction

Genomic DNA was extracted using the CTAB method [17] with necessary modifications. Briefly, the mycelia collected from the cultures were ground using mortar-pestle in liquid nitrogen into a fine powder. 200 mg of mycelium powder was suspended in 1000 μL of DNA extraction buffer. To the above suspension, 200 μL of 5 M NaCl and 100 μL of 10 % cetyltrimethylammonium bromide (CTAB) were added. The resulting mixture was incubated in a water bath for 3 min at 45 °C with occasional inversion. An equal volume of Chloroform: Isoamyl alcohol (24:1; v/v) was added to the lysed mixture and centrifuged at 12,000×*g* for 15 min. The upper aqueous layer was collected gently and incubated with an equal amount of isopropanol overnight at −20 °C for precipitation. Post incubation, tubes were centrifuged at 10,000×*g* for 10 min (4 °C), followed by washing of upper aqueous phase with 70 % ethanol. Finally, the pellet was dissolved in nuclease-free water (30 μL) and stored at −20 °C till further use.

2.3.2. PCR based molecular screening using dbat and ITS genes

The internal transcribed spacer (*ITS*) fragments and 10-deacetylbaccatin III-10-O-acetly transferase (*dbat*) gene were amplified by using universal primers [18] *ITS1* (5′-TCCGTAGGTGAACCTGCGG-3′); *ITS*4 (5′-TCCTCCGCTTATTGATATGC-3′**);** *dbat* F (5′-GGGAGGGTGCTCTGTTTG-3′) and *dbat* R (5′-GTTACCTGAACCACCAGAGG-3′) were purchased from Sigma Aldrich (USA) and Bioservice respectively. The standard PCR reaction of 25 μL consisted of 3 μL genomic DNA (∼100 ng), 1.5 μL forward and reverse primers each (10 μM), 0.4 μL DNA *Taq* polymerase (2 U), 2.5 μL 10X *Taq* buffer (Thermo), 2.5 μL $MgCl_2$ (25 mM), 2.5 μL dNTP mix (2 mM), and 11.1 μL nuclease-free water (Thermo). The PCR reaction was performed by initial denaturation at 94 °C (3 min), followed by 30 cycles at 94 °C (30 s), 55 °C (30 s), 72 °C (1 min) and final extension at 72 °C (5 min) using thermocycler (Eppendorf Mastercycler™, Germany). Besides this, taxadiene synthase (*ts*) and C-13 phenylpropanoid side chain-CoA acyltransferase (*bapt*) genes involved in the taxol synthesis pathway were also screened for PCR amplification using primer sets for *ts and bapt* [19]. The reaction and temperature profile for *ts* and *bapt* gene were similar for *ITS* gene, as mentioned above. Finally, the PCR products of *ITS* and *dbat* genes were analyzed in 2 % agarose gel and visualized using Gel Doc system (*CI50 Azure Biosystem,* USA).

2.3.3. Nucleotide sequencing and phylogenetic analysis

Specific bands of *ITS* and *dbat* genes were sliced from the agarose gel and purified by QIAquick Gel Extraction Kit *(Qiagen, Germany).* Sequencing PCR of the purified product was done by Big-Dye Terminator v 3.1 Cycle Sequencing Kit *(Applied Biosystems USA)* using appropriate sense and antisense primers for *ITS and dbat* gene with standard reaction and temperature profile. Specific amplified products were precipitated, and finally, the samples were loaded into AB 3500 XL Dx Genetic Analyzer (*Applied Biosystem, USA*). Sequence analysis and comparison using the Basic Local Alignment Search Tool (BLAST) was done in ABI 3500 automated DNA sequencer platform. Multiple sequence alignment and phylogenetic tree analysis were done using Clustal X 2.0.11 and Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 *(*www.megasoftware.net*)* software respectively, based on the internal transcribed spacer sequences of similar fungal species.

2.4. Identification and characterization of taxol-producing endophytic fungus

Fungal isolate TPF-06 found positive in molecular screening based on *dbat* gene expression, was further characterized using microscopic and molecular tools. The endophytic fungal strain TPF-06 was grown on 90 mm Petri plates containing MMA medium and characterized based on colony morphology, spores, reproductive structures, and *18S rRNA* sequences.

2.4.1. Morphological characterization using microscopy

Microscopic studies were carried out using fungal mycelia on a glass slide stained with Lactophenol Cotton Blue (LPCB) dye. Fungal mycelia were carefully teased using a needle, and a coverslip was placed onto the thin preparation. Morphology was observed under an upright biological microscope at 40X (Olympus, Japan).

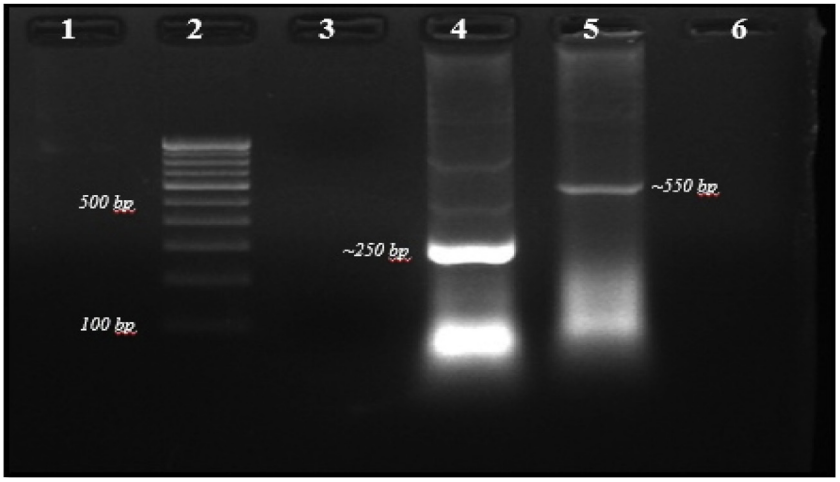

Fig. 2. PCR analysis for the presence of *dbat* gene in *A. fumigatus;* Lane 2: Molecular Marker (100 bp); Lane 4: *dbat* gene (∼250 bp); Lane 5: *ITS* gene (∼550 bp); Lane 1, 3, 6: Empty.

