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Optimum Conditions for Enhanced Production of Podophyllotoxin from *Penicillium* sp. Isolated from Khanspur, Pakistan

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UFG and AM performed the experimental work. HM supervised the project. BM wrote the manuscript.

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ABSTRACT

Podophyllotoxin, a naturally occurring aryl tetralin lignan produced by few plant species and endophytic fungi is used as precursor for the chemical synthesis of the anticancer drugs. In the present study, for the isolation of podophyllotoxin producing endophytic fungi 30 plant (*Podophyllum hexandrum*) samples were collected from different localities of Pakistan. About 261 fungal strains isolated, among them 22 strains had the ability to produce podophyllotoxin. Maximum podophyllotoxin production (88.14µg/ml) was shown by fungal strain P-18, which was later identified as *Penicillium* sp. through 18S rRNA gene sequencing. Optimum growth conditions and parameters such as fermentation medium, pH, temperature, inoculum size, carbon and nitrogen sources were optimized to attain maximum production of podophyllotoxin. The maximum production of 566.23µg/ml was achieved with yeast extract sucrose broth medium with 15% and 5% supplementation of glucose and peptone as carbon and nitrogen source, respectively at pH 5.5 and temperature 25ºC. The endophytic fungi emerging as an alternative source of such medically important secondary metabolites.

INTRODUCTION

Podophyllotoxin, is important bioactive lignin used as a precursor to three anticancer drugs etoposide, tenopside and etopophose phosphate (Kour *et al*., 2012). It has been used as antidot against poisons, vesicant, purgative and as antihelminthic agents (Gordaliza *et al*., 2000). It helps in the treatment of different other disease like colds (MacRae and Towers, 1984; Liu *et al*., 2009), constipation (Li *et al*., 2012; Kumar *et al*., 2015), monocytoid leukemia, non-Hodgkin's and Hodgkin's disease lymphoma (Gordaliza *et al*., 2001; Kumar *et al*., 2015; Li *et al*., 2012; Rajesh *et al*., 2014a).

The podophyllotoxin production from traditional sources is limited due to destruction of wild populations of the primary source plant *Podophyllum hexandrum* and difficulties in the total chemical synthesis lead to search the alternative sources. Endophytic fungi are widespread and ubiquities in their occurrence in plants and have been recognized as a rich source of natural bioactive products (Sterile *et al*., 1995; Eyberger *et al*., 2006). Recently podophyllotoxin have been reported to be produced by endophytes of *Podophyllum hexandrum* (Huang *et al*., 2001; Sivanandhan *et al*., 2012; Nadeem *et al*., 2012). The main podophyllotoxin producing endophytic fungi are *Mucor fragilis, Trametes histuria, Piriformospora indica, Sebacina vermifera, Aspergillus fumigatus, Phialocephala fortinii, F. oxysporum* and *Penicillium* sp. (Rao and Ravishankar, 2002; Kumari *et al*., 2017).

Podophyllotoxin is non-alkaloid and five rings compound mainly drived from plants like *Podophyllum hexandrum* (Arroo *et al*., 2002; Chattopadhyay *et al*., 2002) and *Podophyllum peltatum* (Jackson and Dewick, 1984; Chattopadhyay *et al*., 2002; Guerram *et al*., 2012). The production of podophyllotoxin from plant is not commercially possible due to low yield, it is being extracted from the rhizome and roots of *Podophyllum hexandrum* (Guerram *et al*., 2012).

Here we have attempted to isolate the podophyllotoxin producing endophyte fungi from *Podophyllum hexandrum* and optimize the conditions to enhance the production of podophyllotoxin.

MATERIALS AND METHODS

Collection of plant sample

Thirty samples including root, stem and leaves from *Podophyllum hexandrum* were collected from hilly areas of Pakistan including Murree (9 samples), Khanspur (9 samples) and Mukshpuri (7 samples) brought to the lab

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and stored at 4ºC.

