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Necessity of a Two-Stage Process for the Production of Azadirachtin-Related Limonoids in Suspension Cultures of *Azadirachta indica*

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The effect of major nutrients on growth and azadirachtin-related limonoids (AZRL) production in plant cell culture of *Azadirachta indica* (neem) was studied with the objective to increase the yield of AZRL, one of the major group of pesticidal compounds found in intact neem trees. We report the novel online monitoring of plant cell respiration activities in a new parallel shake flask measuring device. Results obtained using three standard plant cell culture media showed non-growth-associated production characteristics for AZRL. These findings were supported by the oxygen uptake rate data. Further investigations on AZRL production in a modified MS medium with different concentrations of nitrogen and phosphorus sources resulted in 0.25 mg·g^{-1} dry weight of AZRL, compared to no detectable AZRL production in standard MS media. These characteristics suggest the necessity of a two-stage process for the production of AZRL in plant cell culture. Compared to the single-stage process, an almost twofold increase in the volumetric productivity of AZRL was achieved using the two-stage process.

[**Key words:** *Azadirachta indica*, plant cell suspension culture, respiration activity, oxygen transfer rate, specific power consumption, limonoid, azadirachtin]

There are approximately 250,000–500,000 higher plant species on the earth. In these plants about 30,000 different substances have been identified, and an increasing number of investigations are carried out to detect novel bioactive phytochemicals with antimicrobial, antibiotic, insecticidal, molluscidal, hormonal or pharmaceutical relevance. Production of these phytochemicals in plant tissue culture is under intense research because it promises to improve the availability of substances such as taxol, or to save endangered plant species, such as *Podophyllum hexandrum* (1–3).

Azadirachta indica (*A. juss*) (Meliaceae), commonly known as neem, has been well known since ancient times as a source of biopesticide against a variety of insects. Limonoids or tetranortriterpenoids in neem are responsible for these properties. Among these compounds, azadirachtin, which has not been synthesised chemically to date, has the most active insecticidal properties. Its effects are more intense if it is used in a mixture with other limonoids, like nimbin, nimbidin, salanin, etc. (4, 5).

Due to increasing interest in azadirachtin and its complex structure (6), research is going on for *in vitro* production using plant tissue culture techniques. The presence of azadirachtin in callus cultures of leaf explant (7, 8), from leaf and flower explants (9) has been reported. Jarvis *et al.* (10) could successfully quantify the concentration of azadirachtin by NMR and mass spectra in tissue cultured *A. indica* seeds. Different azadirachtin concentrations were observed in different media in callus cultures from Nicaraguan cell lines (11). However, the research work carried out has mainly dealt with basic culture techniques of callus induction and culture maintenance. Only a few reports (7, 8) are available on azadirachtin-related limonoids (AZRL) production in suspension cultures. Kuruvilla *et al.* (8) studied the effect of permeabilizing agents on azadirachtin secretion in suspension culture. In all previous studies the suspension cultures were initiated from callus cultures, which were already producing AZRL. Callus as well as suspension cultures, which do not produce AZRL, could be under catabolite repression, and under favourable conditions they may produce AZRL. Thus, not much is known about the behavior of cells in suspension nor about their ability to produce azadirachtin and AZRL in suspension culture. As a benchmark, the yield of azadirachtin from intact plant (seed kernels) has been reported to be in the range of 2 to 6 mg·g^{-1} dry weight (DW) (12).

In this study, the effect of major nutrients such as nitrogen and phosphorus on growth and production kinetics was studied in suspension cultures in shake flasks. However, for scale-up, power consumption is more evenly distributed in

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shaking bioreactors than in stirred tanks, resulting in lower levels of hydro-mechanical stress (13, 14). For the first time, we performed direct online measurement of the respiration activity (oxygen transfer rate, carbon dioxide transfer rate, respiration quotient) of plant cells in suspension culture (on shake flask level) in eight parallel shaking flasks, using a special device, the respiration activity monitoring system (RAMOS, HiTec Zang, Herzogenrath, Germany) (15). Oxygen transfer rate (OTR) curves can be used to study the growth behavior of microbial cultures as well as plant cell cultures at different conditions and hence to optimise the culture processes (15–18; Losen *et al.*, submitted). Effects of variations in media components, temperature and shaking frequency etc. can be monitored online. Valuable information regarding factors affecting the growth characteristics and kinetics of microbial cultures can be obtained with the help of this device.

