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ISOLATION AND SCREENING OF ENDOPHYTIC FUNGI FOR THE REDUCTION OF TAXOL

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Abstract

Taxol is naturally obtained from the inner bark of several species *Taxus*. But its accumulation in the *Taxus* is very low. Alternative way to produce taxol is through fermentation processes by using taxol producing fungi. In the present study, about 150 endophytic fungal strains were isolated from ten different indigenous plants. These strains were screened for the production of the taxol through static culture in 250ml Erlenmeyer flasks. Out of these 150 strains, about fifteen strains were found to produce the compound paclitaxel (taxol) in their broths. Among these, the strain UH-10 found to produce considerable amount of taxol in the fermentation broth. This strain was isolated from the inner bark of *Taxus sp*. According to characteristics of the spores and morphology of the fungal culture, strain UH-10 was identified as *Cladosporium cladosporioides*. The fungal taxol in the broth was detected through thin layer chromatography and further analyzed by UV spectrophotometer. Further analysis was performed with high-performance liquid chromatography (HPLC) for the quantitative estimation of the taxol. The amount of paclitaxel produced by *Cladosporium cladosporioides* UH-10 was 140µg/L.

Keywords: Paclitaxel, Fermentation, Static culture, Anti-cancer, High-performance liquid chromatography (HPLC).

Introduction

Taxol is an important anticancer drug used widely in the clinical field. It belongs to class diterpenoid. It was first extracted from the inner bark of Taxus brevifolia (Wani *et al*., 1971). It has the ability to kill tumor cells by inhibiting the depolymerisation of the microtubules during the cell division (Schiff *et al*., 1979). Taxol is the first billion dollar anticancer drug and it has wide application for the treatment of the various types of cancers like breast, lung, ovarian and other human tissue proliferating diseases (Wall *et al*., 1966). Taxol is a white to off white crystalline powder (Kohler & Goldspiel, 1994) containing sterocenters with the empirical formula C47H51NO14 having molecular weight 853.9. It is highly lipophillic, soluble in organic solvent like carbon tetra chloride and insoluble in water. Its melting point is at around 216-217ºC.

As the Taxol has ever increasing application in the chemotherapy and it is obtained from the natural sources so the availability and cost of the drug is an important issue (Kwon *et al*., 1998). Taxol used in scientific research and cancer chemotherapy is mostly isolated from Taxus species or semisynthesized from its biosynthetic precursors i.e. baccatin III and 10-deacetylbaccatin III. These precursors are also obtained from same natural plant (Denis *et al*., 1988). However, due to the destructive collection of Taxus bark for Taxol, this natural resource i.e. Taxus is being threatened day by day. Other approaches to obtain Taxol have been under investigation and some progresses have been made in order to protect Taxus in the world and lighten the pressure of Taxol sourcing. There are several other possible routes to industrialize Taxol production such as tissue or cell culture (Hu *et al*., 2003; Christen *et al*., 1989), total chemical synthesis (Nicolaou *et al*., 1994; Morihira *et al*., 1998) and endophytic fungal fermentation (Wang *et al*., 2000; Strobel *et al*., 1996) beside semisynthesis and isolation from the plant sources.

Taxomyces andreanae was reported as the first Taxol producing fungus in 1993 (Stierle *et al*., 1993). But the amount of the Taxol produced by this fungus is very low i.e. 24-50 ng/L. This discovery draws the interest of the researchers toward fungal sources for the production of the Taxol. But there were reports on the isolation of Taxol producing endophytic fungi (Wang *et al*., 2000; Strobel *et al*., 1996) showing that organisms other than Taxus species could produce Taxol. There are many genera of the endophytic fungi such as *Pestalotiopsis microspora, Taxomyces andreanae, Periconia sp., Alternaria alternata, Pithomyces sp., Seimatoantlerium nepalense, Monochaetia sp. and Chaetomella raphigera* are reported to produce Taxol. Recently, a research group has isolated several endophytic fungal strains (more than 100 strains) from varoius medicinal plants in India for the taxol production (Gangadevi & Muthumary, 2008). *Botryodiplodia theobromae* from *Taxus baccata* and leaf spot strain, *Phyllosticta citricarpa* from *Citrus medica* were endophytic fungal strains reported to produce higher amount of the taxol (Raja *et al*., 2008; Senthil *et al*., 2008). There are some other fungal strains that have much high rate of taxol production like *Metarhizium anisopliae*, (846.1µg/L), *Cladosporium cladosporioids* (800µg/L), *Aspergillus fumigatus* (557.8µg/L) and *Asperigllus niger* ver. taxi (273.6µg/L) (Zhao *et al*., 2010). Some of these fungal strains (*Cladosporium cladosporioids* and *Asperigllus niger*) were found to be associated with other plant sources such as sunflower seeds and *Hyoscyamus muticus* (Afzal *et al.,* 2010; Abdel-Motaal *et al.,* 2010)

