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Effect of Aeration on Production of Anticancer Lignans by Cell Suspension Cultures of *Linum album*

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Abstract The effects of aeration within the range of 0.2–0.5 vvm on transformed and high yielding cell cultures of *Linum album* were investigated in a 5-L stirred tank bioreactor equipped with low shear Setric impeller. The kinetics of cell growth, substrate utilization, and production of lignans, namely, podophyllotoxin and 6-methoxypodophyllotoxin, were established. Maximum biomass of 23.2 g/L and lignan accumulation levels of 176.3 mg/L podophyllotoxin and 10.86 mg/L 6-methoxypodophyllotoxin were obtained with initial air flow rate of 0.3 vvm. Specified oxygen demand of cells was estimated to be 1.35 g O_2/g biomass. The optimum oxygen transfer coefficient was found to be 16.7 h^{-1}, which corresponded to aeration rate of 0.3 vvm. The effect of minimum dissolved oxygen (DO) concentration was investigated with respect to biomass and lignan production by comparing identically aerated and agitated bioreactor cultivations at dissolved oxygen concentrations of 10%, 30%, and 50%. Cell growth and podophyllotoxin accumulation were not affected significantly at these DO levels, but 6-methoxypodophyllotoxin production was enhanced when cells were cultivated at 30% DO level. The maximum volumetric productivities of 18.2 mg/L day and 3.2 mg/L day for podophyllotoxin and 6-methoxypodophyllotoxin, respectively, were obtained. These results establish the key role of oxygen on mass scale production of anticancer lignans by cell cultures of *L. album*. It may serve as a suitable parameter for scale-up.

Keywords Aeration · Dissolved oxygen · *Linum album* · 6-Methoxypodophyllotoxin · Podophyllotoxin · Stirred tank bioreactor

Introduction

Plants are known to produce an impressive spectrum of products like pharmaceuticals, flavors, food, cosmetics, and agrochemicals. Plant cell technology has now become a favored tool for further exploitation of plant kingdom for production of commercially

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important chemicals [1]. Despite having potential, commercial applications of large-scale cultivation of plant cells in bioreactors has been successful to a very limited extent for the production of shikonin, berberine, rosmarinic acid, and taxol. Limitation of fundamental understanding of plant physiology and technical problems related to scale-up have impeded development of plant cell culture-based bioprocesses at industrial level.

Podophyllotoxin (PT), a natural lignan present in *Podophyllum* species, is the starting compound for synthesis of anticancer pharmaceuticals such as etoposide, etopophos, and teniposide. Development of alternate methodologies for production of PT by biotechnological means attracted attention of several researchers because of long juvenile phase, pre-harvest period of 5–7 years and endangered status of its natural source, complicated chemical synthesis and very low productivity in plant cell cultures [2–4]. Cell cultures of *Linum album* can provide a suitable system for mass production of these lignans with maximum productivity in comparison to other cell cultures capable of producing podophyllotoxins [5]. In addition, *L. album* is also known to produce another lignan, 6-methoxypodophyllotoxin (6-MPT) with comparable cytotoxicity. To avoid limitations associated with mass production of lignans by native *L. album* like slow growth and low yield, a fast growing transformed cell line of *L. album* with high yield was used in the present study.

Gas transfer phenomenon and hydrodynamic shear sensitivity appear to be the most critical parameters to scale up plant cell cultures. Moreover, very few studies have been made to investigate the effect of aeration on kinetics of plant cell cultivation in bioreactors and to use the same for scale-up to maximize production of desired compounds. It is therefore desirable to know the adequate oxygen requirement of the cells because suboptimal gas transfer may reduce culture performance caused by oxygen limitations and higher aeration rate may have deleterious effect on growth and production in cell cultures. Hence, the objective of present study was to establish the growth and production kinetics of *L. album* at different dissolved oxygen levels in bioreactor to establish its suitability for scale-up of podophyllotoxin and 6-methoxypodophyllotoxin production.