MATERIALS AND METHODS

Cell culture Callus cultures from the shoots of *A. indica* were obtained from the Department of Botany, Delhi University, India. The *A. indica* calli were subcultured every 4 weeks using Murashige and Skoog (MS) basal medium (19) with 0.8% agar and supplemented with growth hormones: kinetin (kn) (0.2 mg·l^{-1}), 2,4-dichloro-phenoxyacetic acid (2,4-D) (0.2 mg·l^{-1}). The pH of the medium was adjusted to 5.8 before sterilisation. Between 50 and 75 ml of the agar medium was used for subculturing in 250-ml conical flasks. The cell lines were maintained at 25°C, with 16–8 h light-dark condition, respectively.

Suspension culture Callus material from the solidified media was used for the initiation of suspension cultures. Fifty ml of the MS media supplemented with concentrations of growth regulators, kn and 2,4-D at 1.5 and 0.91 mg·l^{-1}, respectively was used as subculturing medium in 250-ml normal shake flasks. Cells were collected from cultures grown on solidified agar medium to keep 3 to 4 g·l^{-1} (DW) cell density in each flask. The cultures were grown in a shaking incubator (Kühner AG, Basel, Switzerland) at 150 rpm with shaking diameter of 50 mm, in 16–8 h light-dark conditions. Subculturing was done every 8 d. To study the effect of different media on growth characteristics of *A. indica* callus, cells from standard subculturing medium were centrifuged, washed with sterile water and cultivated in MS (19), Gamborg's B5 (20) and White's media (21). All experiments were performed in triplicate. The data presented are the mean values of triplicate determination with SE less than ±15%.

Effect of nitrogen and phosphorus source Response surface methodology (RSM) was used to optimise the concentrations of nitrogen source (NH_4^+:NO_3^-, 1:2) and phosphorus source (PO_4^{-3}). Four experiments were designed using different concentrations of these two vital nutrients. All the other constituents of the MS media were kept constant. The combinations of the nitrogen and phosphorus sources used in the media were: (i) nitrogen source, 0 mM; phosphorus source, 0 mM; (ii) nitrogen source, 120 mM; phosphorus source, 0 mM; (iii) nitrogen source, 0 mM; phosphorus source, 3 mM; (iv) nitrogen source, 120 mM; phosphorus source, 3 mM.

The cultures were grown in a shaking incubator (Kühner AG) at 150 rpm with the shaking diameter of 50 mm in 16–8 h light-dark conditions. Five hundred-ml shake flasks with 100 ml working volume were used for the cultivation. The experiments were performed in triplicate. Cell mass and AZRL content were analysed after 12 d of cultivation.

Two-stage process The 500-ml shake flasks with 100 ml working volume were used for the two-stage process. The growth medium (GM) and production medium (PM) used for the two-stage process were modified MS medium optimised with respect to nitrogen and phosphorus. The GM comprised of 60 mM nitrogen source (NH_4^+:NO_3^-, 1:2), 1.25 mM phosphorus source (PO_4^{-3}), 30 g·l^{-1} sucrose, 1.5 mg·l^{-1} kn, 0.9 mg·l^{-1} 2,4-D. All the other constituents of this modified MS medium were the same as that of normal MS medium. The composition of the PM was 7.7 mM nitrogen source (NH_4^+:NO_3^-, 1:2), 0.06 mM phosphorus source (PO_4^{-3}), 30 g·l^{-1} sucrose, 1.5 mg·l^{-1} kn, 0.9 mg·l^{-1} 2,4-D. All the other constituents of this modified MS medium were the same as that of normal MS medium.

Initially, the cultures were grown in the GM medium optimised for cell growth at 150 rpm in the shaking incubator (Saveer Biotech, India) with shaking diameter of 50 mm, in 16–8 h light-dark conditions. Samples were taken every 2 d and analysed for cell mass, carbon source and AZRL content. When the carbon source was depleted (8th day) the cells were centrifuged, washed with sterile water and transferred into the PM medium. A single-stage process with PM was run in parallel with the two-stage process.

Chemicals All the chemicals used were of analytical grade (Roth, Karlsruhe, Germany) except 2,4-D, kn, benzylaminopurine (BAP), indole-3-acetic acid (IAA), nepthaleneacetic acid (NAA), azadirachtin standard, Gamborg's B5 media and white's media (Sigma, Taufkirchen, Germany). MS media was purchased from Duchefa (Haarlem, The Netherlands).

