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PRODUCTION OF TAXOL BY *PESTALOTIOPSIS BREVISETA* CR01 ISOLATED FROM THE *CATHARANCEUS ROCEUS* AND ITS GROWTH STUDIES.

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ABSTRACT

As there is a continuous search for the alternative source for taxol, a million dollar drug due to its scarcity scientists have chosen microorganism as one of the alternate sources.*Pestalotiopsis breviseta* CR01 produces taxol invitro which was isolated from the plant *Catharanceus roceus*. The optimum growth of this fungus was analyzed by varying different growth parameters. The presence of taxol in this fungus was confirmed through HPTLC, UV and IR spectroscopic methods. The amount of taxol was quantified through HPLC analysis. This study reveals that *Pestalotiopsis breviseta*CR01 optimum growth can be attained at temperature 25°C and pH 7.5 with the presence of dextrose as carbon source.

KEYWORDS : Taxol, *Pestalotiopsis breviseta, Catharanceus roceus, Coelomycetes*

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INTRODUCTION

Taxol, a powerful antimitotic agent with excellent activity against a range of cancers, was originally isolated from *Taxus brevifolia*[1]. The drug was approved for the treatment of ovarian cancer by the U.S. Food and Drug Administration in 1992.Taxolwas found in extremely low amounts in the needles, bark and roots[2]. The drug was used to treat tumors like the colon, rectum carcinoma, metastatic breast cancer, lung cancer, tumors in head and neck, and malignant melanotic cancer and so on[3].However, a complete treatment for the patient requires approximately 2 g of taxol administered several times over many months. To obtain 1 kg of taxol it requires about 10,000 kg of bark[4] and several thousand trees must be cut to procure this quantity of bark. This scarcity of taxol and the ecological impact of harvesting it encouraged scientists to find alternative methods using microorganisms[5]. A coelomycetous fungus, *Pestalotiopsis microspora*, an endophyte from the inner bark of *Taxuswallichiana* produced taxol in culture[6].There are various studies confirming the presence of taxol producing endophytic fungi in different medicinal plants[7-11]. The taxol isolated from these fungi is biologically active against cancer cell lines and is also spectroscopically identical to authentic taxol. The presence of taxol producing fungus *Pestalotiopsis breviseta* in infected leaf of *Ervatamia divaricate* was first reported by Kathiravan and Sriraman , 2010[12].This study prompted us to continue the search for TAXOL production from fungal sources of infected leaf.
In the present study coelomycetous fungi arescreened for the production of taxol. The aim of the present study is to isolate taxol producing fungus from the infected leaf of the medicinal plant C*atharanceous roceus* and to study the various growth parameter influencing the optimum growth of the fungus and to isolate and quantify taxol from the isolated fungus. This is the first report confirming that this colelomycetous fungi *Pestalotiopsis breviseta* CR01 isolated from C*atharanceous roceus* produces taxol invitro.

MATERIALS AND METHODS

The fungus was isolated from infected leaf of*Catharanceus roceus*. The general laboratory techniques followed in the course of the present investigation were as outlined by Booth[13].

1. Growth Parameters

The following growth parameters were estimated according to Pinkerton and Strobel, 1976[14] to investigate the optimum growth of the fungus.

1.1 ***Radial growth***

Mycelial disc of 7 mm diameter were cut from the growing margin of the culture and were placed in the M1D agar. These plates were incubated at 24±2 °C for 10 days. The growth was measured daily.

*1.**2 Fungal biomass***

The fungus was grown in 250ml Erlenmeyer flasks containing 50ml of MID medium supplemented with 1g soytoneL−1[15]. These cultures were harvested on different days (5, 10, 15, 20, 25 and 30). Both fresh and dry mycelial weight was recorded at regular intervals.

1.3 ***Optimumtemperature****:*

The fungus was grown in 250ml Erlenmeyer flasks containing 50ml of MID medium supplemented with 1g soytoneL−1and was incubated at different temperatures (10, 15, 20, 25 and 30).Fungus was harvested after 20 days. Both fresh and dry weight of the mycelium produced per flask was determined.

