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ISOLATION AND SCREENING OF TAXOL PRODUCING ENDOPHYTIC FUNGI

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ABSTRACT

Taxol is a highly valuable FDA approved anticancer drug broadly used in the treatment of cancers. An efficient alternative source for the production of taxol is the fermentation processes utilizing taxol producing endophytic fungi. In our study, ninety fungal strains were isolated from different *Taxus* plants collected from different areas of Pakistan. The isolated strains were checked for taxol production under static conditions. Out of ninety strains, thirty-one strains showed the production of taxol in their fermentation broth. Among taxol producing strains, the maximum production of taxol (340 ± 0.018 µg/L) was shown by the strain HM27. The strain HM27 was identified as *Aspergillus niger* on the basis of morphological characters such as colony color, texture, and size, etc. The endophytic fungal strain was isolated from *Taxus fauna* bark, collected from Miandam, District Swat, Pakistan. The taxol produced by endophytic fungi was detected by thin layer chromatography and further quantified by using high-performance liquid chromatography (HPLC). The endophytic fungi may be proved as excellent sources of this anticancer drug.

Keywords: Anti-cancer, Taxol, Endophytic fungi, Fermentation, High-performance liquid chromatography (HPLC), Thin layer chromatography (TLC).

INTRODUCTION

Taxol has widely been used for the treatment of cancers because of its low toxicity and high specificity. Taxol was extracted from the bark of *Taxus brevifolia* Nutt. in the early 1960s (Wani *et al*., 1971). The molecule of taxol composed of tetracyclic core (baccatin III) and an amide tail (Zaiyou *et al*., 2013). Its molecular formula is $C_{47}H_{51}NO_{14}$ and molecular mass is 853.9Da (Panchagnula, 1998). Taxol is a highly valuable FDA approved anticancer drug which is broadly used in clinics and hospitals for cancer treatment and advanced forms of Kaposi's sarcoma. The drug has a role in treating various kinds of cancers both in low and high concentration ranges. At lower concentration ranges it induced apoptosis by stabilizing the spindle formation at the G2-M phase during mitosis, results in cell proliferation inhibition.

Paclitaxel, when used in higher concentration increased the polymerization of microtubules led to the inhibition of cell cycle succession to S phase causes cell death resulting in necrosis (Yeung *et al*., 1999). The extraction procedures of paclitaxel from the bark of Pacific yew tree was costly, inefficient and not environment-friendly, as the enormous number of bark needs to be treated in order to obtain a small concentration of taxol.

Keeping in view the increasing demand and declining source of taxol, scientists around the world started looking for an alternative way of obtaining taxol production. Chemical synthesis of paclitaxel could be a sound alternative but unfortunately, low yield and complicated reaction mechanism limit its feasibility (Nicolaou *et al*., 1994). So the scientists were only left with the option of plant tissue culture which is environmentally ecological method and has immense production potential of paclitaxel but long incubation time hindered its practicality (Yukimune *et al*., 1996). Thus considerable interest has been developed in finding suitable sources of paclitaxel production, including fungal endophytes living mutualistically inside the plant tissue (Kusari and Spiteller, 2012).

Endophytic fungi associated with plants through variable complex relationships ranging from antagonistic to mutualistic relationships. However, inducible mutualistic behavior of endophytic fungi is significant in *Taxus* plants. Several efficient taxols producing endophytic fungi were isolated mostly from *Taxus* as well as other plant sources. Globally identified taxol-producing fungi are endophytic in nature and have been isolated from *Taxus* plants (Zhou *et al*., 2007). Different fungal strains that produce taxol includes *Gliocladium* sp. (Sreekanth *et al*., 2009), *Colletotrichum gloeosporioides* (Raj *et al*., 2014), *Aspergillus niger*, *Alternaria alternata*, *Fusarium solani*, *Botryosphaera rhodina*, *Aspergillus terreus*, *Phomopsis* sp. and *Lasiodiplodia theobromae* from *Salacia oblonga* a tropical plant (Roopa *et al*., 2015). The reverse phase HPLC quantifies the amount of the taxol, and also analyse the purity of taxol (Kim *et al*., 2001). Thin layer chromatography (TLC) has also been used as a tool to quantify taxol (Migas and Świtka, 2010).

The main objective of the study was to isolate endophytic fungal strains from *Taxus* sp. to assess their potential for the production of taxol through fermentation. The results showed that these endophytic fungi possess the ability to produce good quantities of taxol in the fermentation broth.

