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REVIEW

Taxol producing fungi: a critical review in experimental aspects

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Taxol (generic name paclitaxel), a potent anticancer drug isolated from specific Yew tree (*Taxus brevifolia*) for the first time is the most profitable chemotherapeutic drug in history. Taxol is widely used to treat various cancers including breast cancer, ovarian carcinoma, prostate cancer, melanoma and lung cancer. A large number of endophytic fungi that reside within healthy plants and many epiphytic, saprophytic and pathogenic fungi have been reported to produce taxol. The different endophytic fungi were isolated and characterized for its potential in taxol production in past two decades. Taxol production was confirmed by various chromatographic and spectroscopic analyses and the fungal derived taxol was compared with authentic taxol (paclitaxel – Sigma grade). The quantitative and qualitative analysis confirmed the presence of Taxol which suggests that the fungus can serve a potential alternative source for the production of the most effective antitumor agent. In this review, we mainly focus on different types of fungal source as a taxol producers, methods used in the extraction of taxol, yield and effectiveness on cancer cells. The review throws light on fungi as the valuable source for commercial production of taxol and can serve as a potential species for genetic engineering to enhance the production of taxol.

Key Words: Taxol, cancer, endophytic fungi, *Taxus*, paclitaxel.

INTRODUCTION

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Cancer is fundamentally a genetic disease: it arises as a consequence of mutation in the information carried by DNA. Cancer causing mutations occur mostly in somatic cells, not in germ line cells. According to World Health Organization (WHO) cancer is leading cause of death worldwide, accounting for 8.8 million deaths in 2015, nearly 1 in 6 deaths is due to cancer (Ferlay *et al*., 2013). Cancers can be grouped according to the type of cell they live in such as carcinoma, sarcoma, leukaemia, lymphoma and brain – spinal cord cancers.

Taxol (pacitaxel) is one of the natural diterpenoid alkaloids firstly isolated from the bark of the pacific Yew tree (*Taxus brevifolia*) (Fig.1).

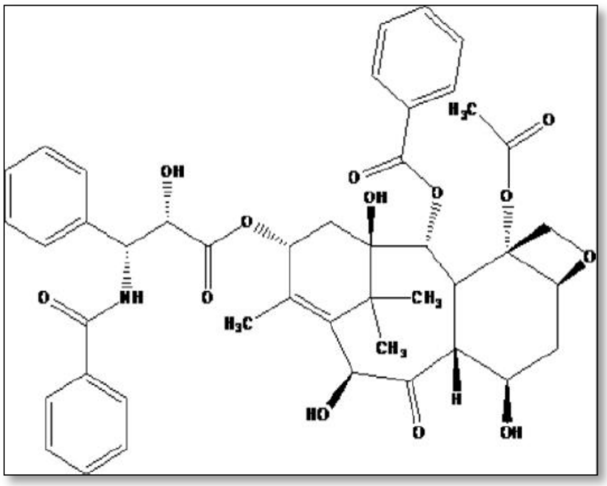

Fig.1 : Chemical structure of Taxol

Taxol interferes with the spindle microtubule dynamics within the cell that ensures the correct alignment of chromosome segregation and cell division leads to mitotic block. It was first commercially developed by Bristol – Myers Squibb Company with the generic name Paclitaxel and

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sold under the trademark Taxol (Weaver *et al*., 2014). However, the supply of taxol has been limited since the discovery of this natural product and with increasing applications in chemotherapy, the availability and cost of the drug will remain an important issue. Taxol is a chemotherapeutic medication used to treat different types of cancers. These include ovarian cancer, breast cancer, lung cancer, Kaposi sarcoma, cervical cancer and pancreatic cancer.

A large number of endophytic fungi from *Taxus* and other plants have been reported to be taxol producers. However, fungal epiphytes, pathogens and saprophytes have also been found to produce taxol. Taxol has been detected in more than 30 genera of unrelated fungal endophytes including *Pestalotia* sp., *Pestalotiopsis* sp., *Fusarium* sp., *Alternaria* sp., *Pithomyces* sp., *Penicillium* sp., *Monochaetia* sp., *Trichothicium* sp. etc (Shrestha *et al*., 2001). Fungi have a considerable potential to be used in economical and environmentally friendly fermentation processes for taxol production. They grow fast in culture media and can be manipulated easily and cultured on large scale. Consequently, fungi could provide a reliable source of taxol through fermentation. Fungal taxol have been detected by various methods like Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Liquid Chromatography – MassSpectroscopy (LC – MS), Nuclear Magnetic Resonance (NMR), UV – Spectroscopic analysis, IR – Spectroscopic analysis, antibodies reactive to taxanes, baccatin III 13 – 0 (3 – amino – 3- phenypropyl) transferase (BAPT), antiproliferation in cancer cell lines (cytotoxicity) *in vitro* apoptotic assay (Flores – Bustamate *et al*. 2010).

