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Taxol production from *Pestalotiopsis* sp an endophytic fungus Isolated from *Catharanthus roseus*

*K. Srinivasan** and *J. Muthumary*
Center for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai – 6000 25, India
**Corresponding Author, Email: vksrini267696@gmail.com*

Keywords

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Abstract
Pestalotiopsis sp an endophytic fungi isolated from the medicinal plant *Catharanthus roseus*, was screened for the production of taxol. The culture filtrate of the *Pestalotiopsis* sp was extracted with methylene chloride and examined for the presence of taxol by chromatographic and spectroscopic analysis. The results of both the analysis confirmed the presence of taxol. The amount of taxol produced by this fungus, quantified by high performance liquid chromatography was found to be 92μg/L in MID liquid medium. The results designate that the fungal endophyte, *Pestalotiopsis funerea* is an excellent alternate source for taxol supply and it is also serves as a potential species in medicinal field.

1. Introduction

Fungi are ubiquitous and morphologically diverse, it occurs in wide spectacular array of shapes, sizes and colors, with unique physiological and biochemical properties. And it is exceptionally important to note that vast majority of fungal diversity remains unexplored. Amongst them, least known so far are, the fungal endophytes. They are ubiquitous, diverse and ecologically specialized group, grow asymptomatically within aerial plant tissue such as leaves and stems [1,2,3,4] and more importantly they were proven to produce a secondary metabolite called taxol, an anticancer drug that gained a great economic value in the medicinal field (Strobel et al.,1996).

Taxol, an anticancer drug is produced by an endophytic fungus, *Pestalotiopsis microspora* [5]. In the early developmental stages of Paclitaxel, the drug was extracted from bark of slow growing yew tree, which caused severe environmental problems. Plant cell culture has been suggested as an attractive alternative technique that could overcome the limitation of extracting useful metabolites from natural resources. In spite of many reports from various groups, commercial Paclitaxel production by plant cell culture has not been successful yet. The major obstacle to commercialization has been the low yield of Paclitaxel from plant cell culture.Hence, the finding of an alternative source is needed to produce this compound in large amount, based on this a study was carried out on extracellular production of Taxol, an anticancer drug from *Pestalotiopsis funerea* isolated from *Catharanthus* roseus, collected from the southern part of India.

2. Materials and Methods

2.1. Endophytic Fungi

Pestalotiopsis funerea an endophytic fungi isolated from the leaves of the medicinal plant *Catharanthus roseus* G. Don. (Apocyanaceae) collected from Chennai. The isolated fungus was identified down to species level using standard monographs [6,7,8,9]. The pure culture was deposited to culture collection centre CAS in Botany, University of madras.

2.2. Screening of Extracellular Taxol from Pestalotiopsis funerea

The test fungus was grown in 2 litre Erlenmeyer flasks containing 500 ml of MID medium supplemented with 1 g soytone l^{-1} [10]. The test fungus was inoculated and incubated for 21 days. After incubation the culture filtrate was extracted and filtered through four layers of cheesecloth to remove mycelia. To the culture filtrate 0.25 g $NaCO_3$ was added with frequent shaking in order to reduce the amount of fatty acids that may contaminate taxol in the culture. Then the culture filtrate was extracted

with two equal volumes of solvent dichloromethane. The organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 35 °C using rotary vacuum evaporator. The dry solid residue was re-dissolved in methanol and the crude extract was examined for the presence of taxol by subjecting to TLC, HPLC, UV and IR. The presence and the amount of taxol present in the crude extract were confirmed by using the authentic taxol.

2.3. Thin layer chromatography (TLC)

The thin layer chromatography was carried out to detect the presence of taxol in the crude extract of *Pestalotiopsis funerea.* The experiment was done on 0.25 mm (10 x 20 cm) aluminum precoated silica gel plates (Merck). Samples were spotted along with authentic taxol (Paclitaxel – SIGMA Grade) as internal standard and the plate was developed in solvent system, chloroform / methanol at 7:1 v/v successively. The R_f values of the samples were calculated and compared with authentic taxol.