Respiration activity Respiration activity of cells in suspension (oxygen transfer rate, carbon dioxide transfer rate, respiration quotient) was monitored online with the respiration activity monitoring system (RAMOS) (15). In this device eight modified shake flasks are mounted on a shaking incubator tray and connected to a forced head space aeration at defined flow rates. Flow rate of air is adjusted such that the head space gas composition remains the same as that of normal shake flasks. Every flask is equipped with a head space oxygen sensor. At set time intervals the inlet and outlet valves of the individual flasks are closed for a specified period of time. During this measuring phase the decrease in oxygen partial pressure is measured and used to calculate the oxygen transfer rate. After the measuring phase, the valves are opened again. Special flasks designed for this device have the same lower part as normal shake flasks, to maintain the same hydrodynamic conditions as in the culture broth. A measuring phase of 10 min and a rinsing phase of 20 min were used for the *A. indica* suspension cultures. Normal shake flasks and modified measuring shake flasks for the device were inoculated simultaneously at the same inoculum concentration. Seven normal shake flasks for each medium experiment were used in parallel. The flasks used in the online measuring device were not disturbed after starting the experiment to collect samples. For sample analysis, normal shake flasks were occasionally harvested and were not replaced on the shaking incubator.

Determination of extracellular sugar concentration The cell suspension was centrifuged at 3000 rpm for 20 min. Residual sucrose, glucose and fructose concentrations in the supernatant were determined using an HPLC system (Dionex, Idstein, Germany) with Chromeleon Software, 232 XL Sampling Injector (Abimed/Gilson, Langenfeld, Germany), UVD 170S (Dionex), Shodex RI71 (Dionex), P 580 Pump (Dionex), 1 mM sulfuric acid, flow 0.6 ml/min, organic acid resin column (RP8, CC125/4 sperisorb 50-5 C8; CS-Chromatographie, Langerwehe, Germany).

Determination of phosphate concentration Phosphate concentration in the supernatant was estimated using a colorimetric assay based on the formation of a blue color complex with molybdate ions. Molybdate reagent was prepared by mixing 2.6 g $(NH_4)Mo_7O_{24}\cdot 4H_2O$, 20 ml deionized water, 0.07 g $K(SbO)C_4H_4O_6\cdot 0.5H_2O$, 60 ml sulfuric acid and 100 ml deionized

water. Hundred μl of sample was taken in a 10-ml vial. Then 9 ml of deionized water were added. After that, 200 μl of ascorbic acid solution (0.1 $g \cdot ml^{-1}$) and 400 μl of molybdate reagent were added. Deionized water was added increase volume to 10 ml. Absorbance was measured at 680 nm, 15 min after addition of the molybdate reagent. To obtain the standard curve of concentration vs absorbance, different concentrations of KH_2PO_4 in the range of 0.061 to 2.45 $mg \cdot l^{-1}$ were used.

Determination of nitrate concentration Nitrate in the supernatant was determined using the nitrate test kit (1.11169.0001) supplied by Merck (Darmstadt, Germany). According to manufacturer's instructions, the nitrate kit reagent was added to the sample, and the concentration of nitrate ions in solution was determined by comparing the color of the sample solution with the standard solution color.

Determination of fresh weight (FW) and DW The cell suspension was centrifuged at 3000 rpm for 20 min. Cell pellets were collected in pre-weighed aluminium trays. Weight was taken again, and the difference in weight indicated FW of the cells. Five g fresh cells were dried in an oven at 60°C until final constant weight was attained. The final constant weight provided DW of cells.

Determination of AZRL Determination of AZRL was carried out using the method developed by Dai *et al.* (22). The colored complexes formed upon the interaction of vanillin with phenolics and other terpenoids exhibits different absorption spectra in comparison to complexes formed by AZRL. This refined multivariate calibration technique eliminates possible interference of other phenolics and terpenoids with the vanillin assay. The mathematical model developed using the different absorption maxima of different complexes gives the concentration of azadirachtin and related limonoids with 95% accuracy. The accuracy of the method was checked by analysing the mixture of the standard solution of azadirachtin and cell extract.