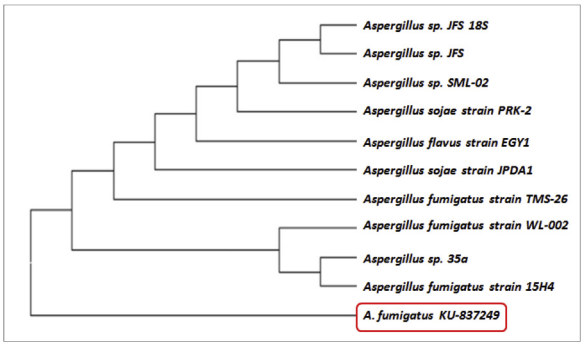

Fig. 3. Phylogenetic dendrogram of selected isolate 18S rRNA sequence based on neighbor joining method.

2.4.2. Molecular identification using 18S rRNA

A fresh plate of the fungal isolate TPF-06 was prepared on MMA medium and outsourced to the Xcelris Genomics, Ahmedabad, Gujarat (India) for *18S rRNA* sequencing. Sequencing data was analyzed and compared with similar sequences from NCBI (USA) using BLAST [20]. Clustal W version 2.0 and MEGA version 7.0 [21] were used to align the partial *18S rRNA* sequences and to build a phylogenetic tree for a selected fungal isolate.

2.5. Hyper-production and extraction of taxol from endophytic fungus

Erlenmeyer flask containing 100 mL modified S7 liquid medium [16] was seeded with 4.79×10^4 spores per mL of the fungal isolate TPF-06 and incubated at 25 ± 1 °C for 21 days with agitation speed at 150 rpm in an incubator shaker. Post 21 days of incubation, microbial biomass was removed from fungal isolates by passing the cultures through four layers of cheesecloth. The fatty acid concentration was minimized by the addition of 0.25 g sodium carbonate to culture filtrate and later extracted with two equal volumes of ethyl acetate. Under reduced pressure at 40 °C (vacuum evaporator), the solvent was removed, leaving behind dry solid residues which were re-dissolved in methanol. The crude extract containing taxol was subjected to TLC, HPLC, UV-spectroscopy, FTIR spectroscopy, MS, and NMR analysis for the presence, quantity, and purity of taxol by comparing with standard Paclitaxel procured from M.P Biomedicals (USA).

2.6. Characterization and analysis of extracted taxol

2.6.1. Thin Layer Chromatography (TLC)

For the detection of taxol, the crude sample was spotted on 0.25 mm (10 × 20 cm) aluminum pre-coated silica gel plates along with standard paclitaxel as internal standard, and the plate was developed in chloroform:methanol at 7:1 (v/v) successively. Taxol was detected by spraying 1 % vanillin (w/v) in sulfuric acid after gentle heating [22] or by using spray reagent consisting of 20 g of antimony trichloride in a mixture of 20 mL glacial acetic acid and 60 mL chloroform [23]. The Retention factor (*Rf*) value of sample was calculated according to the following equation from the chromatogram and compared with standard taxol.

$$\boldsymbol{Rf\,(value)} = \frac{\boldsymbol{Distance\ moved\ by\ the\ compound}}{\boldsymbol{Distance\ moved\ by\ the\ solvent}}$$

2.6.2. Ultra violet (UV) spectroscopic analysis

The UV spectroscopy analysis of the crude extracted sample was performed by scraping off the area of silica TLC plate containing putative taxol at the appropriate *Rf*. After dissolution in methanol, the spectrum of crude taxol samples was plotted in Beckman DU-40 spectrophotometer (USA) and quantified by comparing with that of the standard taxol.

2.6.3. Fourier-Transform Infrared (FTIR) spectroscopic analysis

The extracted sample (crude taxol) was mixed and grounded with potassium bromide (KBr, IR grade) in a 1:10 ratio and pressed under vacuum to form pellet disc using spectra pelletizer. FTIR of the crude taxol was recorded and compared to standard paclitaxel with Nicolet 5700 in transmittance mode with a higher solution ($1\ cm^{-1}$) and a wide scan range of $4000\ cm^{-1}$ to $500\ cm^{-1}$ at Department of Chemistry, HP University, Shimla (India).

2.6.4. Mass spectrometry (MS) analysis

The crude taxol was dissolved in methanol: water: acetic acid (50:50:1; v/v), and at 50 V, 2 μL sample was injected by the loop injection method [1]. Mass spectroscopy (MS) analysis of extracted taxol and standard taxol was performed using Waters Micromass Q-Tof Micro with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APcI) sources having mass range of 4000 amu in quadruple and 20,000 amu in ToF at SAIF/CIL, Punjab

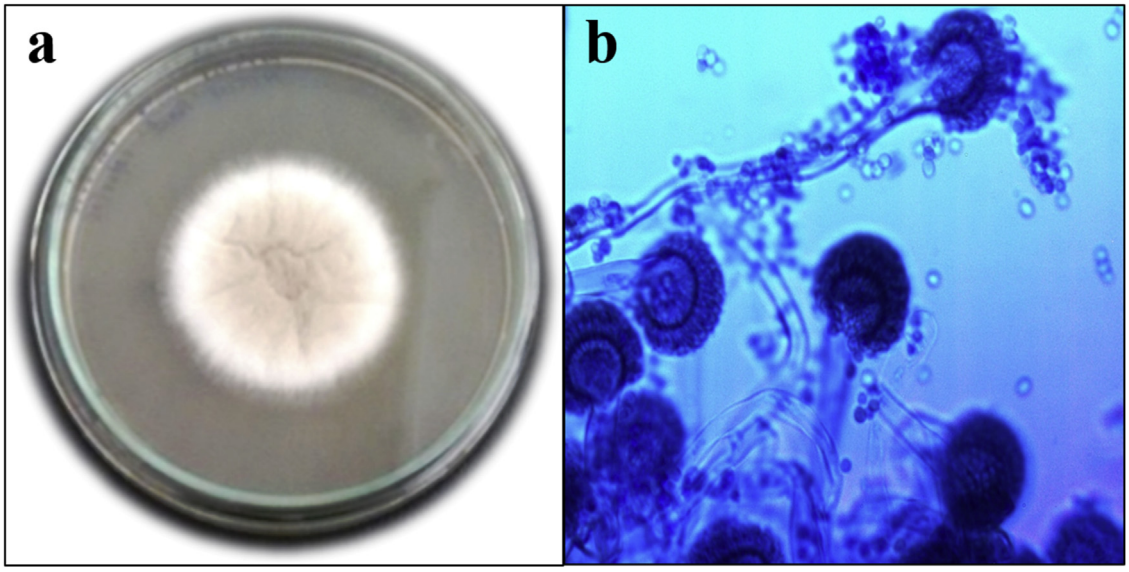

Fig. 4. Morphological characterization of *A. fumigatus* (a) on Modified Mycological Agar Medium (b) under Upright Biological Microscope at 40X.

