Optimization of process parameters for bioproduction, isolation and purification of 4-Ipomeanol from cell suspension cultures of *Ipomoea batatas* (L.) Lam.

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The secondary metabolite, 4-Ipomeanol from Sweet potato (*Ipomoea batatas*) is a potential chemotherapeutic agent for cancer. This study presents an efficient protocol for bioproduction of 4-ipomeanol, in large quantities without disturbing the natural biodiversity of *I. batatas*. Friable Calli from the root tubers of *I. batatas* were used to establish cell suspension cultures in liquid media under agitated conditions and the growth and production kinetics of the same were monitored. The presence of 4-ipomeanol was confirmed by NMR and IR spectroscopy and quantification was done using HPLC. A maximum of 7.39 mg g⁻¹ of 4-ipomeanol was obtained under biotic elicitation of the cell suspension cultures. The elicitors were studied for optimization of the day of addition and maximum bioproduction of 4-ipomeanol was observed when elicitors were added to 4 day old suspension cultures. Further, the 4-ipomeanol thus produced demonstrated cytotoxicity against renal carcinoma cell lines. The root tubers of Sweet potato *Ipomoea batatas* (Convolvulaceae) are known to possess 4-Ipomeanol [1-(3-furyl)-4-hydroxypentanone], a stress metabolite produced in response to microbial infection¹. Its bioactivity in cell culture and animal models suggests its chemotherapeutic potency², and proved sensitive against several human tumor types including lung carcinoma and various cancer cell lines³⁴, and also tested in Phase II studies in patients with hepatocellular carcinoma⁵. Further, 4-ipomeanol has been shown to be a potential prodrug for P450 directed gene therapy in liver and brain cancer⁶⁷. The prodrug converting enzyme P450 4B1 gene has been found to activate 4-ipomeanol to a highly reactive furan epoxide which is responsible for the cytotoxic and anticancer properties of 4-ipomeanol⁸. ¹⁸F labeled 4-ipomeanol could be used as an imaging agent for CYP4B1 gene prodrug activation therapy. Also, ¹⁸F labeled 4-ipomeanol is a promising agent for PET imaging of CYP4B1 transfected cancer cells².

Bioproduction of secondary plant metabolites using elicitation of suspension cultures is well documented for plants producing minute quantities of useful phytochemicals. Isolation of 4-ipomeanol has been reported earlier from the storage roots of *I. batatas* infected with *Fusarium solani*⁹ and the maximum yield in our earlier study with chitin as elicitor was 6.61 mg/g¹⁰. Here, we explored for an efficient protocol for optimal bioproduction of 4-ipomeanol without disturbing the natural population of *I. batatas*.

**Materials and Methods**

**Initiation and maintenance of callus and suspension cultures**

The callus cultures were initiated from root tubers of *I. batatas* and processed and maintained as detailed earlier¹⁰. The fragile 8 wk old callus (4 g) (exponential phase), was aseptically transferred into growth media composed of MS medium (20 mL) supplemented with NAA (11 µM), KIN (1 µM), Sucrose (3% w/v) without agar. The setup was incubated in a rotary shaker (Technico, Chennai) at 25±2°C and a rotation speed of 100 rpm in dark. Within 2 to 3 days of inoculation, the cells from the callus started dispersing in the medium as cell aggregates and free cells. This suspension was passed through stainless steel microseive with mesh size of 0.46 mm and the cells were reinoculated in 100 mL liquid MS medium with the same composition as mentioned above. These suspension cultures were maintained by regular subculturing at 2 wk interval.

**Growth kinetics**

The cells were withdrawn from the suspension cultures at the end of 2nd, 4, 6, 8, 10, 12 and 14th day of incubation and their growth was periodically monitored using haemocytometer. The cell density was calculated using the following formula.

Cell density =

Average number of cells per large square × 10⁴ × 2 (cells mL⁻¹)

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Production kinetics

The cell cultures (in growth media) after three
passages were transferred into the production media
composed of MS medium supplemented with NAA
(11 µM), KIN (1 µM), coconut water (3% v/v),
sucrose (3% w/v) and elicitor [F. solani (1:100);
chitin (1 mg L⁻¹); chitosan (1 mg L⁻¹)] with 25% (v/v)
as inoculum.

Addition of biotic elicitors

Biotic elicitors, namely spore suspension of
Fusarium solani, Chitin and Chitosan were prepared
as described earlier
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. The suspension cultures were
incubated at 25±2°C in dark and 100 rpm in rotary
shaker (Technico, Chennai) and the samples were
withdrawn at the end of 1st, 3rd, 5, 7, 9, 12, 15 and 17th
day, extracted and analyzed for 4-ipomeanol content.