The plant samples were thoroughly washed and sterilized by the method used by Kjer *et al*. (2010). Surface sterilized plant parts i.e. root, stem, leaves were cut into small pieces and placed on sterilized ampicillin added potato dextrose agar plates (Guerram *et al*., 2012).

The plates were incubated at 25ºC for 5 days. The fungi growing out of the plant tissues were screened for the production of podophyllotoxin through submerged fermentation.

Submerged fermentation of fungal isolates and analysis of podophyllotoxin

Spore suspension (1%) was used to inoculate into the fermentation flask. Spore suspension was prepared by adding 10 mL of sterilized distilled water in the fully grown and sporulated fungal slant. The number of spores per ml was counted with the help of hemocytometer and uniform spore number was adjusted.

For Submerged fermentation individual purified fungal strains were inoculated in the set of three 250 ml Erlenmyer flask containing 30 ml of sterilized potato dextrose broth. The flasks were incubated at 25ºC for 7 days.

After 7 days of fermentation, the biomass was separated with the help of muslin cloth crushed with the help of mortar and pestle with the help of sand (used for breaking the cells) in chloroform and methanol solvent (4:1). The homogenate was centrifuged at 6000 rpm for 10 min. and the supernatant was poured in the glass plates and allowed to dry overnight. After 24 h 2 ml of HPLC grade methanol was added to dissolve the plates and stored at -20ºC in eppendorf, for further processing.

The methanolic solution of biomass filtered by 0.45 µm syringe filters was analyzed by HPLC (Perkin Elmer series 200 fUV/VIS Detector) at 254 nm to determine the presence of podophyllotoxin. The mobile phase consisted of methanol: Water (55:45) at a flow rate of 1 ml/min (Chen *et al*., 2016).

Identification of fungi

The fungal colony that had maximum production of podophyllotoxin was inoculated on agar plates and regularly observed for 5 days. The morphological characteristics i.e. colony, color, texture and edges of fungal strain were observed by using a drop of lacto phenol cotton blue stain under the light microscope.

For molecular identification 1680 bp fragment of internal transcribed spacer (ITS) region from genomic DNA was amplified according to Demirel *et al*. (2013). For sequence analysis sample was sent to Advance Bioscience International Lahore, Pakistan. The BLAST tools were used to find out the similarity of the sequenced gene. The phylogenetic tree was built based on these similarities.

Optimization of fermentation conditions

Culture media

Five types of different media malt extract broth MB, 20 g malt extract and 10 g peptone dissolved in 1 L of the distilled water (Guerram *et al*., 2012). Sabouraud dextrose broth SDB, 40 g of dextrose, 10 g peptone dissolved in 1 L distilled water (Puri *et al*., 2001). Yeast extract sucrose roth YSB, 10 g yeast extract and 10 g sucrose dissolved in 1 L distilled water (Puri *et al*., 2001). Potato dextrose broth PDB, 4 g potato infusion and 20 g sucrose dissolved in one L distilled water (Huang *et al*., 2014) and peptone yeast dextrose broth PYDB, 20 g peptone, 2 g yeast extract and 5 g of dextrose dissolved in 1 L distilled water (Amardeep *et al*., 2008) were used for enhanced production of podophyllotoxin.

Physical parameters

Various physical parameters such as inoculum size, pH, temperature and time of incubation were optimized. The effect of inoculum size on podophyllotoxin production was studied by inoculating production media (YSB) with variable percentage of fungal culture ranging from 1 to 5% (v/v), respectively.

Likewise, the effect of pH was studied by varying pH of production media ranging from 4.0 to 7.0. The YSB media of different pH were supplemented with 2% (v/v) seed culture and incubated at 25°C. The effect of different temperatures on podophyllotoxin production was studied by incubating the 30 mL of production media (YSB) in 4 flasks inoculated with 2% (v/v) seed culture at different temperatures of 20°C, 25°C, 30°C, and 35°C, respectively.

For determining optimum time for incubation 30 mL of YSB medium pH 5.5 taken in 5 different flasks was inoculated with 2% spore and incubated at 25 °C. The flasks were taken out after different time intervals viz 5, 6, 7, 8 and 9 days and processed for estimation of podophyllotoxin by HPLC method.