1.4 Optimum pH:

The fungus was grown in 250ml Erlenmeyer flasks containing 50ml of MID medium

supplemented with 1g soytone L−1 with different pH each (2.5, 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5). Fungus was harvested after 20 days. Both fresh and dry mycelial weight from each flask was determined.

1.5 Carbon source:

The fungus was grown in 250ml Erlenmeyer flasks containing 50 ml of M1D broth supplemented with 1g soytone L−1 and amended with different carbon sources such as Dextrose, Fructose, Sucrose, Mannitol and Glycerol and were incubated. Fungus was harvested after 20 days. Both fresh and dry weight of the mycelium produced per flask was determined.

1.6 Estimation of taxol production

1.6.1 Extraction and Isolation

Extraction of taxol was performed according toStierle*etal*, 1993[6]. After incubating the culture for 3–4 weeks, the culture filtrate was passed through four-layered cheesecloth. In order to avoid fatty acid contamination of taxol, 0.25 g of NaCO3 was added to the filtrate. The culture fluid was extracted with two equal volumes of methylene chloride and the organic phase was evaporated to dryness under reduced pressure at 35 °C and the solid residue was re–dissolved in methanol. Taxol content in the methanol sample solution was analysed by High performance thin layer chromatography (HPTLC), Ultra Violet (UV), Infra-Red (IR) and High performance liquid chromatography (HPLC).

1.6.2 High performance thin layer chromatography

The partially purified fungal taxol samples obtained through TLC were further subjected to HPTLC (CAMAG-Planar HPTLC, Anchrom). With Automatic TLC Sampler, 5 Tl of the sample was injected and developed in aCAMAG flat bottom chamber with Chloroform:Methanol, 9:1 for 20 min. Theplate was dried and scanned usingTLC Scanner andfluorescence was recorded at 254 nm.Documentation of the TLC plate was performedand the presence of taxol was visualized byspraying with 1% vanillin sulfuric acid (w/v) andheating the plates gently for 2 minutes. The peak area and peak height of theauthentic taxol and fungal taxol were evaluatedand the Rf values of all the samples werecalculated and compared with authentic taxol.

1.6.3 Ultra Violet (UV) spectroscopic analysis of TAXOL

The purified sample of taxol was analysed by UV absorption, dissolvedin 100% methanol at 273 λmax [15] and compared with authentic taxol.

1.6.4 Infra-Red (IR) spectroscopic analysis of TAXOL

The purified taxol was ground with IR quality potassium bromide (1:10) pressed into discs under vacuum using spectra lab Pelletiser and the spectrum was recorded (4000–500 cm−1 nm) in a Burker 17S 85 FTIR Spectrophotometer.

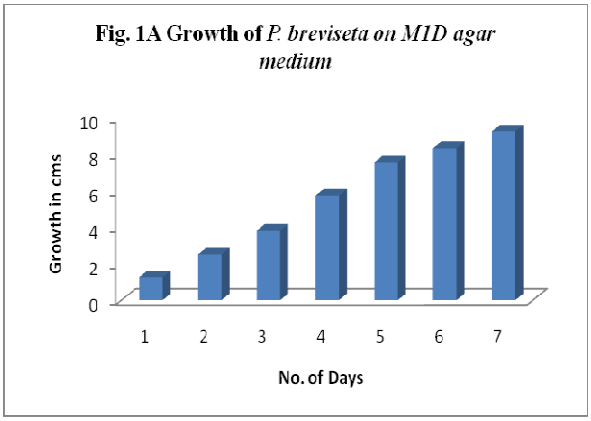
1.6.5 High performance liquid chromatography (HPLC)