In this review, we discuss the potential for production of taxol from fungi, the biology of taxol synthesis in fungi and measures which may improve taxol yields are also discussed.

TAXOL – AN OVERVIEW

Taxol (generic name for paclitaxel) is a oxygenated diterpenoid compound, can kill tumor cells by enhancing the assembly of microtubule and inhibiting their depolymerisation by binding with â – tubulin of the spindle microtubule. It was first extracted from bark of pacific Yew (*Taxus brevifolia*). This compound is the world's first billion-dollar anticancer drug and it is used to treat breast, lung, ovarian cancer and other human tissue proliferating disease. Taxol is the first block buster drug discovered in plant screening programme by National Cancer Institute of United States Department of Agriculture (USDA) in 1962. Taxol had been isolated and identified by Mansukh Wani and Monroe Wall from the bark of pacific Yew tree (*Taxus brevifolia*) in 1967 and named it (Weaver *et al*. 2014). The name taxol is based on its species of origin and the presence of hydroxyl groups.

CHEMICAL STRUCTURE

Paclitaxel is a crystalline white or off-white powder with empirical formula C_{47} $H_{51}NO_{14}$ and known to have a molecular weight of 853.9 units. It is highly lipophilic thus highly insoluble in water. Its melting point is around 216 – 217 R"C. The chemical structure of taxol is classified as taxane diterpenoids or taxoid. Structurally, it can be viewed as the N – Benzoyl – â Phenylisoserine ester of diterpenoid bacentia III with a very characteristic oxetane ring.

BIOSYNTHESIS

The taxol biosynthetic pathway is considered to require 19 enzymatic steps from the universal diterpenoid precursor Geranyl geranyl diphosphate (GGPP), derived from Isopentyl – pyrophosphate (IPP). The pathway committing step is catalysed by taxadiene synthase, yielding the tricyclic diterpenoid pentamethyl 3, 8 tri-cyclo pentadecane, commonly referred to as taxadiene. This parental alefin is then functionalized by a series of eight cytochrome P 450 – mediated oxygenations, three CoA dependent acylations, and several other transformations en route to baccatin III, to which the side chain at C13 is appended to afford final product taxol (Fig.2) (Guo *et al*. 2006; Engles *et al*. 2008).

MODE OF ACTION

The mechanism of action of taxol has been considered to be unique. Taxol inhibits cell proliferation in G2 – M phase of the cell cycle, effectively blocking mitosis. Paclitaxel enhances the polymerization of tubulin to stable microtubules but inhibits depolymerisation of the microtubule, protect it from disassembly . The beta tubulin is known to have the binding site for paclitaxel. The dynamic