MATERIALS AND METHODS

Sample collection and processing

The healthy *Taxus* plant samples of leaves, barks, and stems were collected from hilly areas of Pakistan including Swat, Nathia Gali and Naran (KPK). These *Taxus* plant species were identified and packed into labeled sterilized bags for the isolation of endophytic fungi and further processing for the production, screening and isolation of taxol.

The samples were cut into pieces (5mm x 5mm x 5mm), surface sterilized by using sodium hypochlorite 2.5% (v/v) or ethanol 75% (v/v) and subsequently washed with deionized water. The treated samples were then placed on the surface of potato dextrose agar (PDA) medium supplemented with chloramphenicol (100µg/mL) to avoid bacterial growth. The plates were incubated at 30^{o}C and the fungal growth was observed after 3 days. The hyphal tips were removed, transferred to new PDA medium, and incubated for 14 days at 25^{o}C. The fungi were differentiated on the basis of different morphological characters (size, colour, spore production) of the colony.

Fermentation Experiment

For fermentation, spore inoculum was prepared by using autoclaved water (10mL) to a fully mature fungal slant aseptically, and spores were scratched off to form a homogenized suspension. The suspension formed was further used to inoculate fermentation broth. For taxol screening, fungal cultures were grown separately on 250mL Erlenmeyer flask containing sterilized 30mL potato dextrose broth at 30^{o}C for 21 days under static condition.

Extraction of Taxol

The extraction of taxol from fermentation broth was done using a method developed by Pandi *et al*. (2011). After fermentation, the fungal culture was filtered through muslin cloth in order to remove mycelia. The filtrate obtained was further treated with 0.25g Na_2CO_3 to reduce the amounts of fatty acid that may cause hindrance in the extraction of taxol. Extraction of taxol from culture filtrate was done by adding two equal volumes of dichloromethane with constant shaking resulted in the formation of two layers. Organic phase formed was collected and subjected to rotary vacuum evaporator under reduced pressure at 40^{o}C to remove the solvent. The dry solid residue obtained was again dissolved in methanol for further proceedings.

Thin Layer Chromatography (TLC)

After extraction of taxol from the fungal broth, thin layer chromatography was used to detect its presence by a method developed by Li *et al*. (2000). TLC was performed on pre-coated silica gel plates having a thickness of 1mm. About 10µL of the samples were applied to silica gel plates along with the standard taxol at the baseline and subjected to a series of solvent system which includes: Solvent A- chloroform: methanol (70:10, v/v), Solvent B- chloroform: acetonitrile (70:30, v/v), Solvent C- ethyl acetate: 2 propanol (95:5, v/v), Solvent D- methylenechloride: Tetrahydrofuran (60:20,v/v) and Solvent E- methylenechloride: methanol: dimethylformamide (90:9:1, v/v/v) for the detection of taxol (Visalakchi and Muthumary, 2010). The detecting reagent used was 1% (w/v) Vanillin in sulphuric acid, which showed a blue spot under UV detector.

High-Performance Liquid Chromatography (HPLC)

Quantification of the taxol was done by using High-Performance Liquid Chromatography (HPLC) method, developed by Wang *et al*. (2000). Perkin Elmer HPLC system was used for the quantitative analysis of taxol having C18 reverse phase column (SpherSIL, 5um diameter, column size (250 x 4 mm). The mobile phase was prepared using methanol and water (70:30). The flow rate was adjusted to 1mL/min and its absorbance was taken at 232nm.

Identification of Strain

The fungal strain was recognized by its morphological appearance and more specifically by direct microscopic examination. Colour of the fungal strain, its size and texture were observed visually. Spores and hyphae of the fungal strain were observed under light microscopy for the further confirmation of the fungal strain (Gohar *et al.*, 2015).

Determination of Dry Cell Mass

The cell mass obtained after filtration of fungal broth was dried at 70^{o}C for 12 h in a hot air oven and weighed. The difference between dried and pre-weighed filter paper exhibited dry cell mass content of fungal strain.

Statistical Analysis

Statistical analysis was done using Computer software COSTAT, cs6204W (Wallenstein *et al*., 1980).