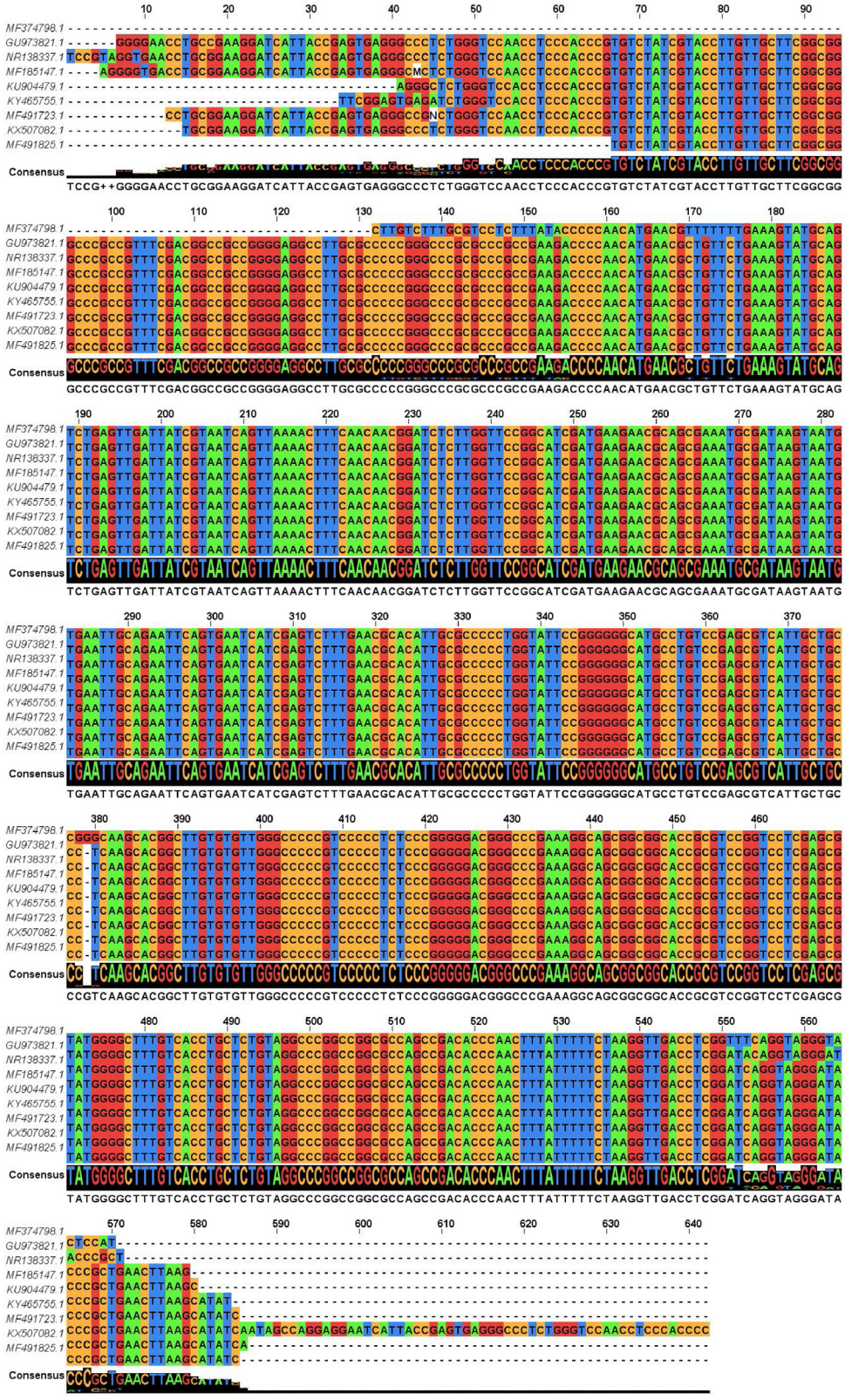

Fig. 5. Conservation of taxol producing *ITS* region in other selected *Aspergillus* spp.

Table 1
Plant tissue culture/cell culture based production of taxolfrom *Taxus* species.

Plant species	Culture type	Taxol yeild	References
Taxus cuspidata	Callus culture in Shake flask	0.020 % DW	[47]
Taxus cuspidata	Callus culture in Shake flask	431 mg/L	[48]
Taxus brevifolia	Callus culture	0.01 % of the dry weight of the bark	[49]
Taxus media	Cell suspension	115.2 mg/L	[50]
Taxus x media	Hairy root culture	221.8 μg/L	[51]
Taxus chinensis	Cell culture in a bioreactor	612 mg/L	[52]
Taxus chinensis	Cell suspension culture in Fed batch conditions	900 mg/L	[53]
Taxus baccata	Cell culture with methyl jasmonate induction	295 mg/L	[43]
Taxus baccata	Cell suspension cultures immobilized within Ca^{2+} alginate beads in Stirred bioreactor	43.43 mg/L	[54]

Table 2
Taxol producing endophytic fungi isolated from different plant hosts.

Endophytic fungi	Plant Host	Concentration (μg/L)	References
Taxomyces andeanae	*Taxus brevifolia*	0.024–0.05	[16]
Pestalotiopsis microspora	*Taxus walachiana*	60–70	[57]
Pestalotiopsis guepinii	*Wollemia nobilis*	0.49	[58]
Periconia sp.	*Torreyagra ndifolia*	0.03–0.83	[59]
Pestalotiopsis microspora	*Maguireothamnusspeciosus*	0.11	[60]
Tubercularia sp.	*Taxus mairei*	185.40	[6]
Trichothecium sp.	*Taxus wallichiana*	0.17	[61]
Phoma sp.	*Taxus yunnanensis*	32.93	[62]
Aspergillus niger	*Taxus yunnanensis*	1000.00	[63]
Nodulisporum sylviforme	*Taxus cuspidata*	392	[64]
Ectosroma sp.	*T. chinensis varmairei*	276.75	[65]
Bionectria sp.	*T. chinensis varmairei*	33.90–430.46	[66]
Aspergillus fumigatus	*Podocarpus* sp.	560.0	[7]
Metarhizlum anisopliae	*Taxus chinensis*	846.1	[67]
Nodulisporum sylviforme	*Taxus cuspidata*	468.6	[68]
Phomabetae	*Gingko biloba*	795.00	[69]
Fusariumredolens	*Taxus baccata* subsp. *wallichiana*	66	[30]
Penicillium aurantiogriseum	*Corylusa vellana*	70–350	[70]
Cladosporium oxysporum	*Moringa oleifera*	550	[71]
Phoma Medicaginis	*Taxus wallichianavar, mairei*	1215	[72]
Aspergillus aculeatinus	*Taxus chinensis var. mairei*	334.92–1137.56	[73]
Aspergillus fumigatus	*Taxus* sp.	1590.00	Present Study

University, Chandigarh (India) and NIPER, Mohali (India) to confirm the presence of taxol.