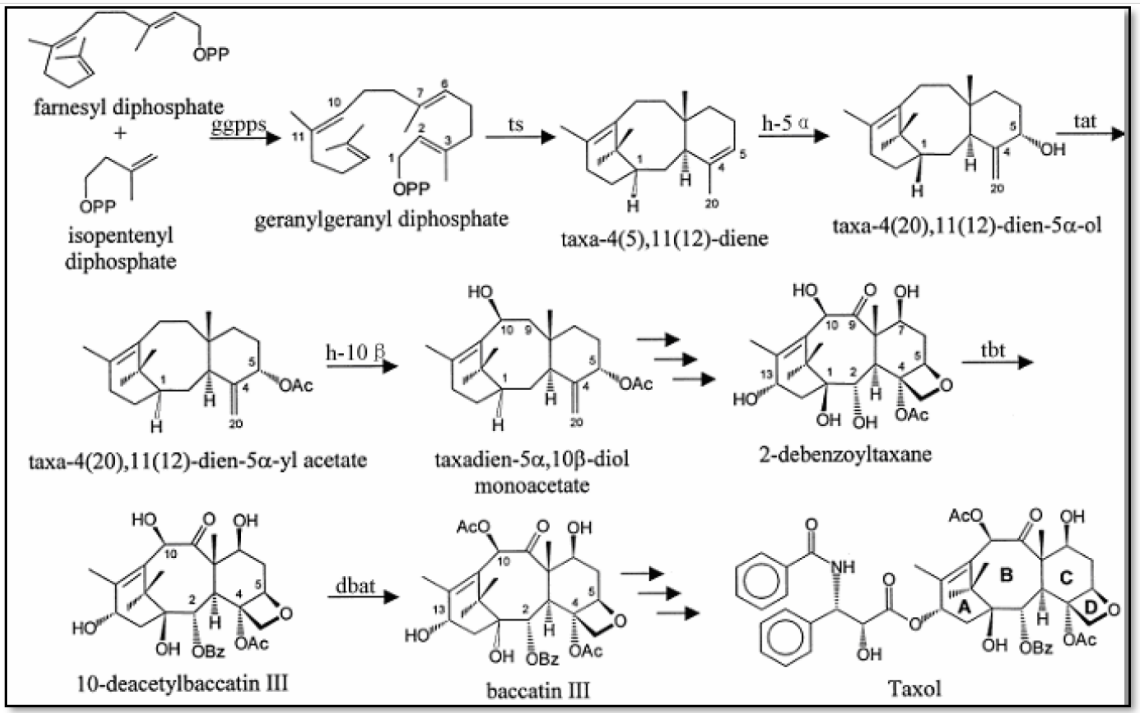

Fig. 2: Taxol biosynthetic pathway. ggpps: geranylgeranyl diphosphate synthase; ts: taxadiene synthase; h-5-: cytochrome P450 taxadiene 5- hydroxylase; tat: taxa-4(20), 11(12)-dien-5a-ol-O-acetyltransferase; cytochrome P450 taxane 10-hydroxylase; tbt: taxane 2a-Obenzoyltransferase; dbat: 10-deacetyl baccatin III-10-O-acetyltransferase. Multiple arrows indicate several as yet undefined steps.

instability of the spindle microtubule facilitates the sister chromatid to move apart in the opposite pole. As taxol inhibits the depolymerisation of the tubulin subunits from the microtubule it impairs the metaphase spindle formation leads to mitotic block. taxol inhibits detachment of the microtubules from centromere of the chromosome (Ganguly *et al*. 2010).

FUNGI AS THE POTENTIAL SOURCE OF TAXOL

Endophytic fungi

In general, endophytes are bacteria and fungi that live within plants. They are defined as “microbes that colonize living internal tissue of plants without causing any immediate, overt negative effects” . Endophytic fungi have been shown to produce compounds with a range of properties, including antibiotics, antivirals, antioxidants, antibiotic agents, immunosuppressive compounds, insecticidal products and anticancer agents (Strobel and Daisy, 2003; Strobel *et al*., 2004; Aly *et al*., 2011). In 1993, the first taxol – producing fungus *Taxomyces andreanae*, was isolated from a taxol producing plant, *Taxus brevifolia*. Since then, many reports have been made of the production of taxol and other anti-cancerous drugs from fungi (Kharwar *et al*. 2011; Suryanarayanan *et al*. 2009; Strobel *et al*. 2004; Yin and Sun, 2011). Since 1993, samples from the inner bark (phloem cambium) and xylem of *Taxus cuspidata* were screened and five new endophytic species were isolated that can produce taxol. These fungi are *Nodulispora sylviforme* (a new genus and new species in China) (Zhou *et al*. 2001). *Pleurocytospora taxi* (Sun *et al*. 2003), *Aternaria taxi* (Ge *et al*. 2004), *Botrytis sp*. (Zhao *et al*. 2008) and *Aspergillus niger* subsp. *taxi* (Zhao *et al*. 2009). Several endophytic fungi have been isolated from *Taxus* species in Asia.

