BIOTRANSFORMATION OF PROGESTERONE TO 17α-HYDROXYPROGESTERONE BY USING PLANT CELL SUSPENSION CULTURE OF CATHARANTHUS ROSEUS

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ABSTRACT

Objective: The present investigation was aimed to perform biotransformation potential of Catharanthus roseus.

Methods: Cell suspension culture of C. roseus were prepared by using Murashige and Skoog medium. Biotransformation of an important bioactive progesterone (4-pregnane-3, 20-dione) was confirmed by cell suspension culture of Catharanthus roseus.

Results: Progesterone, which was used as precursor in cell suspension culture, was found to be bio transformed within three days into a new product through a region selective hydroxylation process. The results confirmed that most of the progesterone added, was hydroxylated at its 17α-position leading to 17α-hydroxyprogesterone as a major biotransformation product after twelve days of incubation with 20% v/v cell density. The optimum biotransformation was achieved with the concentration of 100μg/ml progesterone at pH 5.5. Similarly, glucose was found as the most suitable source of carbon. The optimum incubation time was observed to be 12 days at 20% v/v cell density. Also higher progesterone biotransformation was observed using un immobilized cells as compared to immobilized cells.

Conclusion: The results of present work suggest that C. roseus can successfully biotransformed to the product similar to an original organic substrate, which is entirely synthetic. Such a biotechnological process could be of great interest for production of new chemical compounds like cortisone, hydrocortisone and other important steroids.

Keywords: Catharanthus roseus, Apocynaceae, Cell suspension culture, Biotransformation, Progesterone, 17α-hydroxyprogesterone.

INTRODUCTION

The biotransformation of readily available inexpensive natural products to a more valuable and useful substances through incubation with biological systems has attracted much attention as this approach allows the functionalisation of inactivated carbon atoms, and is still a promising field for the practical application of plant cell culture [1]. In the recent years, much attention has been paid to the ability of cultured plant cells to transfer enantio selectively not only secondary metabolites, but also organic exogenous substrates into biologically active compounds to achieve specific biotransformation [2-4]. Cell suspension cultures of plants have also been efficiently employed for the biotransformation of organic compounds, e.g. steroids [5-7], terpenes [8-10], alkaloids [11] and flavonoids [12]. However, there have been few examples of biotransformation of organic foreign substrates. Many investigators have studied biotransformation of organic compounds by plant cell suspension culture, reporting selective and specific conversion ability including enantio selective oxidation, stereo selective reduction, region selective hydroxylation, enantioselective glucosylation, esterification, methylation and isomerization [13, 10, 14].

Steroidal drugs, a large category of compounds with steroid nucleus, are mainly used to render anti-inflammatory responses, treat cancer and antiretroviral activity for HIV infected therapy clinically [15]. Biological transformation using plant cell suspensions culture allows structural modification on exogenously supplied steroid to provide useful substances [16]. Biotransformation has been used for the production of steroidal drugs universally. Metabolites obtained may be pharmacologically active or can be used in the commercial synthesis of other useful steroid compounds [17, 18, 7]. Some kind of these important biotransformation consisted in 11β, 17α, 16α and 21 hydroxylations of progesterone producing corticosteroids. Hydroxysteroids have been implicated in many physiological conditions. 17α-Hydroxysteroid is an important precursor for medroxyprogesterone acetate production and economically important in the pharmaceutical industry [19-22]. It can be synthesized from progesterone by 17α-Hydroxylation using biotransformation scheme 1.

It has been observed that chemical synthesis is the most common approach despite associated drawbacks. Chemical synthesis of progesterone from various precursors such as stigma sterol, diosgenin and cholesterol involves several critical steps and yield of the final product is also very less. Purification of the product obtained from chemical synthesis is another major issue.

Scheme 1: Production of 17α-hydroxyprogesterone from progesterone by biotransformation

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 MATERIALS AND METHODS

Materials

Progesterone was obtained as a gift sample from Aarage Drug Pvt. Ltd., Parwanoo, India. Plant growth hormones kinetin (Kn) and 2, 4- dichlorophenoxoyacetic acid (2, 4-D) and Murashige and Skoog’s (MS) medium were purchased from Himedia Mumbai, India. 17α-hydroxyprogesterone was obtained as a gift sample from Belco Pharma, Rohtak, Haryana, India. HPLC grade solvents (Methanol, Acetonitrile and Water) were purchased from Sigma Aldrich, St. Louis, U. S. A. Ethyl acetate; chloroform and hexane for TLC were purchased from Sigma Aldrich, U. S. A.