Materials and Methods

Plant Material

Seeds of *L. album* were washed in 1% Savlon, surface sterilized using 70% (*v*/*v*) ethanol treatment for 1 min, and rinsed thrice with sterile double distilled water (SDDW). This was followed by treatment with 0.01% (*w*/*v*) mercuric chloride for 2 min and rinsing with SDDW for four to five times. For germination, the sterilized seeds were placed on Murashige and Skoog medium [6] with 30 g/L sucrose and solidified with 10 g/L agar at 25±1°C in 16/8 h light/dark cycle with a light intensity of 1,200 lx.

Initiation of Cultures

High-yielding cell cultures of *L. album* were initiated by transformation of stem portions of *in vitro* germinated plants with *Agrobacterium rhizogenes* strain NCIM 5140. This strain of *Agrobacterium* was procured from National Collection of Industrial Organisms, Pune, India.

Explants were cultured on sterilized Petri plates comprising solid MS medium supplemented with 30 g/L sucrose and solidified with 10 g/L agar (HiMedia, Mumbai, India). The pH was adjusted to 5.8±0.02. The medium was autoclaved under 15 psig pressure at 121 °C for 15 min. The explants were cocultivated with *Agrobacterium* strain for infection

to induce transformed callus. For this purpose, bacterial colonies were cultured on solid yeast mannitol broth (YMB) at 25±1°C for 2 d. Ten loopful bacteria were then allowed to grow in YMB for 24 h at 25±1 °C. Inoculum (2% *v/v*) of this culture was reinoculated in YMB and grown till an $O.D._{600}$ of one was achieved. The suspension was centrifuged at 6,000×*g* for 10 min. The supernatant was discarded and the pellet was resuspended in 5 mL of the fresh YMB. This concentrated culture was used for infection of plant materials.

The explants were kept in disposable sterile Petri plates, pricked manually with 24-gauge metal needle (~5 wounds/cm^2), dipped in *A. rhizogenes* culture and incubated or 5 min. YMB without bacteria applied to the explants served as a control. The infected explants were incubated for cocultivation at 25±2°C for 48 h on MS media at 25±2°C under 16 h/8 h light/dark regime. The transformed cultures were then transferred to fresh MS medium containing 1 g/L cefotaxime to check the overgrowth of bacteria. Axenic cultures were obtained by subsequent subculture to fresh MS medium containing the antibiotic every 7 d. The transformed cultures were checked for *Agrobacterium* contamination by culturing samples on YMB after every subculture.

Cell suspension cultures were initiated by transferring friable fraction of transformed callus equivalent to 5 g/L dry cell weight (DCW) into 250-mL Erlenmeyer flask containing 50 mL of modified B5 liquid medium [7] supplemented with 45 g/L sucrose, 2.1 mM ammonium, and 3.37 mM calcium choride [8]. The cultures were incubated on a gyratory shaker at 125 rpm and 25±1 °C under 16 h/8 h light/dark regime. Cells (12 days old) were used as inoculum for further experiments at bioreactor level.

Batch Cultivations in Bioreactor

Effect of Aeration

Batch cultivations were carried out in a 5-L stirred tank bioreactor with specially designed low shear Setric impeller (Applicon Dependable Instruments, The Netherlands). *L. album* cells were cultivated in modified B5 medium with 5 g/L inoculum at 125 rpm in a working volume of 3.5 L. The pH of the medium was adjusted to 5.7 before autoclaving and automatically controlled at this value throughout cultivation period by addition of 1 M aqueous NaOH/1 M HCl by biocontroller AD1030 (Applicon Dependable Instruments, The Netherlands). Temperature was controlled at 27±1 °C by circulating chilled water from water circulator into the outer jacket of the bioreactor. The reactor was aerated through a sintered stainless-steel sparger. The dissolved oxygen (DO) concentration was measured using a polarographic oxygen probe (Applicon Dependable Instruments, The Netherlands). The cultivations were done under photoperiod of 16 h/8 h light/dark cycle. Growth and product formation kinetics were studied at different aeration rates (0.2, 0.3, and 0.5 vvm) separately. Samples (25 mL) were collected at a regular interval of 2 days and analyzed for dry cell weight, lignan contents (podophyllotoxin and 6-methoxypodophyllotoxin), and residual sucrose concentration.