RESULTS AND DISCUSSION

Effect of different standard media on growth and AZRL production Three commonly used standard plant tissue culture media (MS, Gamborg's B5 and White's) were chosen to study the growth behavior and AZRL production of *A. indica* calli in suspension culture. These three media have different concentrations of major nutrients. Plant cells require growth hormones, including cytokinins, auxins and more, for growth and reproduction. The growth hormones mentioned in the literature were selected and optimised from the different types of growth hormones available for plant cell cultures, namely, BAP, IAA, NAA, 2,4-D and kn for maximum cell growth (results not shown). The optimised concentrations of 2,4-D and kn were determined to be 0.91 $mg \cdot l^{-1}$ and 1.5 $mg \cdot l^{-1}$ respectively. Figure 1 shows the growth characteristics of *A. indica* cells in all three media.

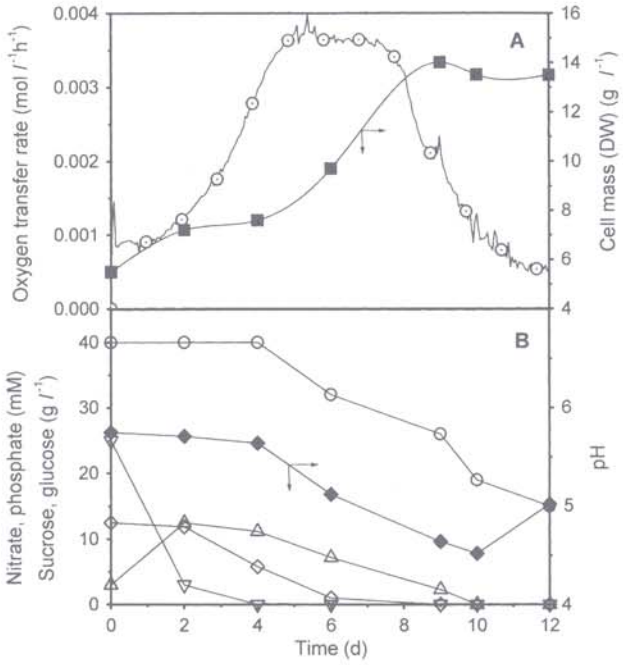
Since the values of carbon dioxide transfer rates were quite similar to those of oxygen transfer rate, only oxygen transfer rates are shown in the diagrams. The oxygen transfer rate of the microbial cultures is between 0.01–0.1 $mol \cdot l^{-1} \cdot h^{-1}$ (13–16; Losen *et al.*, submitted) whereas that of plant cells cultures is between 0.001–0.008 $mol \cdot l^{-1} \cdot h^{-1}$ (17, 18). The fast growing *Nicotiana tabacum* BY-2 cell line showed the highest value of oxygen transfer rate in MS media of 0.008 $mol \cdot l^{-1} \cdot h^{-1}$ using the RAMOS device (unpublished results). The values of oxygen transfer rate curves presented here represent the mean of duplicate determination, and SE is less than ±5%. This confirms that the device is accurate enough to measure small values of oxygen transfer rates in the liquid medium.