The Taxol yield of fungi varies from 24 ng to 70 µg/ L of culture, i.e. very low and unstable. To solve such problems, current studies mainly focuses on the tedious work of finding and isolating fungi with high, stable yield of taxol, as well as optimization of fermentation conditions. The higher taxol producing endophytic fungal strain such as *Botryodiplodia theobromae* from *Taxus baccata* and *Phyllosticta citricarpa* from *Citrus medica* were reported. (Kumaran *et al*. 2008; Raja *et al*. 2005). An endophytic fungus *Pestalotiopsis terminaliae* was isolated from the *Terminalia arjuna* was

produced the highest amount of taxol (Gangadevi and Muthumary, 2009).

A screening for paclitaxel production in endophytic fungi of *Taxus globosa* using a competitive inhibition enzyme immunoassay showed 16 out of 107 isolates producing taxol between 65 – 250 µg/L. The major isolates were species of *Acremonium* sp., *Botryosphaeria* sp., *Fusarium* sp., *Gyromitra* sp., *Nigrospora* sp., *Penicillium* sp. and three novel Pleosporales and Xylariales (Soca – chafre *et al*. 2011).

Epiphytic and pathogenic fungi

Besides endophytic fungi from *Taxus* and non-*Taxus* plants, fungi isolated as epiphytes, pathogenic to plants and even saprophytic fungi have also been reported to taxol producers. A new taxol producing fungus *Pestalotiopsis malicola*, isolated from plant debris in the soil showed high level of taxol yield (186 µg/L) in both the culture and mycelium (Bi *et al*.2011). A leaf spot fungus *Phyllosticta tabernaemontanae* isolated from *Wrightia tinctoria* produced significant amount of taxol on M1D medium (461µg/L) and PDB medium (150µg/ml). The production rate was increased to 9.2× 103 fold than earlier reported fungus, *Taxomyces andreanae* (Kumaran *et al*., 2009). From the diseased leaves of *Citrus medica* leaf spot fungus *Phyllosticta citricarpa* was reported to produce notable amount (265 µg/L) of taxol in M1D medium (Kumaran *et al*., 2008). Another fungus *Pestalotiopsis breviseta* CRO1 also produces taxol in vitro which was isolated from the plant *Catharanthus roseus* (Karthik *et al*., 2012). Not only fungi residing on plant are the taxol producers, evidences from disease related fungi, epiphytic fungi are also important source.

ISOLATION AND EXTRACTION OF TAXOL FROM FUNGAL SOURCE

Isolation of fungi from host plant

For isolation, the healthy plant leaves are washed in running tap water and processed as follows: samples cut into 2mm² segments and surface sterilized by sequentially dipping into 0.5% Sodium Hypochlorite (2 min) and 70% ethanol (2 min), and rinsing with sterile water, then allowed to surface-dry under sterile conditions. The material is then inoculated on to a Petridish containing Potato Dextrose Agar (PDA) amended with Streptomycin. The Petridishes are then sealed with Parafilm and incubated at 25±1°C in a light chamber with 12h light followed by 12h of dark cycles and checked from the second day for fungal growth. Individual fungal colonies were transferred onto other plates containing PDA and continuously monitored for spore formation.

Cultivation and metabolic extraction

The fungus is cultivated on potato dextrose broth by placing agar blocks of pure culture (3mm in diameter) of actively growing culture in 2000 ml Erlenmeyer flask containing 1500ml media. The flask is incubated in BOD shaking incubator for 30 days at 25ºC with periodic shaking at 150 rpm. The fermented broth of the entophyte is then filtered through cheesecloth to remove the mycelia mats. The filtrate is extracted thrice with ethyl acetate at room temperature. The pooled extract after drying over anhyrdrous $MgSO_4$, is to be evaporated in a rotary vacuum evaporator and the dry residue so obtained to be redissolved in methanol for further analysis.

CHROMATOGRAPHIC SEPARATION AND SPECTROSCOPIC ANALYSIS

Thin layer chromatography (TLC) analysis

The thin layer chromatography for the fungal sample containing taxol has been carried out on 0.25 mm (10cm x 20 cm) aluminum precoated silica gel plates (Merk) (Cardellina, 1991). As per report, samples were spotted along authentic Taxol (Paclitaxel, SIGMA grade) as internal standard and the plates are developed in solvent (A), chloroform: methanol (7:1, v/v) followed by solvent (B), Chloroform: acetonitrile (7:3. v/v); solvent (C), ethyl acetate: 2- propanol (95:5, v/v) .The presence of taxol can be detected with 1% w/v vanillin/sulphuric acid reagent after gentle heating, following which, the RF values of the samples are to be calculated and compared with authentic taxol.