Callus initiation

The callus cultures were prepared by transferring the explants in MS media as reported previously with slight modifications [34, 32]. The young leaf buds of C. roseus (L) were collected from Pharmacognosy garden of Department of Pharmaceutical sciences, Dr. Hari singh Gour Vishwavidyalaya, Sagarg (M. P.) India. These leaf buds were washed with tap water followed by mild detergent solution and distilled water respectively. After washings, the buds were sterilized with 90% ethanol (EtOH) for 30 seconds followed by HgCl2 (0.1% of w/v) for 90 seconds. Thereafter, these leaf buds were rinsed with sterile distilled water 2-3 times and cut into 2 mm x 2 mm explants. These explants were transformed on solidified Murashige and Skoog’s (MS) medium containing 3 mg/l 2, 4-dichlorophenoxyacetic acid, 0.3 mg/l k inetin, 3% w/v sucrose and 0.9%w/v agar (pH 5.8). The cultures were then incubated in dark at 25±1°C in B. O. D. incubator (Toshniwal, India). After callus initiation, culture tubes were subjected to illumination (for 16 h light and 8 h dark) at 1500-2000 lux by fluorescent lamp and kept at 25±1°C. The callus cultures were maintained by sub-culturing in the same medium for the duration of four weeks as reported previously [33].

The suspension cultures were prepared by the method reported previously with slight modifications [35]. After subculturing for several generations, the callus tissue was transferred to freshly prepared media (50 ml) supplemented with 3 mg/ml 2, 4-D and 0.3 mg/ml of kinetin and 3% w/v sucrose in 250 ml Erlenmeyer's flask to prepare suspension cultures of C. roseus. Then grown with continuous shaking at 120 rpm on a rotatory shaker (Remi, India) at 25±1°C under 16 h photoperiod (1500-2000 lux) for one week. They were maintained in same medium and sub-cultured every seven days into 250 ml flask [5 ml of cells suspension into 45 ml of fresh MS medium] under the similar culture conditions.

Preparation of immobilized Catharanthus roseus cells

Immobile C. roseus cells in alginate beads were prepared by the method reported previously with slight modification [3]. Suspension of 30%v/v cells in 5% w/v of sodium alginate was prepared. This slurry of sodium alginate cells was dropped through a 1 mm orifice in 50 mM CaCl2 solution to obtain spherical beads. The resulting beads were allowed to stand for 1 h and washed with distilled water. These beads were added to freshly prepared MS medium (50 ml/flask) containing 1 mg/l 2, 4-D and 0.1 mg/l kinetin and 3%w/v sucrose. Thereafter, the beads were shaken on rotatory shaker at 120 rpm in the dark at 25±1°C.

Extraction and identification

After filtration of cell suspension cultures, cells were crushed with fine sand in a mortar and extracted in twice the volume of chloroform. Also, the culture medium was extracted three times by agitator with CHCl3 twice. Combined organic layer was concentrated under vacuum. Then after the residue was analyzed by thin layer chromatography as reported previously [36] (silica gel 60F-254, Merck Art. 5717; solvent systems, EtOAc: CHCl3: 10:90 and EtOAc: Hexane 20:80). Spots were detected by 5% vanillin solution (ethanol: H2SO4: 7:4) or UV light.

High performance liquid chromatography analysis

The chloroform extracts of cells, culture medium or both were analyzed for progesterone and its metabolites using HPLC Shimadzu, (SPD-10MA system, Japan) using reverse phase separating C-18
The biotransformation studies have been performed according to method as reported earlier [36]. The biotransformation experiments were carried out in 250 ml Erlenmeyer’s flask containing 60 ml of freshly prepared MS medium containing 1 mg/ml of 2, 4-D and 0.1 mg/ml of kinetin and 3% w/v sucrose with immobilized cells as well as immobilized cells of *C. roseus*. Then incubated for one week at 25±1°C on rotatory shaker (120rpm). 20% v/v initial packed cell volume was used for biotransformation purpose. To these pre cultured cells, progesterone in concentration of 100 μg/ml was aseptically added using membrane filter (0.22 μm) followed by incubation of cell suspension cultures under similar conditions as mentioned previously in the original culture. At regular intervals, the samples were withdrawn and the cells were removed under sterile conditions for subsequent extraction with chloroform. The extracts were concentrated under the vacuum and thereafter analyzed using TLC and HPLC to identify and quantify products obtained by biotransformation.