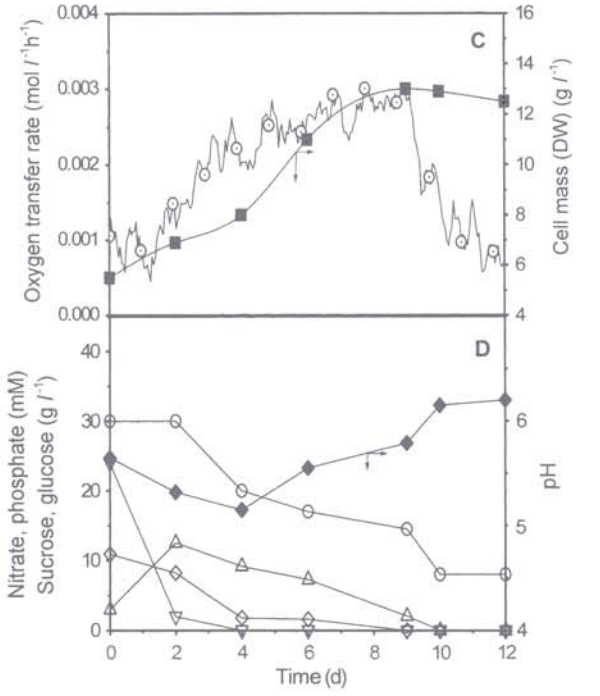
Cells cultivated in MS media showed a maximum oxygen transfer rate of 0.0038 $mol \cdot l^{-1} \cdot h^{-1}$ (Fig. 1A) whereas a minimum oxygen transfer rate of 0.0013 $mol \cdot l^{-1} \cdot h^{-1}$ was observed in White's media (Fig. 1E). There are reports presenting the calculated oxygen transfer rate from dissolved oxygen concentration for *Vitis vinifera* cell culture with a maximum of 0.0038 $mol \cdot l^{-1} \cdot h^{-1}$ and for *Catharanthus roseus* cell cultures with a maximum of 0.0027 $mol \cdot l^{-1} \cdot h^{-1}$ (17, 18). Cells grown in White's medium showed an almost constant oxygen transfer rate during the entire growth cycle. This could be explained by growth rate limitation induced by the lack of nutrients. A maximum oxygen transfer capacity of 0.01 $mol \cdot l^{-1} \cdot h^{-1}$ was calculated for the applied operating conditions of the shaking flasks from the work of Maier *et al.* (23). The measured maximum OTR is much lower than the preceding value, indicating no oxygen-limiting conditions. The total oxygen consumption (TOC) of 25.5 $mM \cdot l^{-1}$ in MS, 23.1 $mM \cdot l^{-1}$ in Gamborg's B5 and 14.6 $mM \cdot l^{-1}$ in White's media were calculated by numerical integration from the OTR data. However, the specific oxygen consumption remained almost the same for the cells cultivated in the three media throughout the growth cycle, with a maximum value of 0.0018 $mol \cdot g^{-1}$ (12th day). The maximum of cell mass accumulation coincided with the decrease in OTR (Fig. 1A, C, E), that is, the start of the stationary phase. Based on the OTR data the following correlation was developed between the TOC and cell mass.

$$\text{Cell mass} = 341.5 \cdot \text{TOC} + 5.5 \quad (1)$$

For the cell culture grown in MS and Gamborg's B5 media, the cell mass could be predicted with 85% accuracy, while the accuracy dropped to 75% in predicting the cell mass of cells cultivated in White's media. Thus, the OTR curves could be used to predict the growth behavior of cells. This could help analysing the desired product at a specific time.

Figure 1B, D and F indicate that phosphate ions and carbon source were the limiting nutrients for the growth of *A. indica* cells. Maximum cell mass accumulation was observed when extracellular phosphate ions and glucose were exhausted. Depletion of phosphate ions and glucose coincided with the decrease in the OTR of cells in MS and Gamborg's B5 media (Fig. 1A–D). During the entire growth cycle, the pH of each of the three media changed in different ways. The presence of ammonium ions influenced the pH of the medium during the growth cycle (24). Since MS medium contains more ammonium ions (20 mM) than Gamborg's B5 (7.44 mM) and White's media (0 mM), there was a decrease in the pH of the MS medium due to uptake of ammonium ions. White's medium contains nitrate ions as the sole nitrogen source, and hence the pH increased during the growth cycle of cells cultivated in this medium. Table 1 shows the growth kinetics data of *A. indica* cell cultures in the three different media. Although there was a difference in the maximum cell mass accumulation in Gamborg's B5 and White's media, the specific growth rates were almost the same for both. The lower average biomass yield and low





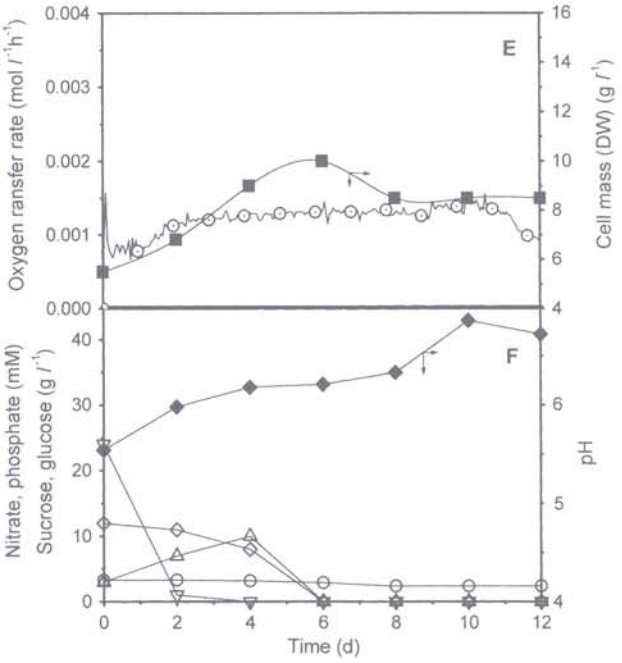


FIG. 1. Growth characteristics of *A. indica* cells in different media. (A, B) MS medium; (C, D) Gamborg's B5 medium; (E, F) White's medium; OTR was measured with RAMOS (dotted circles). Cell mass (solid squares), nitrate (open circles), pH (solid diamonds), phosphate × 10 (open diamonds), sucrose (open inverted triangles) and glucose (open triangles) were determined according to methods described in Materials and Methods.