2.6.5. Nuclear Magnetic Resonance (NMR) analysis

^{1}H NMR of fungal taxol was recorded at 23 °C in $CDCl_3$ using Bruker Advance-II 400 NMR spectrometer (Germany) with a cryomagnet of field strength 9.4 T, to confirm the structure. ^{1}H NMR spectra were obtained at 400 Mhz following standard pulse sequences and phase programs supplied with NMR spectrometer [24].

2.6.6. High-Performance Liquid Chromatography (HPLC) analysis

HPLC was performed to estimate taxol production in the sample extracts. For HPLC analysis, the sample extracts were diluted in the mobile phase and subjected to HPLC (Perkin Elmer, USA), performed using 200 Ic pump (Perkin Elmer) equipped with reverse phase C18 5 μm column (Merck, LiChrosolv) and 785A Absorbance Detector (Applied Biosystem) [16]. Briefly, extracted test samples (crude taxol) were filtered through a 0.2 μm filter. The mobile phase consisted of methanol: water, 80:20 (v/v). 10 μL of the crude sample was injected each time with 1 mL per min flow rate and was detected at 227 nm [25]. NetWin Software (Netel Chromatographs, India) was used to monitor absorbance at 227 nm. Taxol presence was verified by comparing the retention time of the test samples with that of the standard taxol (Paclitaxel, M.P Biomedicals).

2.7. Quantification of fungal taxol

The calibration curve was constructed using HPLC by injecting the different known concentrations of standard taxol. The area under the peak of known concentrations was used for quantification. The average of four independent experiments was used to estimate the concentration of taxol production per liter from the extracted samples.

3. Results and discussion

3.1. Isolation of taxol-producing endophytes from Taxus sp

Endophytes are well recognized as a novel resource of bioactive compounds. Isolation of endophytic microorganisms producing paclitaxel is extensively studied worldwide, and more than 100 taxol-producing strains have been isolated till now [11,22]. However, yield in the reported isolates was extensively low to be explored further for the commercial applications [26,27].

In the present study, an attempt has been made to isolate a hyper taxol-producing endophyte having industrial applications. In total, 34 different endophytes were isolated from the bark, stem, and needle tissue samples of *Taxus* sp. collected from Shimla, Himachal Pradesh (India) (Supplementary information Table 1S). The isolated endophytes include bacterial, fungal, and actinomycetes, which were sub-cultured on MMA medium to eliminate taxol or other taxane traces carried over from the plant tissue. Only

the fungal cultures were further screened, identified, and characterized for taxol production.

3.2. Molecular screening of Taxol-producing Fungi based on dbat and ITS genes

Detection of taxol using biochemical and spectrometric techniques is a time-intensive procedure. A couple of studies have used *dbat*, *ts*, and *bapt* genes encoding for the taxol biosynthetic pathway as molecular markers for screening taxol-producing fungi [9,28–30]. *ITS* gene coding for the nuclear ribosomal internal transcribed spacer region is a universal DNA barcode marker for fungal identification.

Therefore, *dbat* and *ITS* genes were used as molecular markers for screening and identification of taxol-producing endophytic fungi in the present study. CTAB method used for genomic DNA isolation was found to be efficient. The concentration and purity of the DNA were confirmed by nanodrop. PCR amplification confirms that the fungal isolate, TPF-06, was found positive for *dbat* and *ITS* gene. Agarose gel electrophoresis showed a band at approximately ~250 bp and ~530 bp, respectively (Fig. 2). The sequencing of the amplicons was carried out (sequence file provided as Supplementary information in Table 2S). BLAST analysis of the ITS sequence of fungal isolate TPF-06 revealed 93 % similarity with Aspergillus fumigatus (query coverage 97 %). Results of phylogenetic analysis clustered TPF-06 with A. fumigatus species based on the evolutionary distance (Fig. 3). The *ITS* gene sequence is submitted to NCBI with accession number MF-374798.

Similar results were found during the primary screening of taxol-producing endophytic fungi by Zhou et al. [31] and Zhang et al. [32], reinforced the utility of *ITS, ts, dbat* and *bapt* genes as molecular markers [29]. Zhou et al. [33], used a gene coding for taxadiene synthase (*TS*), which is a rate-limiting enzyme in the taxol biosynthetic pathway as a molecular marker to screen for taxol-producing fungi. Jennewein et al. [34], on the contrary, suggested that *dbat* and *bapt* genes are more diagnostic than the *ts* gene because more than ten enzymatic steps after *TS*, are required to reach Baccatin III and taxol itself. Roopa et al. [29] showed the presence of *dbat* and *bapt* gene implicated in taxol biosynthesis and *ITS* gene (~540 bp) in *Alternaria*, *Fusarium* and *A. niger* isolated from *Salacia oblonga*. Xiong et al. [35] isolated and identified *Guignardia, Nigrospora, Phomopsis, and Phoma* from *T. media* based on *ITS* rDNA sequences.

3.3. Morphological Identification and Phylogenetic Analysis based on 18 s RNA

The pure culture of fungal isolate TPF-06, found positive based on *dbat* gene expression, was prepared and maintained at 4 °C. TPF-06 was aerobic, spore former with septate mycelium. The colonies were white-creamish in appearance from the front side and yellowish-white from reverse side (Fig. 4; a) on the MMA plate. Morphological observations of TPF-06 under microscope indicated stipe and conidial head consisted of single series of phialides, and rounded conidia were dispersed in long and parallel dry chains upon staining with LPCB dye at 40X magnification (Fig. 4; b). Based on the morphological features observed, the endophytic fungus was further confirmed to belong to the genus *Aspergillus.*

Sequence analysis of *18S rRNA* elucidated the taxonomic position of taxol-producing fungus. A strong relationship was revealed between selected isolate and members of genus *Aspergillus* using the BLAST comparison of 767 bp sequence with other similar sequences available in the GenBank database [20]. The maximum similarity resulted in a cluster that included many different species of *Aspergillus* was showing a close relationship with the fungus isolated in the present study. Homology comparison conferred 94 %