RESULTS AND DISCUSSION

Ninety plant samples including bark, stem, and leaves from different *Taxus* plants were collected for the isolation of endophytic fungal strains. These samples were collected from hilly areas of Pakistan including Swat, Nathia Gali and Naran (KPK). Sixty fungal strains were isolated from these samples - among them thirty-one were taxol producing strains (Table 1). The presence of taxol was detected using thin layer chromatographic (TLC) technique as shown in (Fig. **1)**, while quantification was done through HPLC analysis. The retention time of the standard taxol (Merck) was 4.5 minutes as shown in (Fig. **2)**. The comparison of retention time between standard taxol and taxol produced by fungal strain provides the concentration of taxol in the fermentation broth. Among the taxol producing strains, the highest production (340 ± 0.018 µg/L) of taxol and biomass (2.56 ± 0.058 g/L) was shown by the strain HM27. The strain was isolated from *Taxus* plant which was collected from Swat Pakistan. The least production of taxol (49 ± 0.023 µg/L) and biomass (2.37 ± 0.040 g/L) was shown by HM25.

Table 1. Screening of endophytic fungal isolates for taxol production using surface culture fermentation.

Sr. No	Strain Code	Amount of taxol (µg/L)	Dry Mycelial Mass (g/L)
1	HM1	200 ± 0.048	2.23 ± 0.032
2	HM2	140 ± 0.071	2.45 ± 0.012
3	HM3	120 ± 0.056	2.2 ± 0.030
4	HM4	195 ± 0.011	2.93 ± 0.045
5	HM5	NIL	2.67 ± 0.023
6	HM6	123 ± 0.057	2.04 ± 0.018
7	HM7	201 ± 0.023	2.47 ± 0.041
8	HM8	100 ± 0.019	2.65 ± 0.009
9	HM9	167 ± 0.067	2.90 ± 0.051
10	HM10	206 ± 0.011	2.25 ± 0.023
11	HM11	130 ± 0.053	2.36 ± 0.041
12	HM12	120 ± 0.022	2.13 ± 0.027
13	HM13	160 ± 0.010	2.23 ± 0.034
14	HM14	172 ± 0.036	2.07 ± 0.018
15	HM15	76 ± 0.056	2.45 ± 0.048
16	HM16	NIL	2.33 ± 0.042
17	HM17	50 ± 0.032	2.09 ± 0.028
18	HM18	NIL	2.12 ± 0.018
19	HM19	50 ± 0.068	2.26 ± 0.098
20	HM20	140 ± 0.071	2.31 ± 0.062
21	HM21	240 ± 0.053	2.09 ± 0.011
22	HM22	50 ± 0.043	2.23 ± 0.022
23	HM23	88 ± 0.067	2.01 ± 0.057
24	HM24	NIL	2.24 ± 0.051
25	HM25	49 ± 0.023	2.37 ± 0.040
26	HM26	59 ± 0.042	2.1 ± 0.023
27	HM27	340 ± 0.018	2.56 ± 0.058
28	HM28	100 ± 0.062	1.8 ± 0.034
29	HM29	160 ± 0.022	1.97 ± 0.019
30	HM30	60 ± 0.058	2.16 ± 0.048
31	HM31	NIL	2.24 ± 0.068

Fermentation conditions:-"Incubation temperature: 30^{o}C; incubation time: 21 days"

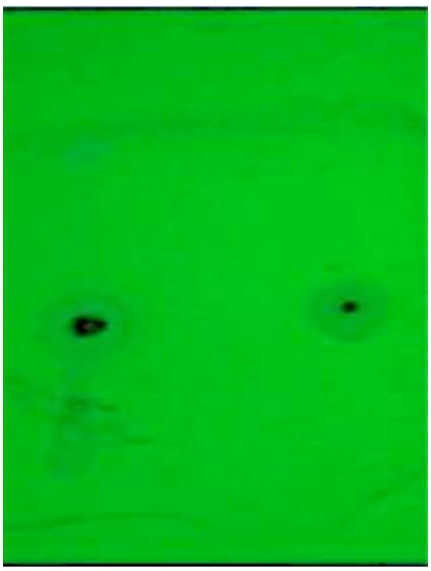

Fig 1. TLC plate for the comparison of standard taxol with sample (fungal culture broth) after visualization under UV.