Optimization of day of elicitor addition

About 1 mL of each dilution (1:10, 1:20, 1:40,
1:60, 1:80 and 1:100) of F. solani spores was added to
100 mL of cell suspension culture at different stages
of growth (0th, 2nd, 4, and 6th day after subculture).
Similarly, various concentrations of chitin and
chitosan (1, 2, 4, 6, 8 and 10 mg L⁻¹) were added to
100 mL of cell suspension culture at different stages
of growth (0th, 2nd, 4, and 6th day after subculture).
All the cultures were incubated in a rotary shaker at
100 rpm at 25±2°C in dark. The cells were harvested
on the 15th day to study the effect on production of
4-ipomeanol.

Isolation, purification and quantification of 4-ipomeanol

The cells grown in suspension culture were
harvested at stationary phase, filtered and freeze dried
using a Lyophilizer (Lyo 4, Borg Scientific, India). Those
cells grown in suspension culture without any
elicitor were treated as control. The lyophilized cells
were subjected to solvent extraction using methanol
and further purified using column chromatography as
described earlier
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 with little modification that 1 g of
the crude extract was dissolved in 2 mL of ethyl
acetate and was eluted at a rate of 20 mL per hour
with 200 mL each of n-hexane, 5% (v/v) ethyl
acetate-n-hexane, 10% (v/v) ethyl acetate-n-hexane,
20% (v/v) ethyl acetate-n-hexane and finally 400 mL
of ethyl acetate. Eluate was discarded until a yellow
pigment appeared, after which different fractions of
the eluent (10 mL each) were collected and analyzed
for the presence of 4-ipomeanol using thin layer
chromatography (TLC). The fractions were chromatographed on precoated silica gel–G plates
(Merck, India) using the solvent system, Methanol:
Benzene (1:10) (v/v). The plates after developing
were viewed under a UV light and the bands parallel
to the standard was scrapped off using a spatula and
pulverized in a glass bottle. The powder was
resuspended in a small volume of ethyl acetate. The
elusion was rechromatographed using a small
glass column packed with silica gel (200-400 mesh
size) and 4-ipomeanol was eluted using 30 mL of
20% ethyl acetate in n-hexane. The eluate was
concentrated and dissolved in 10 mL of ether and
washed thrice with 0.05 N sodium hydroxide. To
remove the base, the extract was washed thrice with
water. The extract was poured in a beaker containing
anhydrous sodium sulfate and then filtered to remove
the moisture. Then ether was evaporated and the
residue was stored and used for further analysis.

Quantification using HPLC

Analytical HPLC was run on a LC-20AT
intelligent pump (Shimadzu, Japan) with different
solvent systems at varying concentrations. Well
resolved peaks were obtained with solvent
A (acetonitrile) : B (water) = 40:60 on Luna C18
column (250×4.6 mm) with a flow rate of 1 mL min⁻¹
monitoring at 220 nm equipped with SPD-20A diode
array detector (Shimadzu, Japan) with an auto
sampler(20 µL). Initially, a standard graph was
plotted with known concentrations of the standard
4-ipomeanol (Futurechem Co. Ltd., Korea, 99% purity)
and was used for the estimation of
4-ipomeanol in the extracts.

Nuclear Magnetic Resonance spectroscopy

1H NMR was recorded on Bruker Avance III 500 MHz
(AV500) NMR and chemical shifts were reported in
parts per million (ppm).

Infra Red Spectroscopy

Further confirmation of the compound was
done using Fourier transform infra red spectroscopy.
FT-IR spectrum was recorded on ALPHA FT-IR
spectrometer (Bruker).

Cytotoxicity assay

Cytotoxicity of 4-ipomeanol on renal carcinoma
cell lines [ACHN cells (ATCC CRL-1611)] was
determined by MTT assay
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. ACHN cells lines were
cultured on Eagle’s Minimal Essential Medium
supplemented with 10% fetal bovine serum and
incubated at 37°C with 5% CO₂. Cells (1×10⁵/well)
were plated (1 mL of medium/well) in 24-well plates
(Costar Corning, Rochester, NY). After the cells reached confluence (48 h after incubation) they were treated with various concentrations of the purified sample (1-10 µM) in 0.1% DMSO for 48 h at 37°C. The sample solution was removed from the wells and washed with phosphate-buffered saline (pH 7.4). About 200 µL of 0.5%, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) in phosphate-buffered saline solution was added to each well. After 4 h of incubation, 0.04 M HCl was added to each well and viability of the cells were determined by measuring the absorbance at 570 nm. The concentration required for a 50% inhibition of viability (IC$_{50}$) was determined graphically. Wells without sample were used as blanks. The effect of the samples on the proliferation of ACHN cell lines was expressed as % cell viability, using the following formula:

\[
\text{% cell viability} = \frac{\text{ABS}_{\text{sample}}}{\text{ABS}_{\text{control}}} \times 100\%
\]

where, \(\text{ABS}_{\text{sample}}\) is the absorbance of cells treated with 4-Ipomeanol and \(\text{ABS}_{\text{control}}\) is the absorbance of control cells (untreated).