Optimization of nutritional parameters

The type and amount of carbon sources i.e. dextrose, glucose, sucrose, fructose and maltose were also optimized for the enhanced production of podophyllotoxin. The fermentation experiment was performed as described earlier. The type and amount of organic (beef extract, malt extract, peptone and yeast extract) and inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, ammonium phosphate and ammonium acetate) were also optimized for the enhanced production of podophyllotoxin.

RESULTS AND DISCUSSION

Fungal isolates

Out of 261 fungal isolates 20 were found to have the ability to produce podophyllotoxin ranging from 2.63µg/ml to 88.0µg/ml (Supplementary Table I). The HPLC analysis demonstrated that the retention time of a single peak (7.55 min) of the chloroform extract is as same as authentic podophyllotoxin (7.55 min) under the same conditions (Supplementary Fig. 1).

One fungal strain P-18 produced 88.14µg/ml of podophyllotoxin. It appeared white and its color turned green when matured, and the velvety colonies grew fast on PDA medium in 5 days (Supplementary Fig. 2). Microscopically chain of single cells conidia was observed, rough walled flask shaped phialides was observed (Supplementary Fig. 3). The morphological characteristics showed maximum similarity with the strain *Penicillium* sp. For further confirmation, ribotyping and sequence analysis of isolated fungal strain P-18 was performed. The sequence analysis of the strain (P-18) showed that it had the maximum similarity to *Penicillum* sp. *Y34*.

Optimum growth conditions

Table I shows that *Penicillium* sp. P-18 had the highest amount of the podophyllotoxin (98.72µg/ml) produced in YSB, while the lowest amount of the podophyllotoxin (0.003µg/ml) was produced in PYD. Guerram *et al*. (2012) and Xiao *et al*. (2018) used similar media and got the similar results.

Table I. Effect of different media for the production of podophyllotoxin by *Penicillium* sp. *P18* under static culture fermentation at optimum pH 5.5 and temperature 25°C.

Types of media	Concentration of podophyllotoxin (µg/ml)
Malt extract broth (MB)	11.27
Sabouraud dextrose broth (SDB)	23.13
Yeast extract sucrose broth (YSB)	98.72
Potato dextrose broth (PDB)	3.98
Peptone yeast dextrose broth (PYDB)	0.5

Optimization of physical parameters

Figure 2A shows the production podophyllotoxin after prolonging period of fermentation at 25°C from 5 to 10 days. As the time proceeded, the amount of podophyllotoxin increased and finally it reached maximum (98.53µg/ml) in fermentation broth after 7 days of fermentation. There was a decrease in the production of the podophyllotoxin, which endeded up to 14.56µg/ml after 10 days of incubation. The amount of podophyllotoxin was found to be related with the growth patterns of the fungus i.e log phase to stationary phase. According to Huang *et al*. (2014) the secondary metabolites are often produced around the arrival of stationary phase of growth. Kour *et al*. (2008) reported the high yield of podophyllotoxin after seven days of incubation.

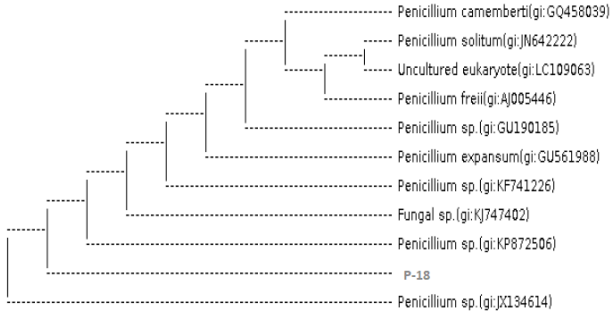

Fig. 1. Phylogenetic tree of endophytic fungi *Penicillium* sp. *P 18* isolated from *Podophyllum hexandrum* based on ITS region with top ten most similar sequences.