**Effect of various parameters on biotransformation**

**Incubation time**

In order to investigate the effect of incubation time on biotransformation of progesterone, un immobilized cells and immobilized cells were pre cultured in freshly prepared MS medium containing (1 mg/l 2, 4-D and 0.1 mg/l kinetin and 3% w/v sucrose) for 1 week at 25±1°C under similar conditions as mentioned previously. Thereafter, 100 μg/ml of progesterone was added to the flask and incubated for 21 days. At a regular interval every 3 days, cells and medium were collected, extracted and analyzed for progesterone and its metabolites.

**Substrate concentration**

The initial experiment was performed to determine the optimum concentration of progesterone that can be fed to give maximal biotransformation yield. In order to investigate the effect of fed precursor (progesterone) concentration on biotransformation, cell suspension culture (20% v/v) was incubated into MS medium containing various concentration viz. 50 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml and 250 μg/ml of progesterone and after 12 days of incubation under similar conditions mentioned earlier, samples were extracted and analyzed.

**pH**

The effect of pH on progesterone biotransformation was determined using immobilized and un immobilized cells with fixed concentration of progesterone. For this purpose pH range of 3.5 to 8.5 was selected and 0.1N HCl or 1N NaOH were used to achieve the desirable pH. In each case, after 12 days of incubation the media was extracted and analyzed as mentioned previously.

**Carbon source**

Various carbon sources glucose, sucrose, fructose, maltose and sodium acetate were used with fixed concentration of progesterone (100 μg/ml) in MS medium to study the effect of different source of carbon on biotransformation. After 12 days incubation period under similar conditions mentioned earlier, samples were extracted and analyzed.

**RESULTS AND DISCUSSION**

**Induction of callus**

After two to three weeks of transferring the callus tissue into MS medium at 25±1°C in the light, the light cream-colored suspension culture was established (fig. 2A). 6.5% v/v and 91.5%, packed cell volume and cell viability respectively were recorded after three weeks.

**Biotransformation**

In an attempt to study the aptitude of cells to metabolize progesterone, several experiments were simultaneously carried out to optimize the cultures; cell suspension culture of *C. roseus* without progesterone (control culture), liquid medium with progesterone in ethanol without cells and cell suspension culture of *C. roseus* with ethanol without progesterone. After incubation for seven days, cells and liquid medium were analyzed separately by TLC. TLC studies revealed that progesterone remains unchanged after seven days of incubation, when it was added into the culture medium without cells. However, it was partially metabolized when added to cell suspension culture. More than one spot was observed in TLC from both cells as well as culture medium which corresponds to the standard samples of progesterone and 17α-hydroxyprogesterone. This type of transformation from progesterone to 17α-hydroxy progesterone using plant cell cultures is critical as well as bear utility, which has also not been reported in earlier literature to the best of our knowledge. This indicated that progesterone was absorbed by cells and converted into various products.

Biotransformation of steroid has been extensively reported. However, the studies on 17α-hydroxylation are rare and the nature of 17α-hydroxylation in microorganism is unknown. In the higher eukaryotes, progesterone 17α-hydroxylase is catalyzed by a cytochrome P450. Cytochrome P450 was expressed in Saccharomyces cervisiae and converted progesterone to 17α-hydroxyprogesterone with the yield of 31.4%. Progesterone is readily metabolized by *Digitalis purpurea* tissue cultures to 5α-pregnan-3, 20-dione and 5α-pregnan-3β-ol-20-one [38-40]. Furthermore, Rosa species tissue cultures were reported to form Δ4-pregnen-20β-ol-3-one and Δ4-pregnen-20α-ol-3-one while *C. roseus* and *L. esculentum* tissue culture form Δ4-pregnen-1α-ol-3, 20-dione [27, 41]. In the present investigation, culture of *C. roseus* column (Phoenix 25 x 0.25 cm). 20 μl of the extract was injected with a Hamilton syringe. The acetonitrile: methanol: water (25:25:50) was used as mobile phase and a flow rate of 0.1 ml/min. Detection was done at absorption maxima 254 nm by photodiode array detector [37].