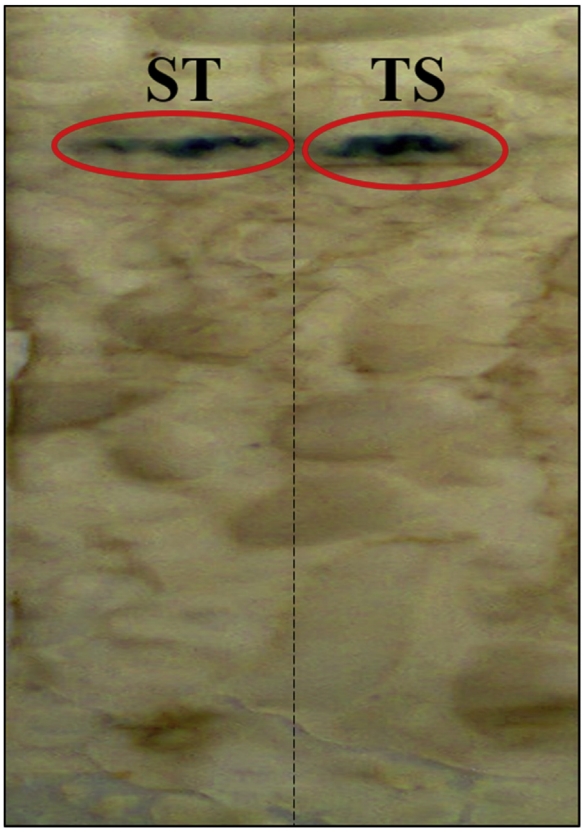

Fig. 6. Detection of taxol using TLC;ST: Standard Taxol; and TS: Test Samples.

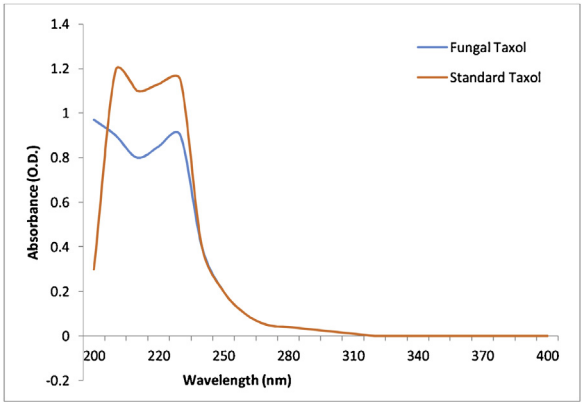

Fig. 7. UV spectrum of standard and fungal taxol produced from *A. fumigatus*.

homology with *A. fumigatus strain*-A (001) and *A. fumigatus* 15H4-PO-P1-1strain (Fig. 5). Based on the morphological features, *18S rRNA* gene sequence homology, and phylogenetic tree analysis, the isolate was identified and designated as *A. fumigatus KU-837249*. Also, the close association of fungal isolate TPF-06 with the endophytic *A. flavus* and *A. neoellipticus* proved the endophytic nature of *A. fumigatus* KU-837249. To the best of author's knowledge and as per the information available in the literature, it is revealed that *A. fumigatus* from *Taxus* sp. of the Northern Himalayan region was not reported for taxol production.

Zhou et al. [33] identified the taxol-producing endophytic fungus as *Mucor* sp. based on 18S rRNA sequence. Similarly, the HD86-9 strain revealed morphological and molecular similarity (98 %) to *A. niger* using *18S rRNA* and internal transcribed spacer (ITS) region analysis [33].

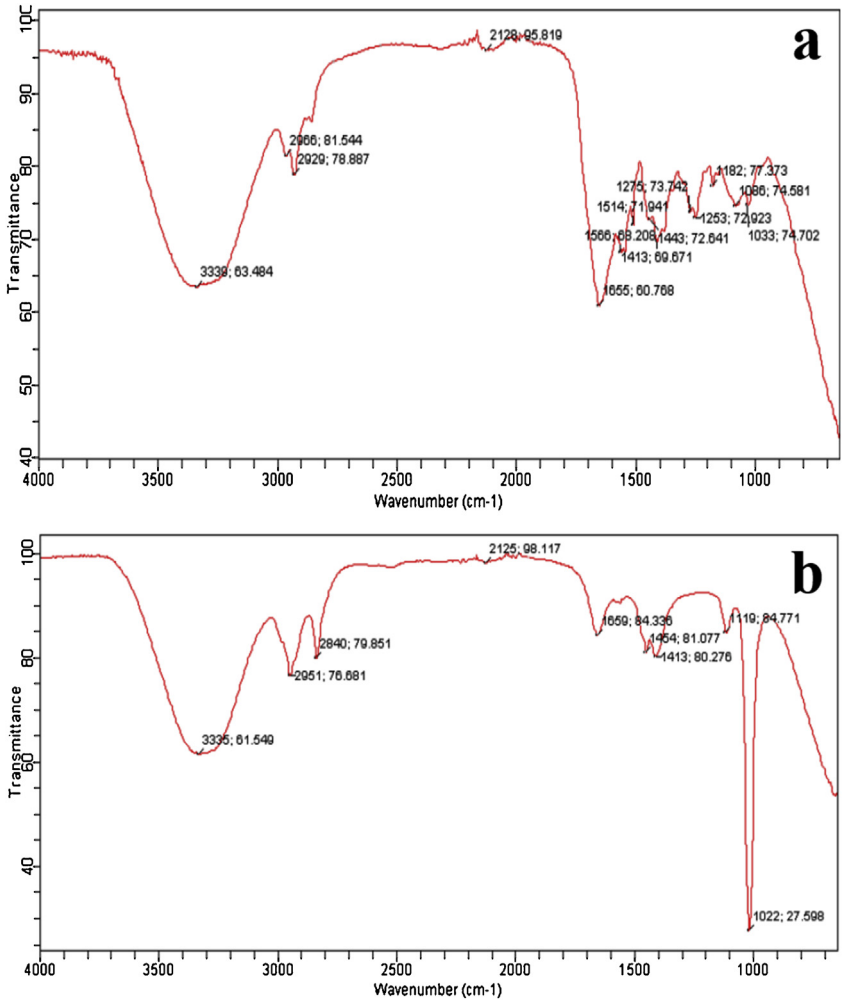

Fig. 8. IR spectrum of (a) standard taxol; and (b) sample taxol from *A. fumigatus*.