FW/DW ratio for cells cultivated in White's media indicate that because of limitation of nitrogen and phosphorus source, cells diverted part of the carbon source for secondary metabolite production. These results are quite comparable with the results obtained for other plant cells after similar cultivation time periods (25, 26).

AZRL were detectable only in White's medium, not in MS or Gamborg's (Table 1). Catabolite repression could be the reason for zero production of AZRL in these two nutritionally enriched media. No AZRL was detected before day 9, which coincides with the beginning of the stationary phase. The highest AZRL concentration (0.8 mg·l^{-1}) was observed in White's medium on day 9 of the cultivation, when the lowest ratio of minerals to sugar was recorded. These results show non-growth-associated product formation characteristics, as observed in the case of other secondary metabolite production from plant tissue cultures (27–30). Carbon and phosphorus sources were totally depleted when the OTR began to decrease, and hence stationary phase began in cultures grown in MS and Gamborg's B5 media. This could be the reason for zero production of AZRL even at the low respiration activity (stationary phase) of the cultures grown in MS and Gamborg's B5 media. There could be AZRL production in MS medium if the

TABLE 1. Growth kinetics data of *A. indica* cells in three different media

	MS medium	Gamborg's medium	White's medium
Max. cell mass (DW) (g·l^{-1})	14	13.2	1
Max. specific growth rate (d^{-1})	0.11	0.094	0.096
Average biomass yield on sucrose (–)	0.44	0.43	0.27
AZRL content (mg·l^{-1})	0	0	0.8 (9th day)
FW/DW ratio (–)	25	25	21

respiration activity of the cells is lowered by changing the major nutrient composition.

Effect of nitrogen and phosphorus source

Compared to Gamborg's B5 and White's media, MS medium contains the highest concentration of nitrogen (60 mM) and phosphorus (1.27 mM) source, while White's media contains the lowest amount of nitrogen (2.06 mM) and phosphorus (0.12 mM) source. There is not a big difference between sulfur source (as SO_4^{-2}) and chloride ions in White's medium (4.3 mM, 0.87 mM), Gamborg's medium (3 mM, 1.03 mM) and MS medium (1.5 mM, 3.01 mM). This indicates that nitrogen and phosphorus concentrations affect cell growth, and azadirachtin and related limonoids production more strongly than the other major nutrients do. Nitrogen is supplied in concentrations of 0 to 60 mM as NH_4^+ or NO_3^- or as a combination of both in most standard plant cell culture media. The concentration of phosphate in cell culture medium is usually kept between 0.05 mM to 3.0 mM (25). The effect of total nitrogen content (NH_4^+:NO_3^-, 1:2) and total phosphorus content (as PO_4^{-3}) of MS medium on growth and product formation was studied and optimised using the RSM (results not shown). RSM allows the investigation of several variables in one experiment, and accounts for possible interactions between variables. It has already been applied successfully in plant cell biotechnology for optimisation of biomass growth and secondary metabolite accumulation (29, 32, 33). Four experiments were designed for optimisation of total nitrogen (NH_4^+:NO_3^-, 1:2) and phosphorus source (PO_4^{-3}) (see Materials and Methods). The optimisation experiments were run in duplicates in the RAMOS device to study the respiration activity of plant cells in parallel. The results of respiration activity of cells, cell mass and AZRL content in the four experiments are shown in Fig. 2. The OTR was less than 0.0018 $mol \cdot l^{-1} \cdot h^{-1}$ in all experiments, which is quite low compared to the value shown by cells cultivated in standard MS medium (Fig. 1A). This could be due to either absence of one of the major nutrients or the higher osmotic pressure that affects the growth of cells. Cells inoculated in media containing elevated nitrogen (120 mM) and phosphorus source (3 mM) (Fig. 2A, inverted triangles) showed similar growth as observed with standard MS medium but lower oxygen uptake rate. The lower oxygen uptake rate could be attributed to nitrate dissimilation. However, no AZRL could be detected in the *A. indica* cells grown with the elevated levels of nitrogen and phosphorus source. The big differences in cell mass and AZRL content (Fig. 2B) and small differences in the OTR show that oxygen does not limit cell growth and AZRL production. However, slow growth rates imposed by absence of nitrogen and phosphorus source (Fig. 2A, circles) favoured the highest AZRL production. The yield of AZRL was 0.25 $mg \cdot g^{-1}$ DW in this modified MS medium. However, the yield is still much less than that found in the seed kernels of intact neem trees of 2–6 $mg \cdot g^{-1}$ DW (10).