Effect of Minimum DO Concentration

Batch cultivations in 5 L stirred tank bioreactor were carried out in similar fashion as described above to establish growth and production kinetics. The experiments were carried out at aeration rate of 0.3 vvm and when DO concentrations had reached 10%, 30%, and 50%, respectively, in separate experiments, DO levels were maintained above these values by increasing the aeration rate manually.

Analysis

Measurement of Dry Cell Weight and Residual Sugar

Samples (25 mL) were aseptically collected at a regular interval of 2 days. To determine dry cell weight, the cells were collected by centrifugation at 5,000×*g* for 15 min, blotted on a filter paper, and dried at 25±1°C until constant weight was achieved. The residual carbon (sucrose) was estimated in spent medium by phenol-sulfuric acid method [9].

Viability Assay

Percentage viability of the plant cells was determined by colorimetric estimation of red color formazan product, produced by living cells using tri-phenyl tetrazolium chloride [10].

Extraction and Quantitative Analysis of Lignans

For extraction of lignans, the dried and powdered cells (100 mg) were sonicated with methanol for 15 min at 4–6 °C. The cells were then soaked in methanol for 24 h at 4–6 °C. The supernatant was removed after centrifugation (5,000×*g*, 10 min) and evaporated to dryness. The extract was redissolved in high-performance liquid chromatography (HPLC) grade methanol, filtered through a 0.22-μm filter and then subjected to analysis by HPLC. Quantitative estimation was done by using Nova Pak RP-C_{18} column (Waters, USA) (250×4.6 mm) in Agilent 1100 series (Agilent Technologies, USA) HPLC system equipped with quaternary pump (G1311A), diode array detector (G1315B), and a temperature controller (G1316A). The separation was carried out by elution with mobile phase (0.01% phosphoric acid in water: acetonitrile (72:28 *v*/*v*) at 0.8 mL/min flow rate. Column temperature was maintained at 30 °C. Lignans (PT and 6-MPT) were detected at 290 nm.

Estimation of Total Oxygen Demand of L. album Cells

Total oxygen demand for complete combustion of biomass was determined by titrimetric analysis of unconsumed dichromate ions with ferrous ions using ferroin as indicator. The method was adopted from Standard Methods for the Examination of Waste and Wastewater [11].

Determination of Volumetric Oxygen Transfer Coefficients (k_La) at Different Aeration Rates

The volumetric oxygen transfer coefficients were determined in the above-described medium as a function of the aeration rates (0.2, 0.3, and 0.5 vvm) at constant rotational speed (125 rpm) as described by Wang and Zhong [12].

Results and Discussion

Effect of Aeration

The effect of different aeration rates on growth and lignan production by *L. album* cells is summarized in Table 1.

Table 1 Effect of different aeration rates on growth and lignan production[a].

S. No.	Parameter	Batch with 0.2 vvm	Batch with 0.3 vvm	Batch with 0.5 vvm
1	DCW (g/L)	14.80 (8)	23.20 (10)	22.80 (10)
2	PT (mg/L)	96.91 (10)	176.32 (10)	166.21 (10)
3	6-MPT (mg/L)	9.92 (8)	8.60 (8)	22.02 (8)
4	Vol. productivity (mg/L day) - PT	9.69 (10)	17.63 (10)	16.62 (10)
5	Vol. productivity (mg/L day) - 6-MPT	1.24 (8)	1.08 (8)	2.75 (8)
6	Cell productivity (g/L day)	1.85 (8)	2.32 (10)	2.28 (10)
7	Specific growth rate (d^{-1})	0.15	0.17	0.17
8	Growth yield coefficient $Y_{X/S}$ (g/g)	0.44	0.54	0.54

[a]The values in parentheses indicate the day at which the maximum value of the parameters was obtained

Almost similar growth kinetics were observed when cells were cultivated at 0.3 and 0.5 vvm with cell productivity of 2.32 and 2.28 g/L day. Maximum PT production levels achieved on the 10th day were 176.32 and 166.21 mg/L respectively. However, at 0.2 vvm, growth of *L. album* cells was limited to 14.8 g/L on the 8th day of cultivation. Total PT production was also comparatively lower with air flow rate of 0.2 vvm (96.91 mg/L) probably because of lesser production of biomass during cultivation. This decrease in biomass and lignan productivity might be caused by oxygen limitation in culture. Comparative growth and lignan production profiles of batch cultivations of *L. album* cells at different initial air flow rates are given in Figs. 1 and 2. 6-MPT accumulation was the highest at aeration rate of 0.5 vvm (22.02 mg/L) among the tested airflow rates. Lower biomass and product accumulation with low oxygen supply have been reported earlier [13–22].