**Fig. 1 A: Explant of *C. roseus* B: Four week old callus culture**

**Fig. 2 A: One week old suspension culture, B: Suspension culture cells entrapped in sodium alginate beads**

metabolite was obtained as ∆\(^{14}\)\_out hydroxylation in the 14\(\alpha\), 11\(\alpha\), and 17\(\alpha\)-positions indicate that certain plant cell cultures are capable of introducing a hydroxyl group apparently without the need for the steroid to be conjugated to a sugar moiety. The stereo specific hydroxylation of non activated positions, as in steroid to be conjugated to a sugar moiety.

Although until recently only reductions of unglycosidated steroids have been reported, apparently some cell cultures can carry out oxidative reactions, namely hydroxylations, on unglycosidated steroids. In addition, reductive and oxidative reactions can be performed by the same plant cell culture. The introduction of a hydroxyl group into one or more positions of progesterone by means of the cultures of C. roseus and L. esculentum was stereospecific. Cultures of L. esculentum yielded hydroxylated in the 6\(\alpha\), 11\(\alpha\), and 14\(\alpha\) positions, whereas C. roseus cultures carried out hydroxylation in the 14\(\alpha\) position [41]. In the present study new metabolite was obtained as ∆\(^{14}\)-pregnene 17\(\alpha\)-ol-3, 20\(-dione by using cell suspension culture of C. roseus upon incubation of progesterone.

Thus, if a new center of asymmetry was formed by hydroxylation, only one of the possible epimers arises. This is similar to the situation found with microorganisms [42-44]. To attain the maximum biotransformation yield, we observed the effects of various parameters viz. precursor (progesterone) concentration, incubation time, pH of the medium and carbon sources on biotransformation of progesterone, which impact the production of metabolites.

**Effect of fed progesterone concentration on biotransformation**

The initial experiment was performed to determine the optimum concentration of precursor i.e. progesterone that showed maximum biotransformation yield. In order to investigate the effect of fed progesterone concentration on biotransformation, cell suspension culture of C. roseus (L) with various concentrations of progesterone; 50\(\mu\)g/ml, 100\(\mu\)g/ml, 150\(\mu\)g/ml, 200\(\mu\)g/ml and 250\(\mu\)g/ml were used in the culture and incubated for 12 days (Table 1). Fig. 3 revealed that the biotransformation product of progesterone i.e. 17\(\alpha\)-hydroxyprogesterone reached to a maximum value and then decreased. The concentration of 17\(\alpha\)-hydroxyprogesterone was dependent on the precursor concentration. As the fed precursor concentration was increased the magnitude of biotransformation initially increased and reached up to optimum level and thereafter decreased. The concentration of 17\(\alpha\)-hydroxyprogesterone was highest at 100\(\mu\)g/ml and then decreased. After the optimum level of precursor concentration, negative effect of progesterone content on biotransformation is evident when more than 100 \(\mu\)g/ml had been added to culture medium. This may be attributed to decline in percent viability of cells. This result was confirmed by microscopic examination of cells by comparison with suspension cultures without progesterone. The dead cell ratio did not increased, except for cultures containing more than 100 \(\mu\)g/ml of progesterone. The maximum biotransformation yield of 17\(\alpha\)-hydroxyprogesterone of 22.33% w/w was observed with optimum fed concentration of progesterone (100\(\mu\)g/ml) over higher concentrations in un-immobilized cell suspension media. At higher concentration, the greater part of fed precursor was accumulated into the cells, which can lead to the lethal effect on the cells. The data suggests an optimal concentration of substrate is required beyond which the cell gradually lose their transformation capacity due to the lethal effect of fed substrate on cells of culture as shown previously [45].

**Table 1: Effect of substrate (Progesterone) concentration on biotransformation by C. roseus after 12 days incubation period**

<table>
<thead>
<tr>
<th>Substrate concentrations [(\mu)g/ml]</th>
<th>Progesterone percent [%]</th>
<th>17(\alpha)-Hydroxy progesterone percent [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>20.25±0.81</td>
<td>15.15±0.92</td>
</tr>
<tr>
<td>100</td>
<td>33.23±0.72</td>
<td>22.33±0.64</td>
</tr>
<tr>
<td>150</td>
<td>38.82±1.0</td>
<td>18.34±1.0</td>
</tr>
<tr>
<td>200</td>
<td>45.13±1.2</td>
<td>11.78±0.82</td>
</tr>
<tr>
<td>250</td>
<td>42.11±0.62</td>
<td>9.18±0.68</td>
</tr>
</tbody>
</table>

\(n=3\)

Fig. 3: Biotransformation yield by C. roseus cell suspension culture at different concentration of progesterone added to MS media after 12 days incubation

