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

Umar Farooq Gohar, Atitia Majeed, Bushra Muneer* and Hamid Mukhtar

Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan 54000.

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 (88.14 µg/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 566.23 µg/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, an 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 antitoxin against poisons, vesicant, purgative and as anthelmintic 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 ubiquitous 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 derived 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...
and stored at 4°C.

The plant samples were thoroughly washed and sterilized by the method used by Kjær 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 25°C 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 Erlenmeyer flask containing 30 ml of sterilized potato dextrose broth. The flasks were incubated at 25°C 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 -20°C 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 IUV/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 broth 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.

<table>
<thead>
<tr>
<th>Types of media</th>
<th>Concentration of podophyllotoxin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt extract broth (MB)</td>
<td>11.27</td>
</tr>
<tr>
<td>Sabouraud dextrose broth (SDB)</td>
<td>23.13</td>
</tr>
<tr>
<td>Yeast extract sucrose broth (YSB)</td>
<td>98.72</td>
</tr>
<tr>
<td>Potato dextrose broth (PDB)</td>
<td>3.98</td>
</tr>
<tr>
<td>Peptone yeast dextrose broth (PYDB)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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 ended 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.
(95.09µg/ml) attained at 25°C. 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 25°C 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 25°C. 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 25°C, 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.
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 0.4% 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.

REFERENCES


Gordaliza, M., del Corral, J.M.M., Castro, M.A.,


Supplementary Material

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

Umar Farooq Gohar, Atta Majeed, Bushra Muneer* and Hamid Mukhtar

*Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan 54000.

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

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

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

*Corresponding author: bushramuneer11@yahoo.co.uk, dr.bushramuneer@gcu.edu.pk

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

Copyright 2022 Zoological Society of Pakistan
Supplementary Fig. 3. The sporangia and spore of isolated fungal strain P-18.