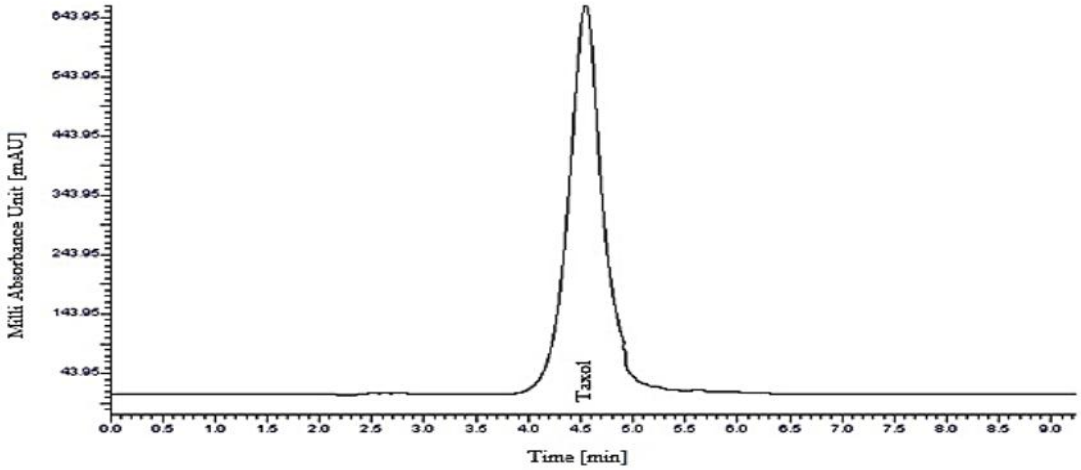

Fig 2. HPLC Chromatogram illustrating retention time for standard taxol.

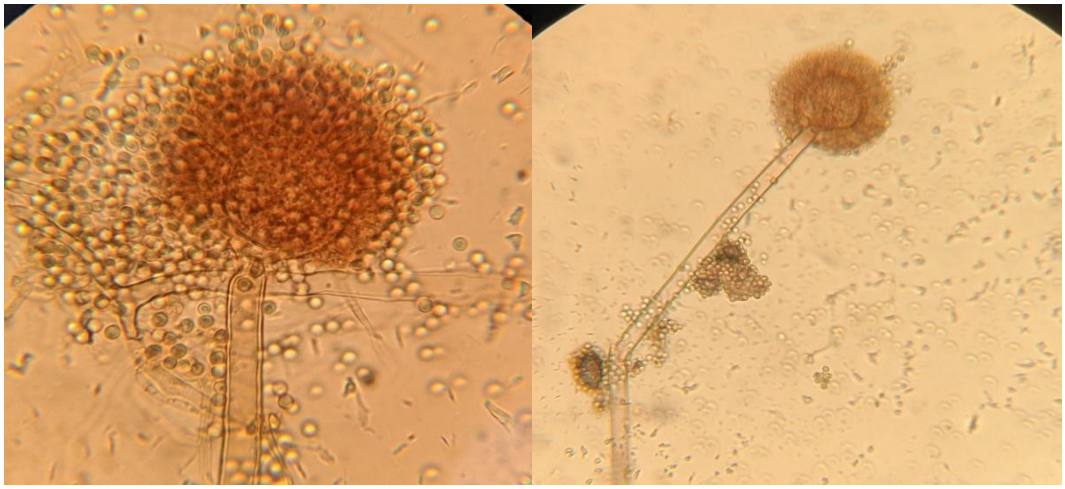

Fig 3. Micrographs of *Aspergillus niger* HM27.

Production of taxol from endophytic fungi has been previously reported by many workers as Ismaiel *et al*. (2017) isolated endophytic fungi from different plant species. Among those isolated fungal strains, the maximum production (307.03ug/L) of taxol was obtained by *Aspergillus fumigatus*. In a recent study, Yang *et al*. (2018) isolated thirty-two endophytic fungi from *Taxus* (yew) plant. The maximum amount of taxol produced was 5.7 mg/l from *Alternaria alternata.* Similarly, Garyali *et al*. (2013) conducted a study in which different endophytic fungi isolated from Himalayan Yew plants, *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger was screened for their ability to produce taxol. The maximum amount of taxol was produced by *Fusarium redolens* (66 µg/L). Qiao *et al*. (2017) isolated endophytic fungus, *Aspergillus aculeatinus* from the bark of *Taxus chinensis* which produced 334.92 µg/L of taxol.

The fungal colonies were morphologically and microscopically characterized and found to be having white basal tuft enclosed by a thick coating of brown-black conidial heads. Conidial heads were large (up to 3 mm by 15 to 20 µm in diameter) and dark brown. Hyphae were septate and hyaline. Conidial heads were large, globose, dark brown, biseriate with the phialides borne on brown, often septate metulae as shown in Fig. **3**. All these morphological characters have close resemblance with *Aspergillus niger*, therefore HM27 was identified as *Aspergillus niger*.

Conclusion

The present study demonstrated that *Aspergillus niger* appears to be promising and attractive producers for paclitaxel, one of the most important anticancer drugs. Further research work is in progress to scale up paclitaxel production by optimizing the medium requirements using response surface methodology and improving the fungal strains using chemical and physical mutagenesis.

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