High performance thin layer chromatography (HPTLC) analysis

The prepurified fungal taxol samples obtained in TLC are further subjected to HPTLC (CAMAG Planar HPTLC, Anchrom). With the automatic TLC Sampler, all the extracted taxol samples and

authentic taxol (5 µL for each samples) are injected into their respective tracks separately, with the data pair technique, with the band length being 5 mm, the distance from lower edge 10 mm, the distance from the side 20 mm and the track distance 6.4 mm, on a pre-coated silica gel 60 F254 (20 cm × 10 cm) plate (Merck, Germany). After loading, the plate is to be dried for 30 s using a plate heater and developed in a CAMAG pre-saturated flat bottom developing chamber with chloroform: methanol (9:1, v/v) for 20 min. Documentation of the TLC plate is performed under a shorter wavelength (254 nm) UV lamp and a longer wavelength (366 nm) UV lamp, prior to derivatization. Presence of the taxol can be visualized after it is sprayed with 1% (w/v) vanillin sulfuric acid and heated gently for 2 min. In HPTLC, the amount of taxol produced by the fungi can be calculated through comparing the area and the height of peaks observed in fungal taxol with that of authentic taxol. The results obtained in HPTLC technique can be used for rapid separation and high throughput screening for taxol producing endophytic fungi and used for quantification of the amount of taxol. The best results have been achieved using visualization after derivatization by spraying a reagent and by identifying with the help of the fingerprint technique. This validated method is found to be simple, reliable and convenient for routine analysis. In HPTLC, the principal requirement for the documentation is the visibility of the chromatogram with or without derivatization. The substances with the absorbance of UV light at 254 nm are visualized as dark zones on the plates, with a fluorescence indicator, which is excited to emit green light under a shorter wavelength (254 nm) UV lamp. A longer wavelength (366 nm) UV lamp is used to excite substances that are able to fluoresce. White light is used to visualize coloured substances. The documentation of the TLC plate isperformed under a shorter wavelength (254 nm) UV lamp.

High performance liquid chromatography (HPLC) analysis

Taxol has been further analyzed by HPLC (Shimadzu 9A model) using a reverse phase C18 column with UV detector. Twenty microliters of the sample is injected each time and detected at 232 - 275 nm. The mobile phase is methanol/acetonitrile /water (25:35:40, by v/v/v) at 1.0 ml/min. The sample and the mobile phase are to be filtered through 0.2 ìm PVDF filter before entering the column. Taxol is quantified by comparing the peak area of the samples with that of the standard taxol. Results of HPLC analysis shows the presence of the compound by recording a peak with a specific retention time. HPLC analysis of the fungal extract gave a peak when eluting from a reverse phase C18 column, with about the similar retention time as authentic taxol and the fungus produced taxol in liquid culture. Taxol detection will not be observed in the blank culture samples, where they show negative results in all the analyses. The fungal taxol yield can easily be quantified accurately with HPLC analysis as evidence by detection of higher quantity in comparison to earlier reports where quantification was carried out with the aid of immunoassay. The biggest problem of using fungi in fermentation is the low level of yield with unstable taxol production. Although the amount of taxol produced by the endophytic fungi associated with yew trees is relatively small, when compared with the host trees, the short generation time and high growth rate of fungi will make it worthwhile to continue investigation on endophytic fungi isolated from the medicinal plants

LC-MS analysis

Further evidence confirming the identity of taxol can be obtained by LC-MS spectroscopic analysis. LC-MS analysis is carried out on the samples dissolved in methanol: water (9:1 v/v). Each sample is injected in Varian LC/MS 1200L Single Quadrupole MS with a spray flow of 2 ìL/min and a spray voltage of 2.2 kV via the loop injection method. Characteristically, standard taxol yielded both a (M +H) + peak at a molecular weight of 854 m/z and a (M +Na) + peak at a molecular weight of 876 m/z. On comparison, fungal taxol also produced peaks (M +H)+ at m/z 854 and (M +Na)+ at m/z 876 with characteristic fragment peaks at 344, 367 and 395 m/z. Major fragment ions observed in the mass spectrum of taxol are divided into three categories, which represent the major portion of the respective taxol molecule. The peaks analogous to taxol exhibit mass-to-charge (m/z) ratios corresponding to the molecular ions (M+H) + of the standard taxol (854 m/z), confirming the presence of endophytic taxol. It is evident that the diterpene taxol is much more complex since its molecular weight from high resolution MS is 854, corresponding to the molecular formula of $C_{47}H_{51}NO_{14}$ as reported earlier. By comparison,