The study is aimed to screen some important indigenous species of plants including Taxus spp. for the isolation of some potent endophytic fungal strains which have the ability to produce high titers of taxol through fermentation.

Materials and Methods

Sampling: Samples from different plant species i.e., *Taxus, Hibiscus rosa-sinensis, Citrus medica, Terminalia arjuna,Dalbergia sissoo,Ficus religiosa,Citrus limonum,*

*Pterospermum acerifolium and Syzygium Cumini*were collected from the different areas. Mostly the softer part of the plant including stem and leaves were collected in sterile polythene bags. The plant parts which were infected by the diseases were also collected for the isolation of the endophytic fungi. The small parts were cut and transferred to the sterile bags. The samples were quickly brought to the lab and were preserved at 4^0C before the isolation of fungal strain.

Isolation of the endophytic fungi: The samples were cut into pieces, surface-sterilized with ethyl alcohol 70% (v/v) and washed with sterilized water. Small pieces of the samples (0.5cm × 0.5cm × 0.5cm) were placed on the surface of medium (PDA). Ampicillin was added to the medium to prevent the bacterial growth. Individual hyphal tips of the various fungal colonies were removed from the agar plates and placed on new PDA medium and incubated at 25°C for at least 10 days. The small hyphae emerging from the piece of plant samples were regularly monitored for the morphological characters (shape size colour etc.) of the colony. Each fungal culture was checked for purity and transferred to another PDA plate by the hyphal tip method (Strobel *et al*., 1996).

Fermentation Experiments: Fungal inoculum was prepared by adding 10mL sterilized distilled water in a fully grown and sporulated slant. The spores were scratched with the help of inoculating needle to make a uniform spore suspension. This spore suspension was used to inoculate the fermentation flasks.

The screening of fungal isolates was carried out in 250mL Erlenmeyer flasks containing 25mL of potato dextrose broth supplemented with 1 g/L soytone. The static fermentation technique was used for the production of taxol. The fungi were individually inoculated to the sterilized media in the flasks and incubated at 30^0C for 21 days statically without any disturbance.

Extraction of taxol: The standard extraction procedure (Strobel *et al*., 1996) was followed for the isolation and extraction of taxol from the fermentation broth. After the incubation, mycelia were removed from culture broth by filtration through four layers of cheesecloth. To reduce the fatty acid in the culture broth 0.25 g Na2CO3 was added as fatty acid on vigorous shaking can contaminate the product. For the extraction of the taxol from the fungal culture two equal volumes of solvent dichloromethane was used. The organic phase was collected. Then the solvent was removed by evaporation under reduced pressure at 35ºC using a rotary vacuum evaporator and a dry solid residue was obtained which was re-suspended in methanol for further analysis and separation. These crude extracts were analyzed for taxol by spectrophotometric and chromatographic methods.

Identification of the fungal strain: The fungal strain with highest amount of the taxol production was identified by observing its morphological characteristics and microscopic analysis. Colony colour, texture and size were visually observed. Fungal spores and hyphae appearing on the fragments of the sample were examined by light microscopy for measurement and identification.

Spectrophotometric analysis of taxol: For the spectrophotometric analysis, different concentrations of the standard taxol in 100% methanol were made to obtain a standard curve. After isolation from fungal cultures, the extracted taxol was dissolved in 100% methanol and observed for the optical density with a Beckman D-50 spectrophotometer. The absorbance was taken at the wavelength of 235 nm.

Thin layer chromatographic (TLC): The thin layer chromatographic (TLC) analysis was performed on 1 mm (20×20 cm) pre-coated silica gel plates. These plates were developed in series of solvents as reported by Visalakchi & Muthumary in 2010. To detect the taxol from the fungal culture, 1% (w/v) vanillin in sulphuric acid was sprayed on the plates. The fungal taxol was identified by comparison with the standard taxol (Paclitaxel, Merck). The fungal taxol showed a blue spot which turned to dark gray after 24 hrs.