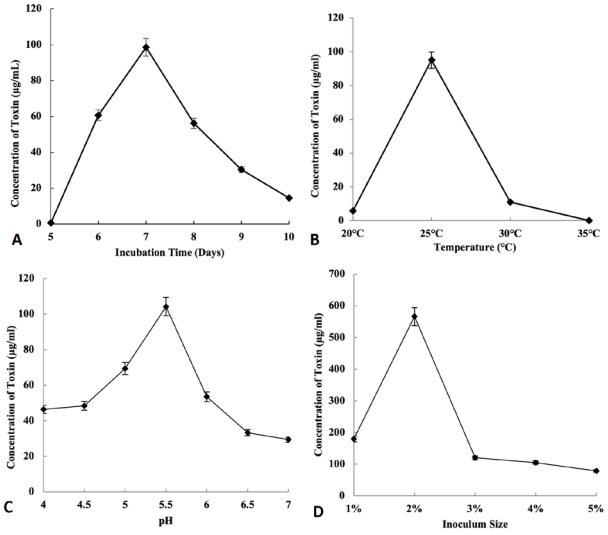

Fig. 2. Physical parameters (A): incubation time; (B): Temperature; (C): pH and (D): inoculum size on the production of podophyllotoxin by *Penicillium* sp. *P-18* using surface culture fermentation in YSB medium.

Figure 2B shows the influence of temperature (20 to 35ºC) on podophyllotoxin production in YSB media pH5.5 with 2% spore inoculum. The amount of podophyllotoxin production increased with the increase of temperature. Maximum podophyllotoxin production

(95.09µg/ml) attained at 25ºC. The temperature influences the growth and metabolite production by the organism. If the temperature is higher than the optimal conditions the enzyme activity would decrease hence the final yield of the product is also decreased (Zhao *et al*., 2011). Huang *et al*. (2014) reported 25ºC optimum temperature for the production of podophyllotoxin in PDA media. Zhao *et al*. (2011) used a similar temperature for the production of another secondary metabolite taxol.

Figure 2C shows the amount of podophyllotoxin production by strain *Penicillium* sp. P18 at various pH i.e. 4, 4.5, 5, 5.5, 6, 6.5 and 7. The maximum amount of podophyllotoxin (104.22µg/ml) was produced at pH 5.5 while minimum amount of podophyllotoxin (29.4µg/ml) was produced at pH 7. Bhagat *et al*. (2016) determine the maximum production of secondary metabolites at pH 5.5.

Figure 2D shows the effect of inoculum size in YSB media pH 5.5, on the production of podophyllotoxin at 25ºC. The maximum amount of podophyllotoxin (566.23µg/ml) was produced when 2% of the spore suspension was used while the minimum amount of podophyllotoxin (78.87µg/ml) was produced with 5% of spore inoculum. Inoculum size had a great impact on the growth of fungal cells. Large inoculum size lead to the production of self-inhibitory substances that can inhibit the germination of the spores (Foster *et al*., 1945; Liu *et al*., 2009; Hornby *et al*., 2004; Gohar, 2013).

Optimization of nutritional sources

Table II shows the effect of carbon, organic nitrogen and inorganic nitrogen on the production of podophyllotoxin. The maximum amount of the podophyllotoxin (150µg/ml) was produced when glucose was used as carbon, peptone as organic nitrogen source and ammonium phosphate as inorganic nitrogen source in the YSB media at optimum pH and temperature i.e., 5.5 and 25ºC, respectively

The amount of glucose that showed the maximum productivity of podophyllotoxin was also optimized. Figure 3A shows the maximum amount of podophyllotoxin (450.92µg/ml) production in the presence of 15% of glucose while the minimum amount of podophyllotoxin, (10.62µg/ml) was produced when 30% glucose, was supplemented to the medium.

For the optimization of concentration of peptone, different concentrations of the peptone ranging from 5 to 25% were used (Fig. 3B). About 517µg/ml podophyllotoxin was produced when 5% peptone was used in the medium. On further increasing the amount of peptone amount of podophyllotoxin decreased and reached 58.76µg/ml when 25% peptone was used.