RSM was used to optimise the total nitrogen and phosphorus content of MS media for maximum biomass growth and AZRL production. All other media constituents were not changed. However, no medium resulted in higher volumetric productivity of AZRL, presumably because of impaired cell mass generation. Therefore, a two-stage process was designed. The idea of two-stage process has long been suggested for the production of plant secondary metabolites. Zenk *et al.* (34) were the first to develop a two-stage process using two different media for the production of indole alkaloids from *C. roseus* cell suspension culture. The indole alkaloids production increased 10 times using the two-stage strategy (35). In this process the *C. roseus* cells were first cultivated in the growth medium followed by the Zenk's alkaloid production medium (35). The suspension cultures of *Lithospermum erythrorhizon* were incapable of producing shikonin in Linsmaier and Skoog medium (36) but there was significant production of shikonin in optimised M9 medium (37). Also Fujita *et al.* (37) successfully developed a two-stage process for shikonin production from cell suspension cultures of *L. erythrorhizon*.

In the present study, two different media, namely, GM

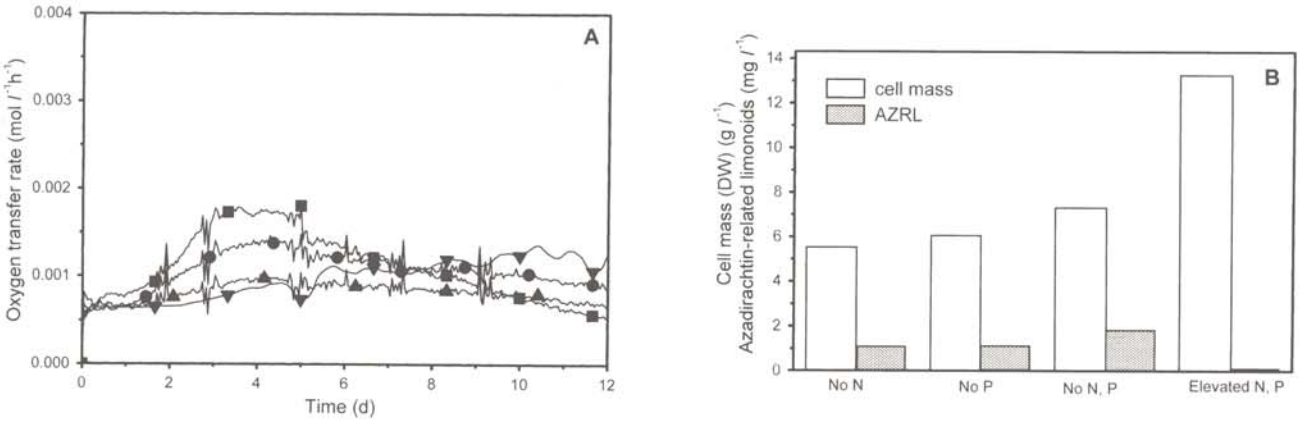


FIG. 2. (A) Respiration activity (measured by RAMOS) of *A. indica* cells in modified MS medium for optimisation with respect to nitrogen and phosphorus source (see Materials and Methods). Symbols: squares, only phosphorus source (3 mM) and no nitrogen source; triangles, only nitrogen source (120 mM; NH_4^+:NO_3^-, 1:2) and no phosphorus source; circles, no nitrogen and phosphorus source; inverted triangles, with nitrogen (120 mM; NH_4^+:NO_3^-, 1:2) and phosphorus (3 mM) source. (B) Cell growth and AZRL production in four modified MS media formulations; no P, only nitrogen source (120 mM; NH_4^+:NO_3^-, 1:2) and no phosphorus source; no N, only phosphorus source (3 mM) and no nitrogen source; no N, P, no nitrogen and phosphorus source; elevated N, P, with elevated concentrations of nitrogen source (120 mM; NH_4^+:NO_3^-, 1:2) and phosphorus source (3 mM).