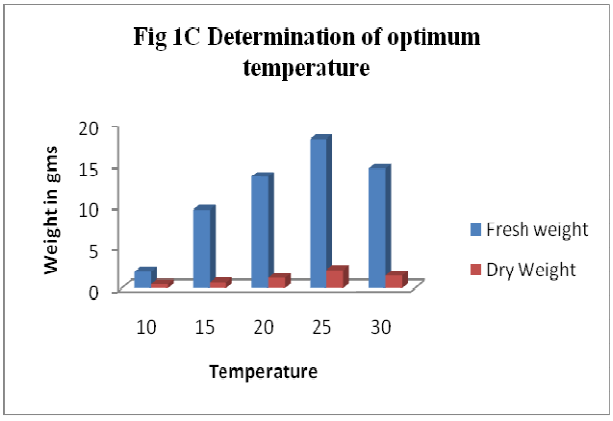
To further confirm the presence of taxol in the fungal extracts, the culture filtrate of the fungus was extracted with chloroform: methanol, 7:1 v/v and subjected to TLC with the solvent system chloroform: acetonitrile, 7:3 v/v. The putative taxol was scraped and eluted with acetonitrile. The resulting residue was subjected to micropore HPLC separation for optimum sensitivity. The study was conducted on a reverse phase 1 mm×150 mm×5 mm particles with isocraticmobile phase consisting of methanol:water, 80:20 v/v at a flow rate of 50 µl/min. Subsequent analysis of 2µl of the sample prepared from 100µl of the dissolved samplewas again conducted. Registration of peak and retention time should be identical to taxol.