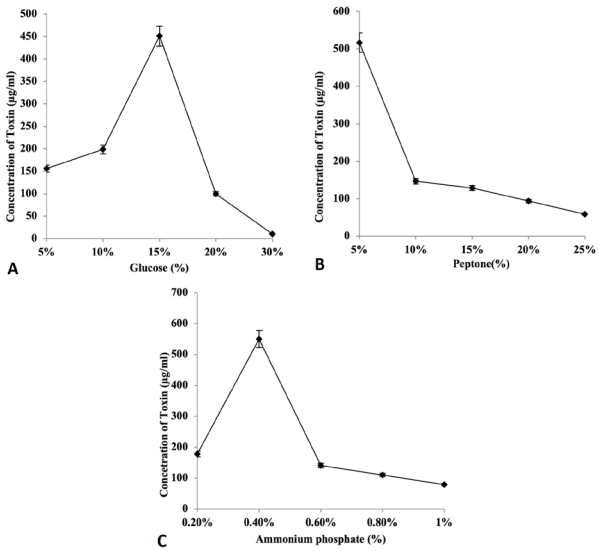

Fig. 3. Effect of different concentrations of (A) Glucose, (B) Peptone, and (C) Ammonium phosphate on the production of podophyllotoxin by *Penicillium* sp. *P-18* by using surface culture fermentation in YSB medium pH5.5 at 25°C.

Table II. Effect of different nutrient sources (carbon source, organic nitrogen source and inorganic nitrogen source) for the production of podophyllotoxin by *Penicillium* sp. *P-18* by using surface culture fermentation in YSB medium pH 5.5 at 25 ºC.

Different nutrients sources	Concentration of podophyllotoxin (µg/ml)
Carbon source	
Dextrose	106
Glucose	150
Sucrose	15.52
Fructose	22.47
Maltose	10.1
Organic nitrogen sources	
Beef extract	98.73
Malt extract	140
Peptone	510
Yeast extract	45
Inorganic nitrogen sources	
Ammonium nitrate	100
Ammonium phosphate	178
Ammonium sulphate	520
Ammonium acetate	0.53

To optimize the amount of ammonium phosphate in the culture medium, different amounts of ammonium phosphate (0.2 to 1%) were added into the medium and the fermentation was carried out at optimum conditions (Fig. 3C). The maximum amount of podophyllotoxin (550µg/ml) was produced when 0.40% of the ammonium phosphate was added to the medium, whereas minimum amount of podophyllotoxin was produced in the presence of 1% of the ammonium phosphate i.e., 78.87µg/ml.

Gohar (2013) and Merlin *et al*. (2013) reported that carbon sources like glucose, fructose, lactose and maltose, organic and inorganic nitrogen sources like yeast extract, soy tone, peptone and beef extract in culture medium. could influence the production of primary and secondary metabolites. Structure of carbon sources from monosaccharide (glucose) to polysaccharide (sucrose, maltose) can affect the production of secondary metabolite. Radu and Kqueen (2002) reported that the amended medium that contain glycerol, yeast extract and carbon source enhanced the production and antitumor activity of the product. In the present study, maximum amount of podophyllotoxin was produced when 15% of glucose was used as the carbon source. Nithya and Muthumary (2009) optimized carbon sources and found the similar results for the production of secondary metabolites. Nitrogen is the basic component of amino acids and it is also required for the enzymatic system and biomass formation.

CONCLUSION

It is concluded that endophytic fungus *Penicillium* sp. P18 isolated from *Podophyllum hexandrum*, was a good producer of podophyllotoxin during submerged fermentation at static conditions. The fungal isolate produced 88.01µg/ml which was enhanced to 6.5 folds (566.23µg/ml) under the optimum conditions of fermentation.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20210401130420

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Optimum Conditions for Enhanced Production of Podophyllotoxin from *Penicillium* sp. Isolated from Khanspur, Pakistan