3.4. Hyper-production and extraction of taxol from endophytic fungus A. fumigatus

Current sources of Taxol production include extraction from cultivated *Taxus* spp., chemical semi-synthesis, *in vitro* plant tissue and cell culture, metabolic engineering in bacterial and fungal endophytes. The strategy to extract taxol from natural bark source is limited at the global scenario because of the slow growth of *Taxus* spp. and low-yield [36]. Thus, chemical semi-synthesis [37–41] and *in vitro* plant tissue culture [42–45] techniques prevail as the primary source for taxol supply at clinical level. Chemical semi-synthesis of paclitaxel from 10-deacetylbaccatin III (10-DAB) was reported for the first time in 1988 [46]. Plant tissue culture/cell culture-based strategies used for the production of taxol from *Taxus* spp. has been summarized in Table 1. However, low and unstable product yield, high production costs [55] and dependence on the yew tree material are burning problems in these widely used methods [56].

Since the discovery of first taxol-producing fungi in 1993 [16], continuous interest from researchers all over the world to explore a different approach to produce the drug from fungal endophytes. More than 50 taxol-producing fungal endophytes have been isolated over the past decades. A comprehensive list of fungal endophytes isolated from different plant species with the taxol yield has been highlighted in Table 2. Numerous issues had delayed the fungal production of the drug at the commercial scale. One of the most highlighted challenges is the low yield in fungal strains. Besides this, the stains will loose their taxol-producing capabilities after long-term culturing [74]. To overcome this issue, the heterologous expression of genes from taxol biosynthetic pathway has been attempted to produce the compound using genetic engineering techniques [13,75]. An *E. coli* and yeast strain were engineered to produce taxadiene in high titers of 1 g/L and 8.7 ± 0.85 mg/L respectively [76,77]. However, the lack of availability of a complete set of genes involved in paclitaxel biosynthesis is at present a limiting factor, especially in case of endophytic microorganisms [26].

In the present study, taxol from *A. fumigatus* (TPF-06) was produced by growing fungal isolate in modified S7 medium incubated at $25 \pm 1\,^{\circ}C$ for 21 days with sucrose as carbon and beef extract as a nitrogen source. S7 medium was chosen for fungal fermentation because the sugar ratio in the S7 medium was identical to the inner bark of *Taxus* sp. [16]. Since the benzoyl ring in plant-derived taxol is from phenylalanine [78,79], modified S7 medium was supplemented with phenylalanine, and sodium acetate to act as precursors during the metabolism of these endophytic fungi.

Similarly, in earlier investigations [80,81], S7 medium have been used for taxol production. Xu et al. [82], reported taxol yield of 20 μg/L with *Fusarium maire* in the basal medium consisted of glucose (80 g); NH_4NO_3 (5 g); $MgSO_4$ (0.5 g); KH_2PO_4 (0.5 g); $ZnSO_4$ (1 mg); $Cu(NO_3)_2$ (1 mg); $FeCl_3$ (2 mg); NaOAc (1 g), vitamin B1

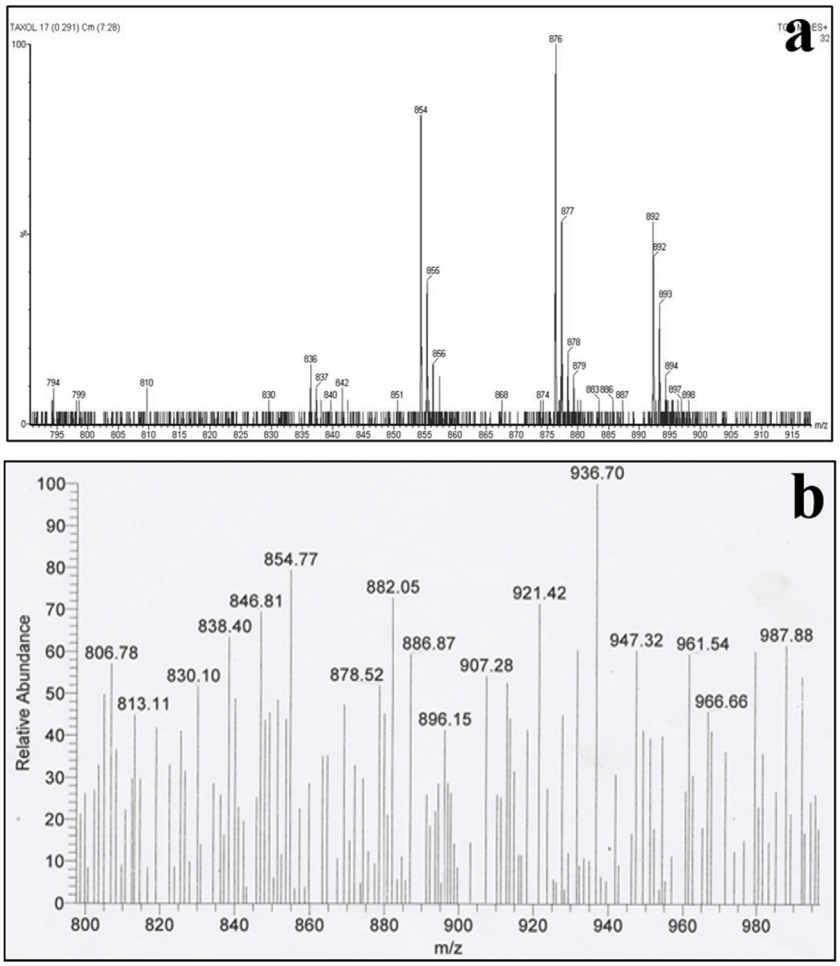

Fig. 9. MS chromatogram of (a) standard taxol; and (b) sample taxol from *A. fumigatus*.

(50 mg) and L-tyrosine (5 mg). Chakravarthi et al. [83] used potato dextrose liquid medium for *Cladosporium cladosporioides* MD2 (*T. media*) and *Fusarium solani* (*T. celebica*) for better taxol yield.

After 21 days of fermentation, culture filtrates were extracted with equal volumes of ethyl acetate, and the organic phase was collected. The solvent was evaporated under vacuum to get dry solid residues of fungal extracts. These extracts were dissolved in methanol and examined for taxol production by TLC, UV-Spectroscopy, FTIR, MS, and HPLC techniques. The results were compared with the standard taxol from MP Biomedical (USA) to confirm the presence of taxol.

3.5. Characterization and analysis of extracted taxol

3.5.1. Thin-layer Chromatography (TLC) analysis

TLC analysis detected crude taxol on 0.25 mm silica gel plates developed in chloroform:methanol (7:1, v/v) with 1 % (w/v) vanillin in sulphuric acid reagent after gentle heating. The spot appeared blue and faded to dark grey after 24 h (Fig. 6), indicated the presence of taxol in the sample mixture when compared with standard. *Rf* value was found to be 0.90, which was similar to standard taxol [84]. A similar chromatographic result was also reported for taxol production from *Pestalotiopsis malicola* and *P. pauciseta* VM1 [85,86]. Gangadevi and Muthumary [87], using TLC confirmed taxol production by *Colletotrichum gloeosporioides* JGC-9 isolated from medicinal plant *Justicia gendarussa*.