2.4. High Performance Liquid Chromatography (HPLC) analysis

To confirm and to quantify the presence of taxol in the fungal sample was subjected to high performance liquid chromatography. Taxol was analyzed by HPLC (Shimatzu 9A model) using a reverse phase C_{18} column with a UV detector. Twenty µl of the sample was injected and detected at 232 nm. The mobile phase was methanol / acetonitrile / water (25:35:40, by vol.) at 1.0 ml min^{-1}. The sample and the mobile phase were filtered through 0.2 µm PVDF filter before entering the column. Taxol was quantified by comparing the peak area of the samples with that of the authentic taxol using the formula:

$$\text{Amount of Taxol} = \frac{\text{Standard concentration} \times \text{Total area of sample peak}}{\text{Total area of the standard peak (authentic taxol)}}$$

2.5. Ultra Violet (UV) spectroscopic analysis

After chromatography, the crude sample of taxol was analysed by UV absorption, dissolved in 100% methanol at 273 nm [11] in a Beckman DU-40 Spectrophotometer and compared with authentic taxol (Paclitaxel – SIGMA Grade).

2.6. Infra-Red (IR) spectroscopic analysis

The IR spectra of the compound were recorded on Shimadzu FT IR 8000 series instrument. The purified taxol was ground with IR grade potassium bromide (KBr) (1:10) pressed in to discs under vacuum using spectra lab Pelletiser and compared with authentic Taxol. The IR spectrum was recorded in the region 4000 – 500 cm^{-1}.

3. Results and Discussion

The test fungus *Pestalotiopsis funerea* grown in MID media was screened for the production of taxol as described by Strobel *et al.,*[5]. The culture filtrate extracted and examined for the presence of taxol by chromatographic and spectroscopic analyses were given below. The TLC profile of the crude extract *Pestalotiopsis funerea* when sprayed with the reagent consisting of 1% vanillin (w/v) in sulfuric acid after gentle heating [12]. The appearance of bluish spot having particular Rf value identifies the presence of taxol. Similar, observation was made in authentic taxol. Therefore, it was evident that this fungus showed positive results for taxol production [13]. Then the fungal samples were further analyzed using HPLC, UV spectroscopy and IR.

The fungal extract analyzed by HPLC to confirm the presence of taxol. The fungal extracts gave a peak when eluting from a reverse phase C_{18} column, with about the similar retention time as authentic taxol. The quantity of taxol produced by fungi was calculated based on the area of the sample peak and concentration and peak area of authentic taxol (Fig. 1). This fungus produced 92 µg/L taxol in MID liquid medium.

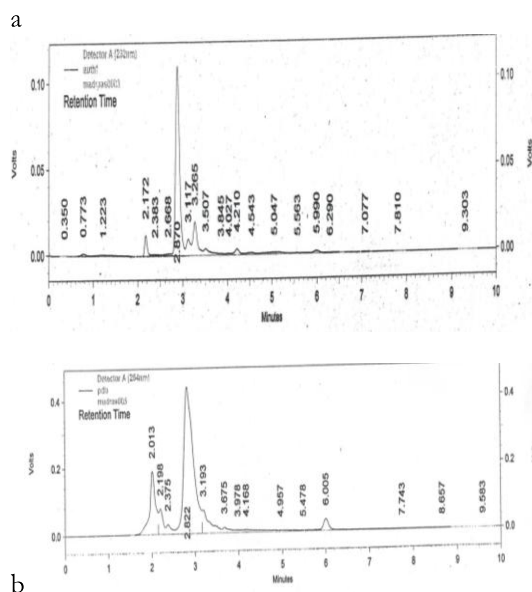

Fig. 1 HPLC analysis of fungal taxol isolated from *Pestalotiopsis* sp. grown in MID Medium. a)Authentic taxol; b)Taxol from *Pestalotiopsis funerea*