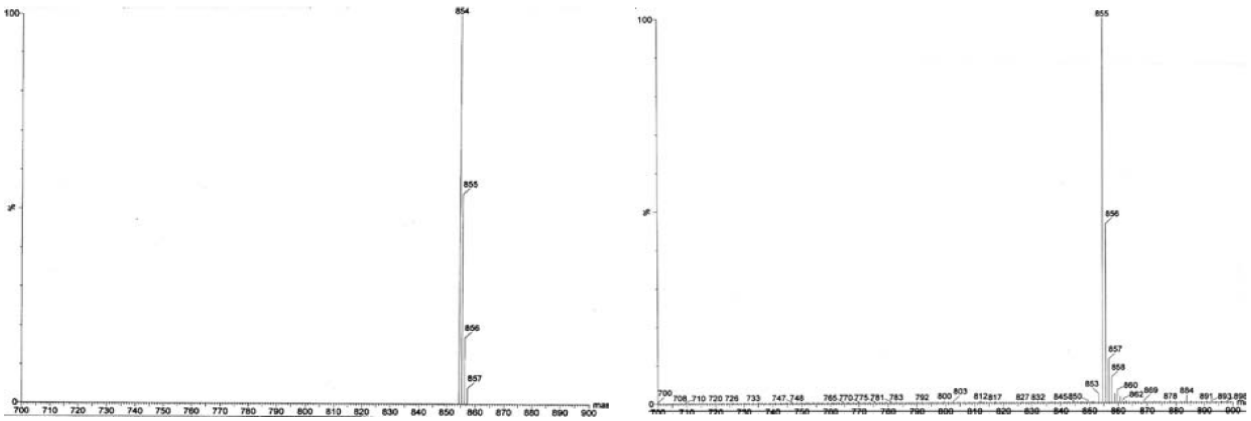

Fig. 3 : (a) Liquid chromatography mass spectrum of authentic Taxol; (b) Liquid chromatography mass spectrum of fungal extracted taxol

fungal taxol also yielded a peak MH+ at m/z 854 with characteristic fragment peaks at 569, 551, 509, 464, 286 and 268. Major fragment ions observed in the mass spectrum of taxol can be placed into three categories which represents major portions of the molecule 15. The peaks corresponding to taxol, exhibit mass-tocharge (m/z) ratios corresponding to the molecular ions (M+H)+ of standard taxol (854) confirming the presence of taxol in the fungal extracts. As detailed report33, the esterified position was found to be the allylic C13 hydroxyl moiety.

Ultraviolet (UV) spectroscopic analysis

The presence of taxol in the fungal extract is further confirmed by UV spectroscopy. After the TLC method, the area of plate containing putative taxol is carefully removed by scrapping off the silica at the appropriate Rf and exhaustively eluting it with methanol. After the elution, the crude taxol is used for the qualitative and quantitative analyses. The taxol samples dissolved in 100% methanol when analyzed by UV absorption (Beckman DU-40 Spectrophotometer), exhibit a characteristic absorption peak at 235–273 nm, similar to the previous reports.

Infrared (IR) spectroscopic analysis

For determination of IR spectrum of the fungal taxol (on Shimadzu FT IR 8000 series instrument) the partially purified fungal taxol is ground with IR grade potassium bromide (KBr) (1:10) pressed into discs under vacuum using spectra lab Pelletizer and compared with authentic Taxol. The IR spectra are recorded in the region 4000– 500/cm. The IR spectrum shows a broad peak at 3434.99/cm which is assigned for the presence of the O group in the parent compound, as evidenced by its OH stretch. The aliphatic CH stretch is observed at 2927.74/cm. The C=O (keto group) stretch is positioned at 1724.24 and 1656.74/cm. The registration peak observed at 1485.08 and 1450.37/cm are due to the NH stretching frequency. The COO stretching frequency is observed at 1371.29 and 1242.07/cm. The peaks in the range between 1070.42 and 979.77/cm are due to the presence of aromatic C and H bends. Fungal taxol can be further confirmed by IR fingerprints recorded between 1000 and 3500/cm, which are also identical in comparison to the standard taxol. Therefore, it is evident that the endophytic fungus showed positive results for taxol production in M1D medium. For example, the IR spectral data of fungal taxol from *C. raphigera* showed a broad peak in the region 3417.6/cm was described to hydroxyl (–OH) and amide (– NH) groups stretch. The esters and ketone (C=O) groups stretch was observed in the region of1,724.2/cm. The aromatic ring (C=C) stretching frequency was observed in the region 1,658.7/cm. A peak observed in the region 1026.1/cm is due to the presence of aromatic C, H bends.