Specific growth rate was also slightly lower in cultivation with 0.2 vvm (0.15 d^{-1}) in comparison to cultivation at other air flow rates (0.17 d^{-1}). Generally growth rate of plant cells increases with increase in oxygen supply until it reaches a maximum [15–18]. No further increase in growth during batch cultivation at 0.5 vvm was mainly caused by higher

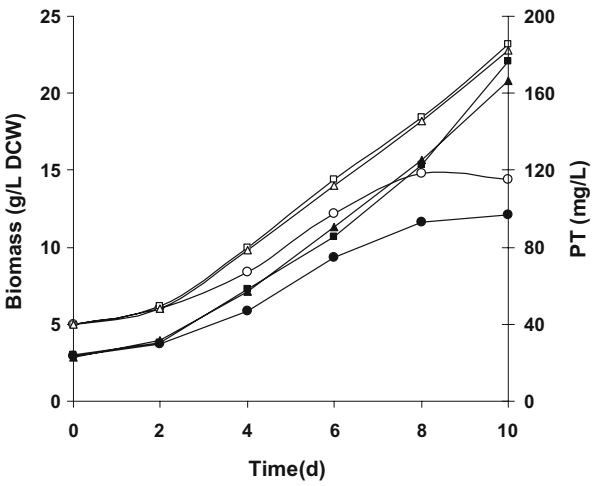

Fig. 1 Effect of aeration rate on *L. album* cell growth and PT production in stirred tank bioreactor (average values are given; open circle, open square, and open triangle: cell growth pattern at 0.2 vvm, 0.3 vvm, and 0.5 vvm, respectively; filled circle, filled square, and filled triangle: PT production profiles at 0.2 vvm, 0.3 vvm, and 0.5 vvm, respectively)

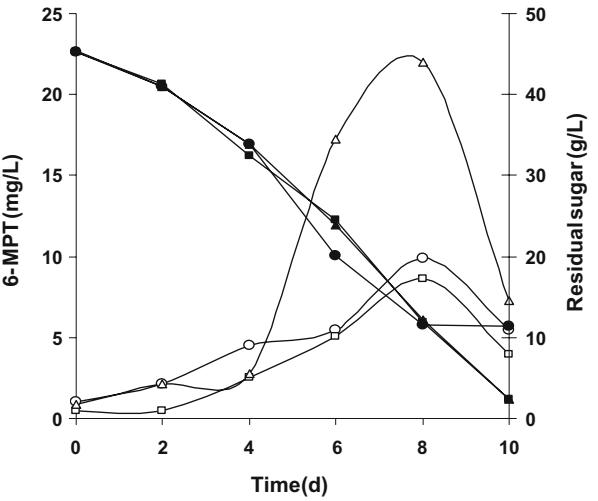

Fig. 2 Effect of aeration rate on 6-MPT production and sugar consumption pattern by *L. album* cells in stirred tank bioreactor (average values are given; open circle, open square, and open triangle: 6-MPT production profiles at 0.2 vvm, 0.3 vvm, and 0.5 vvm, respectively; filled circle, filled square, and filled triangle: sugar consumption profiles at 0.2 vvm, 0.3 vvm, and 0.5 vvm, respectively)