The UV spectral analysis of the fungal extracts isolated from two media were examined with a Beckman DU-40 spectrophotometer and the spectra were superimposed on that of authentic taxol at 273 nm (Fig. 2). The appearance of bands in IR spectra convincingly illustrates the identical feature of the extracted samples with the authentic taxol. A broad peak in the range of 3431 cm^{-1}, which was due to hydroxyl (-OH) and amide (-NH) groups stretch. The aromatic ring (C=C) stretching frequency was observed in the range of 1590 cm^{-1}. The registration of peak observed in the range of 1040 – 1060 cm^{-1} is due to the presence of aromatic C, H bends. The IR spectra of the fungal samples extracted from the two media were superimposed on the spectrum of authentic taxol (Fig. 3). The techniques like UV, TLC, IR, HPLC, are the tools applied in the confirmation test for the antitumor compound taxol isolated from fungi and are supported by many workers [13,5].

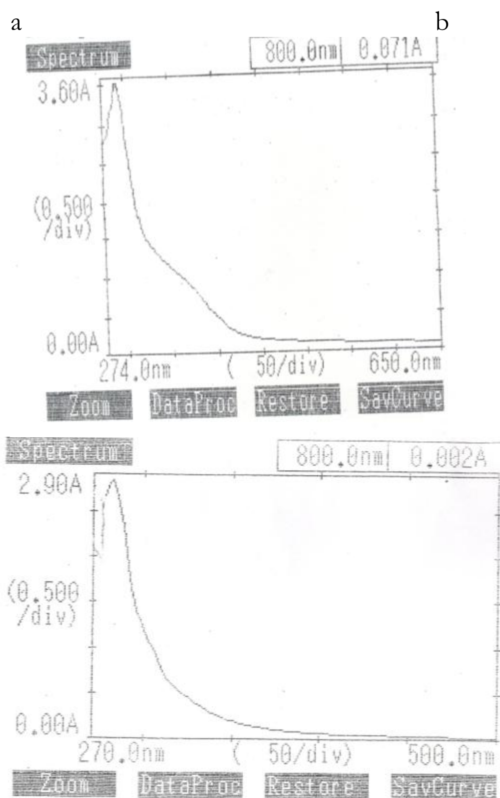

Fig. 2 UV analysis of fungal taxol isolated from *Pestalotiopsis* sp. grown in MID
Medium. a)Authentic taxol; b)Taxol from *Pestalotiopsis funerea*

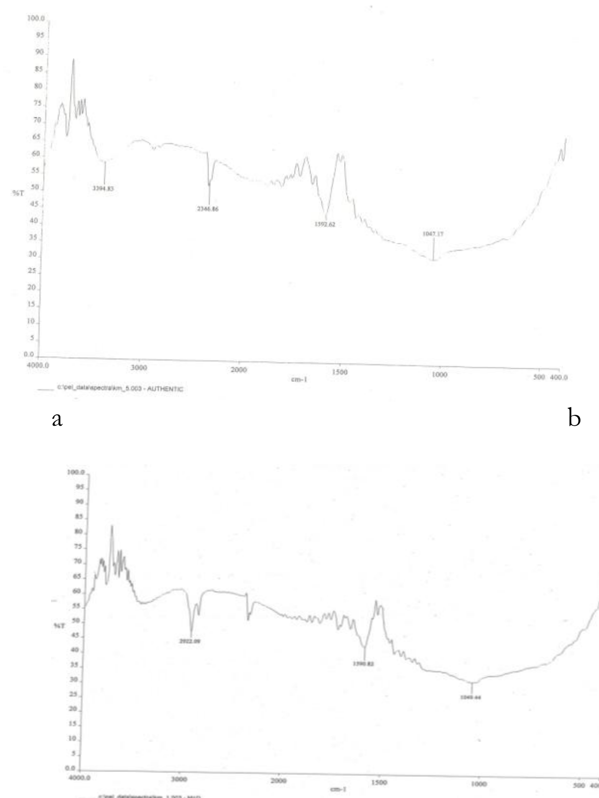

Fig. 3 UV analysis of fungal taxol isolated from *Pestalotiopsis* sp. grown in MID
Medium. a)Authentic taxol; b)Taxol from *Pestalotiopsis funerea*

In conclusion, if the endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price.

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