High performance liquid chromatographic analysis: Further confirmation and quantification of the taxol in the fermentation broth was done by the HPLC. A C-18 reverse phase column was used to quantify the amount of taxol in the fermentation broth. The methanol: water (70:30 v/v) was used as mobile phase. Samples in methanol were injected and elution was done. The HPLC analysis was performed at flow rate of 2ml/min and detection was done at the wavelength of 232nm. The peaks of the fungal taxol were compared with standard taxol.

Dry Cell Mass: The fermentation broth was filtered using pre-weighed filter paper (whatman-44). The cell mass of the fungus was dried in oven at 80Cº for 24hrs and weighed again. The dry cell mass was calculated by subtracting pre-weight from after weight.

Results and Discussion

Two hundred samples belonging to 10 different plant species from different sites were collected for the isolation of taxol producing endophytic fungi. The plates were regularly checked for subsequent growth. One hundred and fifty different endophytic fungal strains were collected from ten different plants. Maximum number of fungal strains were isolated from the *Taxus sp*. i.e., 40 fungal strain. The number of fungal isolates from other species was as *Hibiscus rosa-sinensis*, 20; *Citrus medica*, 15; *Terminalia arjuna*, 15; *Dalbergia sissoo*, 7; *Ficus religiosa,* 5; *Alstonia sp*., 13; *Citrus limonum,* 10; *Pterospermum acerifolium*, 14 and *Syzygium cumini*, 11.

These 150 endophytic fungal strains were screened for the production of the taxol. Table 1 shows that 6 out of 40 fungal strains isolated from *Taxus sp.* had the ability to produce taxol while *Ficus* species had only one fungal strain to be able to produce the taxol. *Hibiscus rosa-sinensis* had two fungal strains (UH-73 and UH-87) and *Citrus medica* also had two fungal strains (UH-141 and UH-147). Four fungal strains i.e. UH-93, UH-108, UH-116 and UH-132 were screened from *Terminalia arjuna*. The maximum biomass (3.45gm/L) was produced by UH-10 while the minimum amount of the biomass (2.04mg/L) was produced by the UH-108.

Table 1. Screening of the endophytic fungal strains for the production of the taxol through static culture fermentation*

S. No.	Strain	Plant source	Amount of Taxol produced (µg/L)	Dry mycelial mass (gm/L)
1	UH-6	*Taxus sp.*	20	2.67
2	UH-10	*Taxus sp.*	140	3.45
3	UH-18	*Taxus sp.*	10	2.99
4	UH-23	*Taxus sp.*	43	2.23
5	UH-37	*Taxus sp.*	27	2.56
6	UH-40	*Taxus sp.*	19	2.90
7	UH-62	*Ficus sp.*	28	2.10
8	UH-73	*Hibiscus rosa-sinensis*	58	2.87
9	UH-87	*Hibiscus rosa-sinensis*	34	2.98
10	UH-93	*Terminalia arjuna*	25	2.87
11	UH-108	*Terminalia arjuna*	6	2.04
12	UH-116	*Terminalia arjuna*	12	2.32
13	UH-132	*Terminalia arjuna*	55	2.97
14	UH-141	*Citrus medica*	25	2.90
15	UH-147	*Citrus medica*	49	2.10

*Fermentation conditions:- Medium; PDA,Temprature;30^{0}C, Incubation period; 21 days

Fig 1: TLC plates showing taxol in fungal culture broth

The presence of the taxol in the culture broth was confirmed by the thin layer chromatography. The blue spots on silica gel plates had indicated the presence of the taxol as shown in the Fig. 1. Further the presence of the taxol was confirmed by the HPLC method. The quantity of the taxol in the fermentation broth was also determined by HPLC method. The retention time for the taxol was 3.7 minutes (Fig. 2). The retention time of the taxol produced by the fungal strain was compared with the standard taxol obtained from Merck. It was found that the maximum amount of the taxol (140µg/L) was produced by the strainUH-10 which was isolated from *Taxus* plant while the minimum amount of the taxol (6 µg/L) was produced by the UH-108 which was isolated from *Terminalia arjuna*.