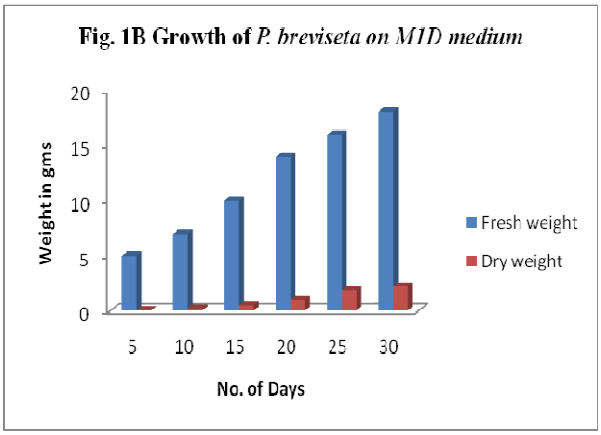
RESULT AND DISCUSSION

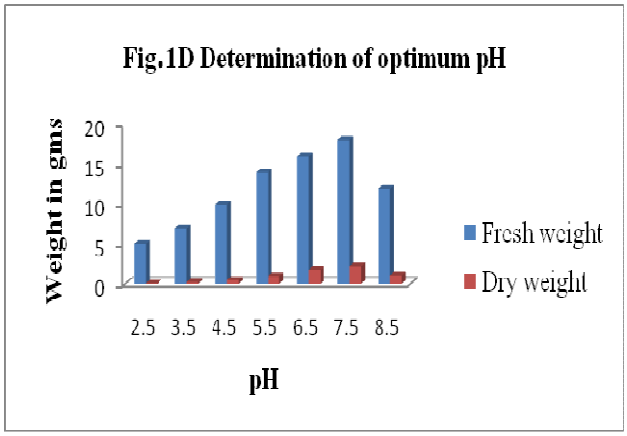
Based on the macroscopic and microscopic appearance the fungus was identified as *Pestalotiopsis breviseta*. Fungi in the culture could be described as Pustules, punctiform, gregarious, 90–150µm diam. Conidiomataacevular, flat. Conidiogenous cells lining the bark of the conidioma, discrete, ampullifrom, hyaline, 2–5×3–4 µm with 0–1 annellation. Conidia, 5 celled, elliptic fusiform, 20.2×4–5 µm, hardly constricted at septa, intermediate coloured cells olivaceous, concoloures, sometimes slightly contrasted, 9–10.8 µm long, apical appendage 2–3, sometimes 5–8 µmlong end cells hyaline, short, apical cells conic cylindric,the basal cells broad conic, basal appendage short 1–2 µm long. In the present growth study the fungus was found to grow well in M1D agar medium and attained a maximum growth on 7th day of harvesting (Fig 1A) and in M1D medium the maximum growth was on 20th day (Fig 1B).*C. gloeosporioides* isolated asendophytic fungus from Artemisia annua was found toattain a maximum growth of 40-50mm in diameter in PDA medium at 28°C for 5 days[16]. The optimum temperature required for the mycelial growth is 25°C (Fig 1C) and optimum pHwas found to be 7.5(Fig 1D).This result coincides with the growth parameter studies of Coleomycetes,*C. gloeosporioides*isolated from the plant*P. acutifolia* grew well inM1D medium with pH of6.5 at 25°C[17].The carbon source for the optimum growth of the fungus was found to be dextrose followed by sucrose (Fig 1E).With the optimum growth parameters the fungus was subjected for taxol production in M1D medium for 20 days at 25°C with pH 7.5.The extract of the fungal culture was examined for the presence of taxol by chromatographic and spectroscopic analyses The selected fungi showed a blue–grey colour reaction with the vanillin/sulphuric acid reagent[15]which is identical to that of authentic taxol(Fig.2). The UV spectroscopy analysis of authentic taxol and fungal taxol was performed. The λmax for authentic taxolwas observed at 273 nm similarly the λmax for sample taxolwas also found at 273 nm range. This confirms the existence of taxol molecule in the sample (Fig.3). Further presence of taxol was confirmed by IR analysis.Coincidence of a single broad intense peak at 3500 cm^{-1}in both authentic taxol and sample substantially proved the existence of secondary amine (Fig. 4a and b) Band at 3150cm^{-1}was observed due to the existence of O–H group.An intense peak was observed at the range of 1680–1720cm^{-1} where 2 weak bands at 1680cm^{-1} and 1690cm^{-1} and a strong band at 1715cm^{-1} . The strong band was due to the existence of carbonyl group (3 in number) as ketones. The shift in frequencies was due to overlapping of CO frequencies and –N–H frequency. It was also due to the presence of bulky electron with drawing group to the carbonyl carbon.The sharp intense peak at 2200 cm^{-1} clearly provedthat it was due to C–N stretch.The existence of aromatic ring by (benzene derivatives) was witnessed by peaks at 1600 and 1475 cm^{-1}. The N–H group of amide and amine also gave absorption peaks at 1550–1640cm^{-1}and this overlapped with be benzene ring peaks.The presence of taxol was further confirmed by using HPLC. The HPLC column was a C18. The sample solutions of *P. breviseta* for HPLC analysis were filtered through a 0.2 µm membrane before injection. The mobile phase consisted of methanol:water, 80:20 v/v. The flow rate was 1 ml min−1.The quantification of fungal taxol was produced by the fungus was based on the area of the sample peak, concentration and peak area of authentic taxol (Paclitaxel purchased from Sigma) (Fig. 5a and b).The present investigation showed that the production of taxol from *P. breviseta* CR01 showed 0.056 mg/l in M1D medium and this was little low when compared to that of *P. breviseta* isolated from infected leaf of *Ervatamia divaricate* was found to be 0.064 mg/lreported by Kathiravan and Sriraman 2010[12]. Host variance might be reason for the low yield for the production of taxol. Taxol production from callus cultures of

Taxuscuspidata and *T. canadensis*had also been demonstrated to be slightly better than that of *T. brevifolia* (0.02%) [18]. The significance in the discovery of fungithat produce taxol indicated that there areabundant resources of fungi that produce taxol[19].When compared to the production of taxol from plant tissue culture, the yield was definitely higher in the present study from coelomycetous fungi. The tested fungi, *P. brevisetaCR01*, had a great potential for commercial exploitation in the future, for better taxol production by enhancing the cultural conditions since the taxol production was very low in these fungi at present.