Nuclear magnetic resonance (NMR) spectroscopic analysis

1H NMR spectra were recorded to confirm the structure of fungal taxol at 23°C in CDCl 3 using a JEOL GSX 500 spectrometer (operating at 499.65 MHz) and were assigned by comparison of chemical shifts and coupling constants with those of related

compounds. Chemical shifts were reported as ä values relative to tetramethyl silane (TMS) as internal reference and coupling constants were reported in Hertz. Samples dissolved in CDCl3 (Sigma) were used for the analysis. Proton spectrums were assigned by comparison of chemical shifts and coupling constants with those of related compounds.

Chemical shifts are reported as ä-values relative to TMS as an internal reference and coupling constants were reported in Hertz. In ^{1}H NMR spectroscopic analysis, almost all signals were well-resolved and distributed in the region between 1.0 and 8.5 ppm. The strong three proton signals caused by the methyl and acetate groups lie in the region between 1.0 and 2.5 ppm (H17, H19, H18, H6â, 10-OAc, H14, 4-OAc and H2Oá), together with multiplets caused by certain methylene groups. Most of the protons in the taxane skeleton and the side-chain are observed in the region between 2.5 and 7.0 ppm (H3, H_2O â, H2Oá, H7, H2 2 , H5, H2, H32 , H13, H10 and NH) and the aromatic proton signals caused by C-22 benzoate, C-32 phenyl and C-32 benzamide groups appear between 7.0 and 8.3 ppm. The 1H NMR spectrum of the fungal taxol was identical in comparison with standard taxol. The taxol assignments obtained was confirmed with the earlier reports. The method has been established for isolation, identification and characterization of a novel fungal endophyte (*Trametes hirsute*) that produces aryl tetralin lignans detected by HPLC, LC-MS, LC/MSMS and NMR21.

ANTICANCER AGENTS FROM ENDOPHYTES

In vitro apoptotic method of assay

Cytotoxicity effect of fungal taxol isolated from the endophytic fungus was detected and quantified using apoptotic assay on various cancer cells. The taxol-producing endophytic fungi had previously tested for their cytotoxic activity via an apoptotic assay against different cancer cell lines. They showed strong cytotoxic activity in the presence of BT220, H116, Int407, HL251 and HLK210 human cancer cells in vitro. Previous report of the endophytic fungus showed the strong cytotoxic activity towards BT 220, H116, Int 407, HL 251 and HLK 210 human cancer cells *in vitro*, tested by Apoptotic assay. Recently, taxol was tested using an *in vitro* cytotoxicity assay against human cancer cell lines (A-549 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer) in comparison with the standard authentic example, resulting in comparable activities (Puri *et al*. 2005).

ENGINEERING AND STRAIN IMPROVEMENT FOR HIGH YIELD OF TAXOL IN FUNGI

Protoplast Fusion

Protoplast fusion and mutation are commonly applied techniques for strain improvement. A mutant *Fusarium maire* K178 was developed from the endophytic strain *F. maire* Y1117 by protoplast mutation using UV radiation and diethyl sulfate (DES) with high production of paclitaxel (taxol). After strain improvement and optimization of the media for important components like NH_4NO_3, NaOAc and $MgSO_4$ the yield of taxol increased from 20 to 225.2 mg/L in mutant Fusarium maire K178 (Xu *et al*. 2006). The development of high yielding mutant strains by protoplast fusion was also done in Nodulisporium sylviforme fermentation. The protoplasts of N. sylviforme strains UV40-19 and UL50-6 were fully inactivated prior to fusion by heating at 54 ºC for 5 min and by UV irradiation (30WUV light and vertical distance of 30 cm) for 85 s. The mutant strain HDF- 68 was able to produce 468.62 ±37.49 mg/L taxol, which was increased by 24.51% and 19.35% compared to parental strains UV40-19 and UL50-6, respectively (Zhao *et al*. 2011).