To identify the fungal strain, the morphology of the fungal strain was observed. The colonies were appeared to be olivacious green to olivicious brown in colour. These colonies were turned black when mature. The colony diameter was upto 5.6 cm of 10 days old colony. The colonies appeared velvety in texture when young (Fig. 3a). The colonies grew very well on PDA medium. The hyphae appeared septate, hyaline and upto 5 µm wide. The conidiophores appeared to be straight or flexuous, simple or branched, intercalary or terminal, smooth. The conidiophores appeared to be septate. The conidiophores were up to 360 µm long and upto 4 µm wide. The conodia were in heads of densely crowded, profusely branched chains. The conidia were oblong, limoniform, ellipsoid or fusiform in shape (Fig. 3b). All these morphological and microscopic characteristics were very close to the *Cladosporium cladosporioides* so the fungal strain UH-10 was identified as *Cladosporium cladosporioidesa.*

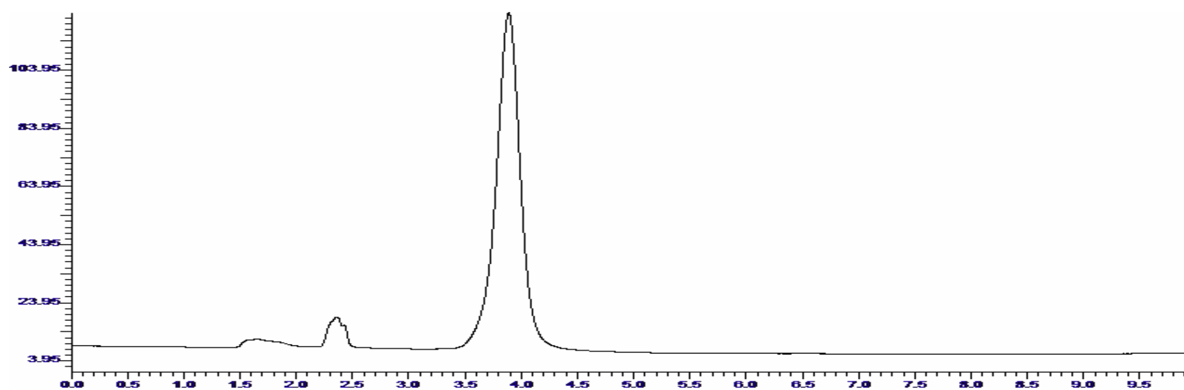

Fig. 2. HPLC analysis of the fungal broth showing the retention time for taxol

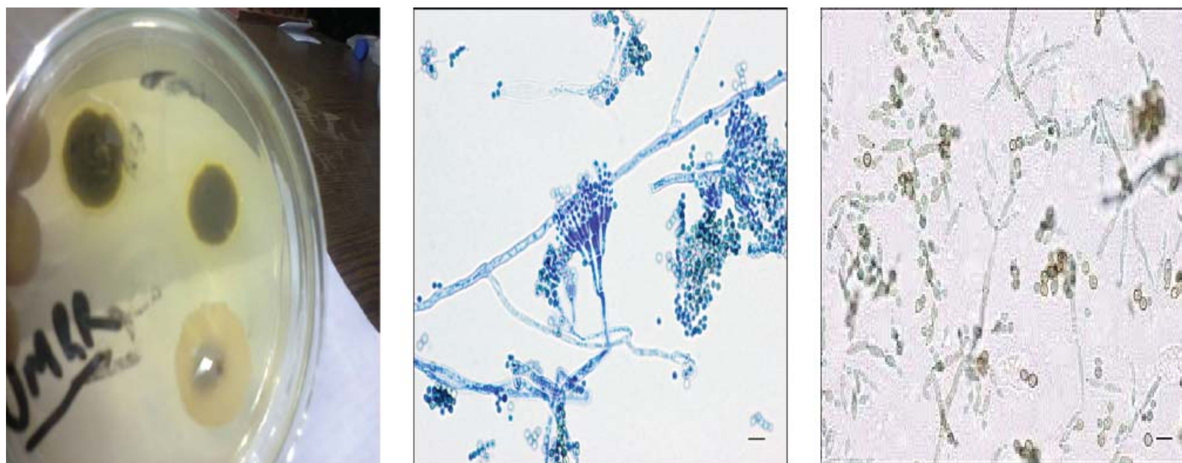

Fig. 3. Microscopic structure of hyphae conidiophores and conidia of *cladosporium cladosporioides* (a) Colony growth (b) conidia and conidiophores

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