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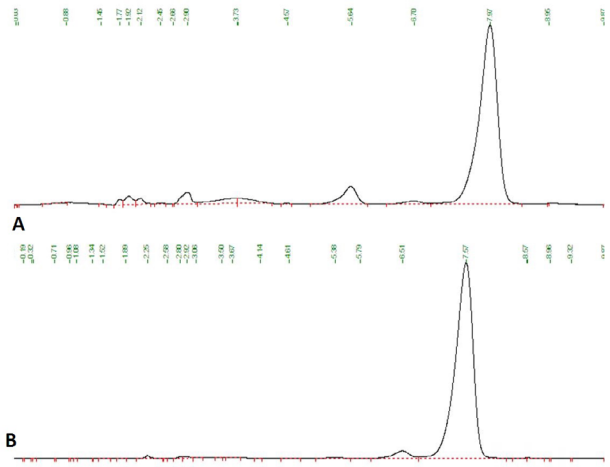
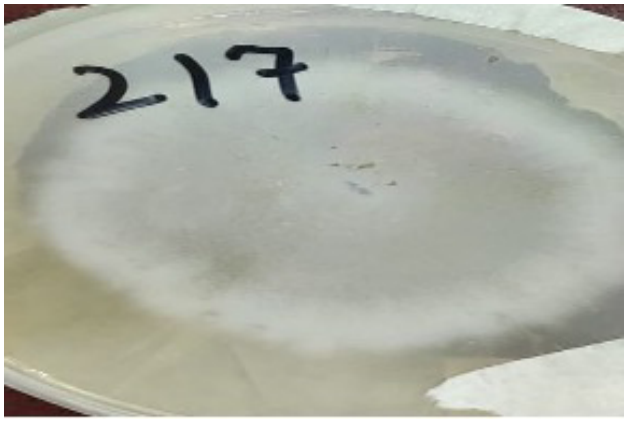
Supplementary Table I. Isolation and screening of the fungal isolate for the production of podophyllotoxin through surface culture fermentation.

No. of samples	Fungal isolates	Amount of Podophyllotoxin produced (µg/ml)
MR-1	P*-15	10.799
	P-16	7.64
	P-18	88.14
	P-8	54.0
	P-1	3.8
	P-2	7.31
	P-3	5.68
MR-2	P-13	1.67
	P-12	2.63
MR-4	P-5	49.77
	P-7	14.71
KH-2	P-4	0.043
	P-6	0.65
	P-17	3.64
KH-3	P-19	7.9
	P-9	6.14
	P-10	12.8
MK-1	P-20	22.05
	P-14	3.64
MK-3	P-11	21.92

*MR, Murree; KH, Khanspur; MK, Mushkpuri. *P stands for podophyllotoxin.



Supplementary Fig. 1. HPLC chromatogram of podophyllotoxin (A) Standard (B) Sample.



Supplementary Fig. 2. Colony of isolated fungal strain P-18.

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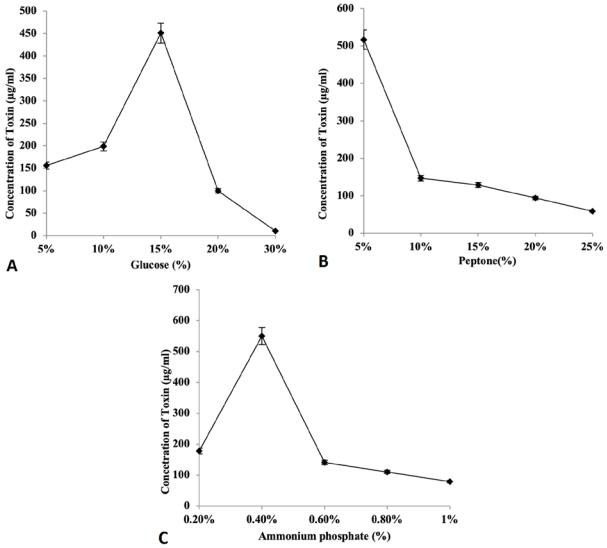
0030-9923/2022/0001-0001 $ 9.00/0



Supplementary Fig. 3. The sporangia and spore of isolated fungal strain P-18.