**Statistical analysis**

A set of data derived from 10 replications were processed by analysis of variance (ANOVA) using SPSS (Version 10) statistical software. Differences between the treatments were compared at a significance level of 5% using Duncan’s multiple range test (DMRT).

**Results and Discussion**

The optimal medium for callus initiation was found to be MS media supplemented with NAA (11 µM), KIN (1 µM), Sucrose (3% w/v) and the maximum amount of callus produced was 4 g (Fresh weight) as reported in our previous paper. Suspension cultures were initiated from 8 wk old callus cultures in the same media without agar. Stationary phase was observed from 15th day onwards (Fig. 1).

Biotic elicitors like spores of *F. solani*, chitin and chitosan were added to the suspension culture, to study their effect on the production of 4-ipomeanol. The maximum concentration of 4-ipomeanol was obtained when cells were harvested at stationary phase (Fig. 2). Addition of *F. solani* spores to 4 day old suspension culture enhanced the production of 4-ipomeanol to a maximum of ~2 folds [1.89±0.18 mg g$^{-1}$ DW] over the control culture. However, *F. solani* at higher concentrations (1:10, 1:20, 1:40) interfered with the growth of plant cells.

When chitosan was used as an elicitor, it doubled the production of 4-ipomeanol (~4 folds, 4.06±0.17 mg g$^{-1}$ DW) compared to that of *F. solani* spores. However, the presence of chitin increased 4-ipomeanol content to ~7 folds and did not interfere with the cell growth. The production was maximum (7.39 mg g$^{-1}$ DW), when chitin (4 mg L$^{-1}$) was added during the 4th day of growth (Table 1).

The cells were extracted with methanol and purified by column chromatography. On co-chromatography (Thin layer) with standard 4-ipomeanol, the fractions containing 4-ipomeanol had the same Rf (0.65) as that of pure sample and showed a pink spot up on treatment with Elrich’s reagent.

Satisfactory separation in HPLC was obtained with the mobile phase acetonitrile and water (40:60 v/v). The retention time of standard 4-ipomeanol was found to be 9.4 min. A protocol has been standardized for the quantification of 4-ipomeanol using HPLC. The standard graph obtained with standard 4-ipomeanol is shown in Fig. 3. The purified extracts were analyzed...
under the same conditions to quantify the amount of 4-ipomeanol in them. The chromatograms are shown in Fig. 4.

The structural characterization details of 4-ipomeanol using NMR and IR are as follows:

\[ ^1H-NMR \text{ (CD}_3\text{OD, 500MHz)} \delta \text{ (ppm) } = 1.23 \text{ (d, 3H, CH}_3), \ 1.81 \text{ (m, 2H, CH}_2), \ 2.90 \text{ (t, 2H, CH}_2), \ 3.86 \text{ (m, 1H, CH), } 6.78 \text{ (dd, 1H, aromatic CH), } 7.44 \text{ (t, 1H, aromatic CH), } 8.07 \text{ (m, 1H, aromatic CH).} \]

FT-IR \ ν cm\(^{-1}\) = 3543(OH), 1636(C=O), 1441 & 1375(C=C), 1038(C-O-C), 919-500(C-H). The spectral analysis confirmed that the isolated molecule is 4-ipomeanol and the molecular formula of the compound is C\(_{9}\)H\(_{13}\)O\(_{3}\).

Though there are previous reports on the cytotoxicity of 4-ipomeanol, we report here the cytotoxicity of 4-ipomeanol produced from cell culture on renal carcinoma cell lines (ACHN). It was found that the compound was cytotoxic to cell lines with an IC\(_{50}\) of 4 µM (Fig. 5).