hydrodynamic stress as reflected by slightly lower cell viability (87.8%) in comparison to batch cultivation at 0.3 vvm (92.8%). Similar phenomenon of decreased cell growth at higher air flow rates has been reported [13]. The growth yield (g cell mass generated/g sugar consumed) was also lowered by oxygen deprivation at 0.2 vvm to 0.44 g/g in comparison to 0.54 g/g, when cells were supplied air at a flow rate of 0.3 vvm. It means that carbon flux toward biomass was altered by aeration rate. A similar phenomenon has also been reported for cultures of *Catharanthus roseus* [17] and *Panax notoginseng* [21]. Higher air flow rate did not affect the growth yield. Substrate consumption profiles in these experiments are given as Fig. 2. Utilization of substrates also got affected greatly at low air flow rate, i.e., 0.2 vvm, as about 26% of sucrose remained unconsumed when the maximum dry weight and lignan production was achieved on 8th day of cultivation. In contrast, better and almost complete utilization of sucrose was observed when cells were cultivated at higher aeration rates. These observations also suggested that under oxygen limited conditions, *L. album* cells did not utilize carbon source efficiently. Almost similar substrate consumption profiles were observed at 0.3 and 0.5 vvm, suggesting that cultivation at air flow rate of 0.3 vvm was adequate for cultivation of *L. album* cells under above culture conditions. Cultivation at 0.7 vvm resulted in slight decrease in cell growth and lignan accumulation (data not shown). Problem of cell deposition on the bioreactor wall was observed in later phase of the cultivation at higher aeration rate. Aeration rate of 0.5 vvm also resulted in substantial deposition of cells around drive shaft, probes and on wall area above the liquid during late exponential phase. This accumulation was reduced by maintaining 0.3 vvm aeration rate at 125 rpm. Similar phenomenon with respect to oxygen dependence was also observed during cultivation of sandalwood cells in bioreactor with cell-lift impeller [23].

Estimation of Total Oxygen Requirement and Optimum Air Flow Rate

Oxygen transfer coefficients $k_L a$ (h^{-1}) were calculated for different air flow rates. The values were found out to be 6.0, 17.5, and 24.0 h^{-1} for air flow rates of 0.2, 0.3, and 0.5 vvm, respectively. As 23.2 g/L biomass was produced in 10 days during batch

cultivation of *L. album* cells in a bioreactor, overall maximum productivity achieved was found to be 0.096 g DCW/L h. Total oxygen demand for complete combustion of biomass was determined to be 1.35 g O_2/ g biomass. Hence total oxygen supply of 0.13 g/L h was required to produce desired biomass. To supply this amount of oxygen, the minimum k_La was found out to be 16.70 h^{-1}. Therefore, optimum airflow rate required was equal to 0.297 vvm as calculated from relationship between volumetric oxygen transfer coefficient and air flow rate. This also further proved that lower biomass and lignan productivity during cultivation at 0.2 vvm aeration rate was because of limitation of oxygen.

Effect of Minimum DO Concentration

The effect of minimum DO concentration on lignan productivity at initial air flow rate of 0.3 vvm was then studied. Results are summarized in Table 2.

The growth and production profiles of batch cultivations of *L. album* cells at different dissolved oxygen concentrations (10%, 30%, and 50%) were established. No significant effect of minimum DO concentration on biomass and PT accumulation was observed. This might be because of the fact that even at 10% level, oxygen was available in plenty than the requirement of plant cells. Apparently, there was sufficient energy for carbohydrate uptake and metabolism even at low DO concentration. The highest biomass of 24 g/L on a dry cell weight basis and PT productivity of 18.36 mg/L day were obtained at 50% DO level, which was almost similar to that obtained with 30% DO level (23.8 g/L and 18.19 mg/L day, respectively). Almost similar biomass (23.2 g/L) and PT (17.63 mg/L day) were obtained at minimum DO concentration of 10%. It had been reported by several authors that a low DO concentration with a minimum of 10% had no effect on biomass yield [14, 17, 24, 25]. Substrate consumption patterns were also found almost similar at different DO levels as approx. 94% sugar was utilized during cultivation. In contrast to dry weight and PT, accumulation profile of 6-MPT was greatly affected by minimum DO levels. Oxygen seemed to affect conversion of deoxy-PT to 6-MPT significantly as maximum 6-MPT accumulation of 31.51 mg/L was observed at 30% minimum DO level than at other DO concentrations. At minimum DO concentrations of 10% and 50%, lower 6-MPT production of 8.60 and 7.20 mg/L, respectively, were obtained. This alteration in 6-MPT production might be caused by involvement of oxygen in the biosynthesis of 6-MPT in biosynthetic pathway. Deoxy-PT is the precursor for the biosynthesis of PT and 6-MPT. Hydroxylation can occur either at position 7 by deoxy-PT-7-hydroxylase (DOP7H) to give PT or at position 6 to β-peltatin by the cytochrome P450 dependent deoxy-PT-6-hydroxylase (DOP6H). β-Peltatin can be methylated to β-peltatin methyl ether (PAM) by β-peltatin-

Table 2 Effect of minimum DO levels on growth and lignan production[a].