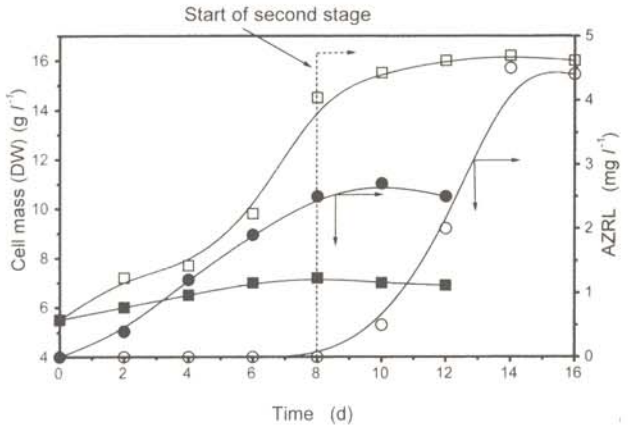


FIG. 3. Comparison of single-stage (in PM) and two-stage process (started with GM and on 8th day the medium was replaced with PM). Symbols: solid squares, cell mass in single-stage process; open squares, cell mass in two-stage process; solid circles, AZRL content in single-stage process; open circles, AZRL content in two-stage process. See Materials and Methods for media composition of PM and GM.

and PM were chosen using the RSM to get maximum cell mass and maximum AZRL production, respectively. The composition of both media is given in materials and methods. The cells were cultivated first in PM and GM separately to study the validity of the RSM used. The predicted value of AZRL in the optimised PM was 3.2 $g \cdot l^{-1}$ whereas, the experimental value of maximum AZRL produced in PM was 2.7 $mg \cdot l^{-1}$ (Fig. 3). The predicted value of cell mass in the optimised GM was 15 $g \cdot l^{-1}$, whereas the experimental value of maximum cell mass obtained in GM was 13.8 $g \cdot l^{-1}$ (result not shown). A two-stage process comprised of media mentioned above was designed to increase the volumetric productivity of AZRL. A single-stage process comprised of PM was also run in parallel with a two-stage process. Figure 3 shows the comparison of single-stage and two-stage process. Since the PM was poor in major nutrients, the increase in cell mass in the single-stage process was not more than 2 $g \cdot l^{-1}$ during the entire growth cycle. The AZRL production started on the 2nd day. Higher cell mass (16 $g \cdot l^{-1}$) as well as higher AZRL production (4.5 $mg \cdot l^{-1}$) was achieved in the two-stage process (Fig. 3). The specific productivity was almost the same in both the processes (0.022 and 0.023 $mg \cdot l^{-1} \cdot d^{-1}$, respectively). However, volumetric productivity increased 1.8 times in the two-stage process (0.32 $mg \cdot l^{-1} \cdot d^{-1}$). This implies that the two-stage process could be used to achieve higher productivity for AZRL production.

We report direct online measurement of the OTR in a special shake flask device (RAMOS) for plant cell suspension culture for the first time. Measurement of OTR in parallel with all experiments in normal shake flasks was shown to provide valuable information on culture conditions. Investigations which were until recently performed in more complicated stirred tank fermentors with exhaust gas analysis can now be carried out efficiently in up to eight parallel shake flasks.

This study shows that nitrogen and phosphorus have a significant influence on cell growth characteristics and AZRL production of *A. indica* cells in suspension. In the absence of these vital nutrients, cells use the carbon source for production of secondary metabolites. There was 0.25 $mg \cdot g^{-1}$ DW AZRL production in modified MS medium as compared to no detectable AZRL production in standard MS medium. However, a batch process with minimum nutrients can hardly be economically feasible due to low cell mass generation and hence low overall productivity. Almost two-fold increase in the volumetric productivity of AZRL was achieved using the two-stage process as compared to a single-stage process. Our results indicate that a two-stage process is indispensable for the production of AZRL. These findings could also be applicable to other secondary metabolite production processes using plant tissue culture. There are possibilities for the optimisation of other media constituents and culture conditions. Effects of elicitors or precursors and other aspects of optimised plant cell fermentation can also be analysed and observed by online measurement of the respiration activity in shake flasks.

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