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**Table 1**—Effect of elicitors on the production of 4-Ipomeanol added at different days after subculture

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Concentration of 4-Ipomeanol [mg/g] produced at different days of elicitor addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chitin</td>
</tr>
<tr>
<td></td>
<td>0(^{th}) day</td>
</tr>
<tr>
<td>1</td>
<td>0.34(^{a})</td>
</tr>
<tr>
<td>±0.11</td>
<td>±0.21</td>
</tr>
<tr>
<td>2</td>
<td>0.30(^{b})</td>
</tr>
<tr>
<td>±0.21</td>
<td>±0.12</td>
</tr>
<tr>
<td>4</td>
<td>0.24(^{b})</td>
</tr>
<tr>
<td>±0.08</td>
<td>±0.26</td>
</tr>
<tr>
<td>6</td>
<td>0.19(^{c})</td>
</tr>
<tr>
<td>±0.17</td>
<td>±0.31</td>
</tr>
<tr>
<td>8</td>
<td>0.14(^{c})</td>
</tr>
<tr>
<td>±0.21</td>
<td>±0.27</td>
</tr>
<tr>
<td>10</td>
<td>0.02(^{d})</td>
</tr>
<tr>
<td>±0.01</td>
<td>±0.13</td>
</tr>
</tbody>
</table>

[Dilution: F. solani: 1 indicates 1 mL of spore suspension (1.9e5 spores/mL) diluted to 10 mL; 2 indicates 1 mL of spore suspension diluted to 20 mL and so on. Chitin and Chitosan: 1 indicates a concentration of 1 mg L\(^{-1}\); 2 indicates a concentration of 2 mg L\(^{-1}\) and so on. Values are mean ± SE from 10 replicates per treatment. Difference between the means was calculated using one way ANOVA followed by DMRT at 5% significance level]
Production of secondary metabolites through plant tissue culture is found to be an attractive alternative approach to traditional methods of production as it offers controlled supply of the biochemical independent of plant availability. In our previous report, we had described the protocols for the production of 4-ipomeanol from the root tubers and root tuber derived rhizogenic callus of sweet potato. Compared to callus cultures, suspension cultures allow recovery of large quantities of cells from which the desired product can be easily isolated. 4-Ipomeonal, which is a furanoterpenoid produced in response to stress or infection in whole plants can be simulated in suspension cultures of plant tissues using elicitors. In the present study, maximum production of 4-ipomeonal was obtained when the cells were grown in the presence of the biotic elicitor, chitin. Chitin as an elicitor has proved to be beneficial in the production of various secondary metabolites from undifferentiated cells. Our results have shown that simulation of 4-ipomeonal in sweet potato suspension cultures using chitin is concentration dependent with optimum concentration at 4 mg L⁻¹. An increased concentration of elicitor did not enhance the 4-ipomeonal production. This could be attributed to the fact that over-dosage of elicitor may fail to increase or allow product accumulation since dose-response curves may have sharp optima.

In the present investigation, maximum production of 4-ipomeonal was observed when the cells were harvested at the stationary phase of growth. Earlier studies revealed that high levels of secondary metabolites were obtained from suspension culture of Sweet potato at the stationary phase. During the present investigation, we had made an attempt to optimize the day of addition of the elicitors. It was found that addition of elicitor at the 4th day after inoculation yielded maximum amount of 4-ipomeonal. This observation is supported by previous reports that addition of precursors at the log phase of growth enhances secondary metabolite yield.

In the present study, acetonitrile: water (40:60) was found to be the best solvent system for analysis of 4-ipomeonal in reverse phase HPLC and the retention time was found to be 9.4 min. It has been reported previously that ¹⁸F labeled 4-ipomeonal was analyzed in normal phase HPLC with the help of ethyl acetate: hexane (35:65). Another furanoterpenoid, ipomeamarone was eluted in reverse phase HPLC using methanol: water (70:30) as mobile phase.

Cytotoxicity of 4-ipomeonal has been documented in lung, liver, breast and brain tumor cell lines. Several studies have reported cytotoxicity of various phytochemicals from plants. Though, the extracts of *Cynara cardunculus*, *Laurus nobilis*, *Sideritis perfoliata* also exhibited cytotoxicity to ACHN cell lines with an IC₅₀ of 18 µg/mL, 78.24 µg/mL, 98.58 µg/mL, the results of our experiments indicate that 4-ipomeonal (IC₅₀ value 4 µM) from sweet potato is more efficient in destroying renal cell carcinoma. The results of the present study is encouraging because 4-ipomeonal produced from cell suspension cultures of *I. batatas* has shown significant inhibition of renal cell carcinoma cells in vitro. Further studies with in vivo trials are warranted.

**Conclusion**

The present study provides a valuable and economically viable protocol for the production and screening of 4-ipomeonal from suspension cultures of *Ipomoea batatas*.

**Acknowledgement**

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