S. No.	Parameter	Batch with 10% DO	Batch with 30% DO	Batch with 50% DO
1	DCW (g/L)	23.20 (10)	23.80 (10)	24.00 (10)
2	PT (mg/L)	176.32 (10)	181.97 (10)	183.60 (10)
3	6-MPT (mg/L)	8.60 (8)	31.51 (8)	7.20 (8)
4	Vol. productivity (mg/L day) - PT	17.63 (10)	18.19 (10)	18.36 (10)
5	Vol. productivity (mg/L day) - 6-MPT	1.08 (8)	3.93 (8)	0.90 (8)
6	Cell productivity (g/L day)	1.85 (8)	2.32 (10)	2.28 (10)

[a]The values in parentheses indicate the day at which the maximum value of the parameters was obtained

6-*O*-methyltransferase (βP6OMT) from which 6-MPT is formed by hydroxylation at position 7 by PAM7H [26]. The enzyme DOP6H, being cytochrome P450 dependent, requires NADPH and molecular oxygen. The low amount of oxygen during cultivation at 0.3 vvm air flow rate with a minimum DO concentration of 10% was probably not sufficient to induce the enzyme and was used primarily for cell growth and maintenance than for enzymes involved in biosynthesis. However, sufficient supply of oxygen at 30% DO level might be the reason for induction of the enzyme and consequent enhanced production of 6-MPT. Similar effect of DO was observed in *C. roseus* cultures for production of ajmalicine as its content was low at 15% DO level because of limitation of molecular oxygen as a substrate to enzymes involved in terpenoid biosynthetic pathway. However, increased ajmalicine accumulation was observed at 85% DO level [20].

These results provide new dimension to mass production of PT and related lignans by plant cell cultures and need more detailed study. A change in product pattern during batch cultivation due to oxygen was also reported earlier [18]. Schlatman et al. [20] had also reported that ajmalicine accumulation was enhanced during batch cultivation of *C. roseus* cells in high DO conditions. Production of berberine-type alkaloids by *Berberis wilsonae* was also enhanced when the DO concentration was increased from 20% to 50%, while growth was not affected [15]. Spieler et al. [24] also reported a positive correlation between DO concentration and biotransformation with *Digitalis lanata*. No further increase was observed over 50% DO concentration. Moderate oxygen level equivalent to 30% were also reported to be optimum for phenolics accumulation in sandalwood cell suspension cultures in a cell-lift bioreactor [23], high density cultivation of *Anchusa officinalis* in stirred tank bioreactor [27], for cell growth and L-DOPA (3,4-dihydroxyphenylalanine) production in cell cultures of *Stizolobium hassjoo* [28] and for production of rosamarinic acid by *Lavandully vera* MM cell line [29]. Combined growth-associated and nongrowth-associated kinetics for lignan production was observed in the present investigations. A similar phenomenon was observed in lignan accumulation in *P. hexandrum* [30] and *L. album* [31] cell suspension cultures. Moreover, the podophyllotoxin accumulation achieved in present investigation was almost four times higher than those reported earlier [31]. This significant enhancement was caused by selection of high-yielding cell line and its cultivation in optimized medium and environmental conditions.

Conclusions

The aeration rate and DO concentration had indisputable effect on cell growth and lignan productivity by *L. album* cells in 5-L stirred tank bioreactor. Cell growth and lignan productivity were affected at 0.2 vvm because of oxygen limitation. Aeration rate of 0.3 vvm was found to be optimum for biomass accumulation and lignan production. Significant increase in production of 6-MPT was observed at minimum DO concentration of 30% Maximum volumetric productivities of 18.2 and 3.15 mg/L day were obtained for PT and 6-MPT production, respectively, which were about four times higher than those reported earlier. These studies will be considered useful in developing an integrated bioprocess for mass production of anticancer lignans in bioreactors from cell cultures of *L. album*.

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