Genome Shuffling

Genome shuffling was done to improve taxol production by endophytic *Nodulisporium sylviforme*. A high taxol producing strain of *N.sylviforme* F4-26 was obtained which produced 516.37 mg/L taxol. This value was 64.41% higher than that of the starting strain NCEU-1 and 31.52%e44.72% higher than that of the parent strains (Zhao *et al*. 2008).

Genome mining

Genomes of filamentous fungi reveal that they contain far more gene clusters for secondary metabolite biosynthesis than estimated from the previously identified metabolites. These gene clusters encode enzymes for different classes of secondary metabolites such as non-ribosomal peptide synthetases, terpene synthases and

polyketide synthases, known as “signature” genes/ enzymes. These genes are presumed to be the founders of secondary metabolic gene clusters. The secondary metabolic gene clusters are silent under standard laboratory conditions in filamentous fungi, due to which no product can be formed (Brakhage and Schroeckh, 2011). The majority of successful approaches to activate the gene clusters are based on the generation of gene “knock outs,” over expression of transcriptional factors, promoter exchange and other pleiotropic regulators. Other strategies such as epigenetics and simulation of the natural habitat of the same ecosystem will promote the activation of silent gene clusters and the production of novel metabolites.

Screening of endophytic fungi using molecular markers

Various enzymes of biosynthetic pathway of paclitaxel (taxol) and related taxanes are well characterized and genes encoding these enzymes are cloned (Kusari *et al*. 2013).It has been reported that fungi showing amplification of DNA fragments specific to genes involved in taxol biosynthesis namely,taxa-4(5),11(12)-dienesynthase (ts), debenzoyltaxane-22 -a-O-benzyoltransferase (dbat) and the gene encoding final step in taxol biosynthetic pathway i.e., baccatin III13-O-(3-amino-3-phenylpropanoyl) transferase (bapt) were able to produce taxol (Zhou *et al*. 2007; Zhang *et al*. 2008; Xiong *et al*. 2013).

The screening of endophytic fungi for the production of paclitaxel using some of the biosynthetic genes as molecular markers is reported. The nucleotide sequence of these genes and amino acid sequence of encoded enzymes of endophytic fungi showed high homology with related genes of *Taxus* species. The PCR amplification and cloning of genes of taxol biosynthetic pathway from different taxol producing endophytic fungi facilitated the potential alternative and sustainable source of taxol (Kusari *et al*. 2014).Therefore, PCR based molecular markers specific to taxol biosynthetic pathway genes could be effectively used for the screening of large number of isolated endophytic fungi.

CONCLUSIONS AND FUTURE PROSPECTIVE

The discovery of fungi as a source of taxol offers many future possibilities. The aim of this review clearly mentions about the isolation and characterization of taxol producing fungi. The basic challenge is to enhance the rate of taxol production by fungi. Fungal fermentation offers the best hope for efficient and sustainable production of taxol. The review also depicts the fungal taxol analyzed by spectroscopic and chromatographic estimation are identical to standard taxol. The significance of the discovery is the abundant fungal source for taxol production which can serve as a potential species for genetic engineering to enhance the production of taxol. Genetic engineering of endophytic fungi known to produce taxol, both by gene over expression and random mutagenesis coupled with genome shuffling, have been attempted in only a very limited number of fungal isolates. Reports on the PCR amplification and cloning of many genes of this pathway from several Taxol-producing endophytic fungi facilitate a decisive re-evaluation of their ‘true’ biosynthetic potential, and in turn their potential as alternative and sustainable sources of Taxol. The rate of production of fungal taxol is still under the milligram per liter of culture medium level. Manipulation of culture conditions and bioengineering techniques to develop fungal overproducers of taxol is the future goal. It has been estimated that the commercial production from fungi would be viable when taxol yield is at the level of 1mg/L of fermentation. Genetic engineering and recombinant DNA technology may add a new dimension to the goal of maximizing yield of taxol from fungi. These techniques are most commonly applied in fungi for the production of homologous as well as heterologous enzymes, biochemicals, pharmaceuticals at the commercial level.

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