3.5.2. Ultra violet (UV) spectroscopic analysis

UV spectroscopic analysis showed λ_{max} for sample taxol at 227 nm ranges as comparable to λ_{max} for standard paclitaxel is observed at 227 nm range with minor variations in absorbance [88]. This confirmed the existence of taxol molecule in the sample mixture (Fig. 7). The λ_{max} corresponds to the existence of the benzoyl group. The fungal compound isolated from *Tubercularia* sp. TF5 showed a similar UV absorption spectrum to that of standard taxol at 228 nm [6,82]. The UV absorption spectrum of fungal taxol isolated from *Pestalotiopsis pauciseta* VM1 was similar to that of standard taxol with maximum absorption at 235 nm, and 232 nm [86] whereas Gangadevi et al. [89], reported similar UV spectrum with maximum absorption at 273 nm.

3.5.3. Fourier Transfer Infrared (FTIR) spectroscopic analysis

The IR spectra of taxol produced by *A. fumigatus* was almost superimposed on the spectrum of standard taxol with small variations in peaks (Fig. 8; a,b). A broad peak in the range of 3336 to 3436 cm^{-1} was observed due to hydroxyl (–OH) and amide (–NH) groups stretch in addition to aliphatic CH stretch in the range of 2920 to 2939 cm^{-1}. The registration peak was observed in the range of 2356–2364 cm^{-1} and 1045 to 1068 cm^{-1}, due to amine (NH) group and aromatic C and H bands stretching frequency. The aromatic ring (C=C) stretching frequency and the esters and ketone (C=O) groups stretching was observed in the range of 1590 to 1735 cm^{-1}. Based on the IR analysis, this fungus showed a

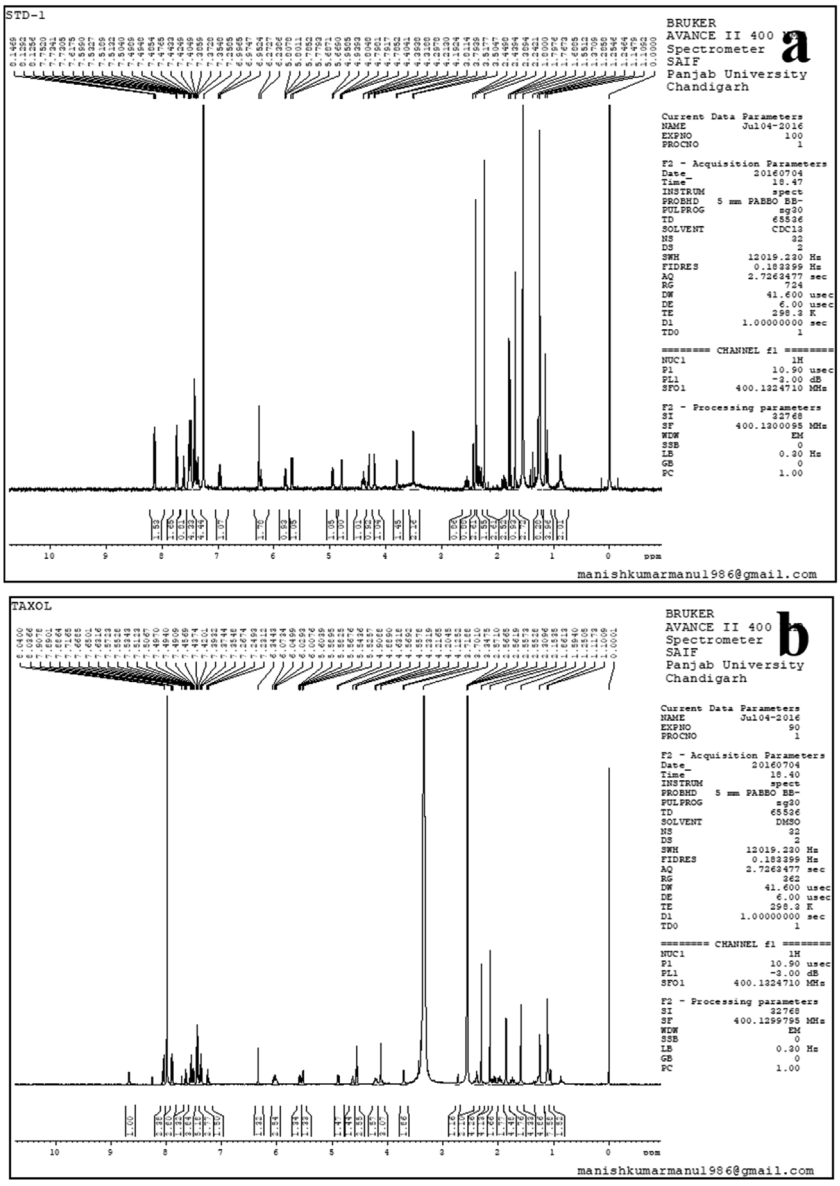

Fig. 10. ^{1}H NMR spectrum of (a) standard paclitaxel; and (b) crude paclitaxel from *A. fumigatus*.

positive sign for the production of taxol in the culture medium in comparison to the standard taxol, which was also reported in the earlier work of Kumaran et al. [90] and Gangadevi and Muthumary [91]. A similar IR spectrum with identical group stretching was obtained for taxol obtained from an endophytic fungus *P. pauciseta* VM1 [86].

3.5.4. Mass spectrometry (MS) analysis

High-resolution MS revealed the complex structure of crude taxol produced from *A. fumigatus* with empirical formula $C_{47}H_{51}NO_{14}$ and molecular weight of 853.9. Chromatogram analysis of standard taxol yields both $(M+H)^+$ and $(M+Na)^+$ peak at an 854 *m/z* and 876 *m/z*, respectively (Fig. 9; a). Crude taxol sample also produced both peaks $(M+H)^+$ and $(M+Na)^+$ at 854.77 *m/z* and 878.52 *m/z*, respectively (Fig. 9; b), with small variations and confirmed the presence of taxol in test samples. These results showed that *A. fumigatus* produced taxol in the appreciable amount [57]. Electrospray mass spectra of fungal taxol isolated from *A. niger* from *Taxus cuspidate* and *A. candidus* MD3 showed $(M+H)^+$ and $(M+Na)^+$ peak at 855 *m/z* and 876 *m/z* respectively and was similar to the standard taxol [33,92].