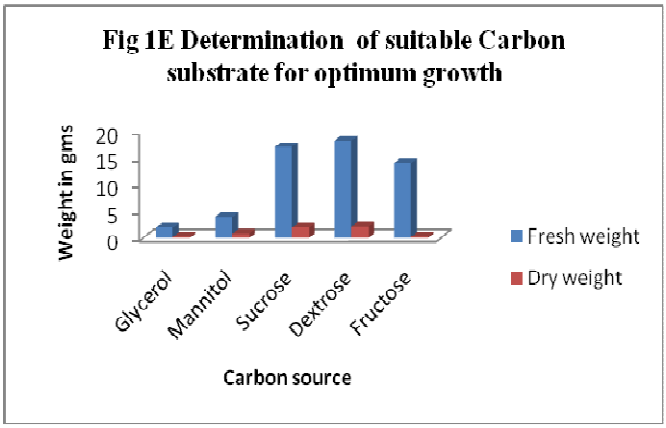


Figure 2
HPTLC Analysis: S1-Authentic taxol ; U7: Fungal taxol

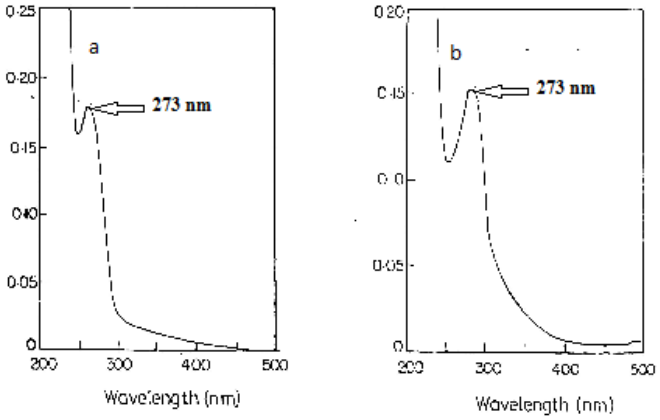


Figure 3
UV Spectroscopic analysis of a. authentic taxol fungal taxol

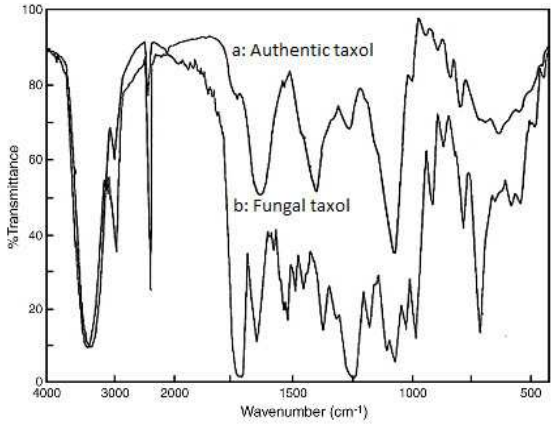


Figure 4
IR Spectrum of (a) authentic taxol (b) fungal taxol

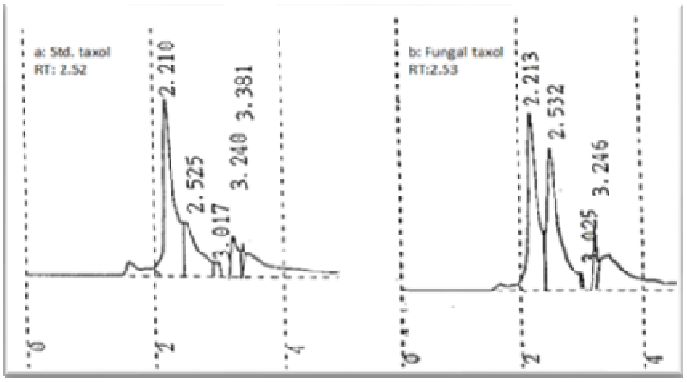


Figure 5
HPLC analysis (a) authentic taxol (b) fungal taxol

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