Effect of incubation time on progesterone biotransformation

To investigate the effect of incubation time on biotransformation of progesterone, the cell suspension cultures of C. roseus containing 100 \(\mu\)g/ml progesterone was incubated for 21 days and at regular interval every 3 days, samples were analyzed for progesterone and its metabolite. The time course study of progesterone, indicated that biotransformation product i.e. 17\(\alpha\)-hydroxyprogesterone, was detected from 3rd day of the experiment in culture medium (fig. 4). Its yield increased gradually from 3rd to 6th day followed by rapid increase from 9th to 12th day. The yield of 17\(\alpha\)-hydroxyprogesterone was found to be 13.92\(\mu\)g/ml and 12.70\(\mu\)g/ml at 9th and 12th day respectively. Beyond that almost stationary biotransformation were recorded until 21 days.

Fig. 4: Biotransformation yield by C. roseus cell suspension culture at different time period with 100 \(\mu\)g/ml progesterone was used
Effect of pH on progesterone biotransformation

Fig. 5 showed that at pH below 5.0 the biotransformation yield was very poor and negligible amount of 17α-hydroxyprogesterone was obtained. However, maximum biotransformation was observed between pH 5.5 to 6.0 with 20.70 %/w/w and 18.89 %/w/w yield of 17α-hydroxyprogesterone, respectively. Beyond pH 6.0 no noticeable change was observed. There was a steady but slow decline in the rate of progesterone transformation over the range pH 5.5 to 6.0. The marked drop in hydroxylation activity at pH 7.5 could be correlated with the loss of cell viability at this pH. The poor biotransformation rate below pH 5.0 and beyond pH 7.5 might be attributed to enzymes inactivation at these pH ranges.

![Fig. 5: Effect of pH on biotransformation of progesterone. After 12 days incubation with 100 μg/ml progesterone, samples were analyzed](image)

Effect of carbon source on progesterone biotransformation

In order to investigate the effect of carbon source on biotransformation of progesterone, various additives such as glucose, sucrose, fructose, maltose and sodium acetate were used in the culture medium. After constant incubation period (12 days), highest biotransformation yield of 17α-hydroxyprogesterone was recorded with glucose as carbon source. The yield of 17α-hydroxyprogesterone was found to be 20.70 μg/ml, 18.71 μg/ml, 16.9 μg/ml and 15.18 μg/ml with sucrose, fructose, maltose and sodium acetate respectively. Carbon sources are invariably present in the tissue culture media especially for its energy values; however, the type of such source is of paramount importance. Replacement of glucose with sucrose, fructose, maltose, and sodium acetate lead to considerable decrease in biotransformation (Fig.6). Although, sucrose is most commonly used in tissue culture work, the present study indicates that glucose is favorable carbon source for progesterone biotransformation. Substitution of fructose, maltose and sodium acetate brought about a decrease in biotransformation rate in the following order i.e. fructose > maltose > sodium acetate.

![Fig. 6: Effect of various Carbon source on biotransformation.12 days after addition of progesterone (100μg/ml), the pattern of 17α-hydroxyprogesterone were determined](image)

Biotransformation by immobilized cells and unimmobilized cells

To investigate the effect of immobilization, separate set of studies were performed. As evident from fig. 7 un immobilized cell suspension culture of C. roseus showing lesser progesterone biotransformation observed from 3rd to 6th day, suddenly increased progesterone biotransformation from 9th to 12th day. After 12 days of incubation, there was almost stationary biotransformation observed until days 21st. In case of immobilized cells system, (fig. 2B and 7) slower biotransformation rate was observed following the same pattern then after stationary biotransformation recorded post 18th day instead of 12th day. Both type of suspension cultures, immobilized cells and un immobilized cells, retained the ability to hydroxylate progesterone over this period and remained constant in each case. In these preparations, the difference indicates that the immobilized cells show slow biotransformation for prolonged period of time. However, the biotransformation rate was greater with un immobilized cells suspension. The yield was found to be 20.70% w/w and 11.40% w/w after 12 days in case of un immobilized cells and immobilized cells respectively. These might be due to a retarded uptake of progesterone and slow release of biotransformation products by entrapped cells.

![Fig. 7: Biotransformation yield by freely suspended cells and immobilized cells of C. roseus at 20%v/v cell density (free or Calcium alginate beads) incubated in 50 ml MS media with 100 μg/ml progesterone](image)
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