3.5.5. Nuclear magnetic resonance (NMR) analysis

^{1}H NMR spectra of taxol showed good distribution and resolution of all the signals in the 1.0 ppm–8.5 ppm range. The strong three-proton signals caused by the methyl and acetate groups contributed to the strong three-proton signals and lie in the range of 1.0 ppm–2.5 ppm along with multiplets caused by few methylene moieties. The taxane skeleton and the side chain are distributed by majority of the protons and observed in the region between 2.5 ppm and 7.0 ppm, whereas C-2 benzoate, C-30 phenyl, and C-30 benzamide groups contributed the aromatic proton signals between 7.0 ppm and 8.3 ppm. The characteristic chemical shifts of taxol are shown in Fig. 10 (a, b). Similar, ^{1}H NMR characteristic chemical shifts of taxol were obtained from the previous findings of Zhang et al. [92] and Gangadevi and Muthumary [91] from the *A. candidus* MD3 (*T. media)* and fungus *Bartaliniaro billardoides* (*Aegle marmelos)* respectively. Pandi et al.

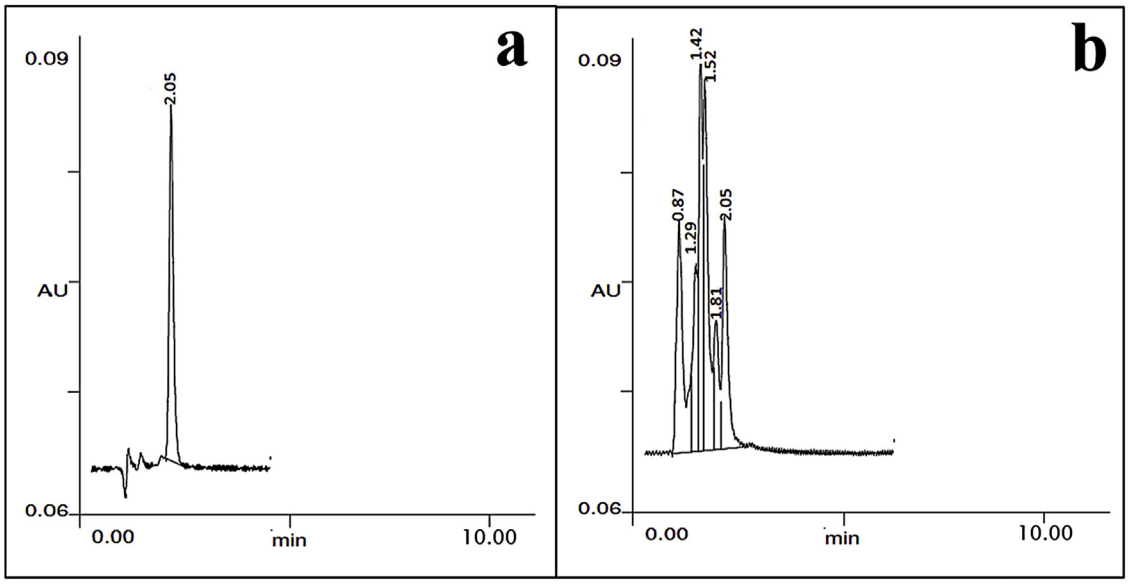

Fig. 11. HPLC Chromatogram of (a) standard paclitaxel (retention time = 2.05); and (b) sample taxol (retention time = 2.05).

[3] have also noticed an identical NMR spectrum of taxol produced from an endophytic fungus of *Lasiodiplodiatheo bromae* isolated from *Morinda citrifolia* medicinal plant.

3.5.6. High-Performance Liquid Chromatography (HPLC) analysis

First taxol-producing fungus *Taxomyces andreanae* with a very low yield of 24–70 ng/L was reported by Sterile et al. [16]. Since then, few reports on the isolation of taxol-producing endophytic fungi [57,81,82,85,93] have been documented. However, the unstable production and lower yield is the major problem for taxol production using fungal fermentation. HPLC analysis recorded a peak with a specific retention time of 2.05, which is identical to standard paclitaxel and confirmed the presence of taxol in test samples (Fig. 11; a, b).

Srinivasan and Kathiravan [84,94] also reported taxol yield of 92 μg/L and 0.064 mg/L from *P. funereal* and *P. breviseta* fungus and quantified with HPLC with a similar retention time of 2.822 and 2.210, respectively as standard taxol. Even *Metarhizium anisopliae* and *Cladosporium cladosporioides* MD2 fungal strains are very promising taxol producers with up to 800 mg/L yield quantified by HPLC [95].

3.6. Quantification of fungal taxol

The taxol content from the stain TPF-06 identified as *A. fumigatus* after fungal fermentation in S7 media for 21 days was quantified using HPLC. The area under the peak of different known concentrations of standard taxol served as the standard curve for taxol quantification (Supplementary information Fig. 1S). The total amount of taxol produced was recorded after an average of multiple measurements from samples of single cultivation and found to be 1.60 g/L, which proposed the utility of this fungus for the production of taxol in the culture medium. Although taxol production from *Aspergillus* sp. has been reported previously. However, this is the first report for the isolation, identification, and characterization of *Aspergillus fumigatus* from *Taxus* spp. from the Northern Himalayan region, India which has ability to produce taxol at a high amount of 1.6 g/L. To boost the supply and to bring down the price of cancer treatment, different research strategies including plant cell culture, strain improvement in endophytes using metabolic engineering should be employed in future to satisfy the demand.

4. Conclusion

Endophytes associated with the tissues of higher plants are emerging as a promising alternative and a novel source for microbial taxol production. However, botanical resources and chemical semi-synthesis are not able to satisfy the huge demand for the anti-cancerous drug taxol. In the present work, a new source for microbial taxol production has been explored from the Northern Himalayan region, India, based on the genes involved in taxol biosynthesis and nuclear ribosomal internal transcribed spacer (ITS) region. From the study, it was revealed that identical results exist between the fungal taxol and standard taxol in all the respective spectroscopic and chromatographic techniques. Taxol produced by fungal endophyte *Aspergillus fumigatus* was found 1.60 g/L, which is so far the highest yield of microbial taxol production recorded till date according to the literature cited. Thus, fermentation processes using taxol-producing endophytes may be an alternative yet promising way to boost the supply of this multibillion-dollar drug (taxol), thereby reduce the increasing cost of drug therapy.

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Declaration of Competing Interest

There is no conflict of interest among the authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00395.

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