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Production of Biopesticides in an *In Situ* Cell Retention Bioreactor

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Abstract The seeds of *Azadirachta indica* contain azadirachtin and other limonoids, which can be used as a biopesticide for crop protection. Significant variability and availability of seed only in arid zones has triggered biotechnological production of biopesticides to cope up with its huge requirement. Batch cultivation of *A. indica* suspension culture was carried out in statistically optimized media (25.0 g/l glucose, 5.7 g/l nitrate, 0.094 g/l phosphate and 5 g/l inoculum) in 3 l stirred tank bioreactor. This resulted in 15.5 g/l biomass and 0.05 g/l azadirachtin production in 10 days leading to productivity of 5 mg l^{-1} day^{-1}. Possible inhibition by the limiting substrates (C, N, P) were also studied and maximum inhibitory concentrations identified. The batch kinetic/inhibitory data were then used to develop and identify an unstructured mathematical model. The batch model was extrapolated to simulate continuous cultivation with and without cell retention in the bioreactor. Several offline computer simulations were done to identify right nutrient feeding strategies (with respect to key limiting substrates; carbon, nitrate and phosphate) to maintain non-limiting and non-inhibitory substrate concentrations in bioreactor. One such continuous culture (with cell retention) simulation was experimentally implemented. In this cultivation, the cells were propagated batch-wise for 8 days. It was then converted to continuous cultivation by feeding MS salts with glucose (75 g/l), nitrate (10 g/l), and phosphate (0.5 g/l) at a feed rate of 500 ml/day and withdrawing the spent medium at the same rate. The above continuous cultivation (with cell retention) demonstrated an improvement in cell growth to 95.8 g/l and intracellular accumulation of 0.38 g/l azadirachtin in 40 days leading to an overall productivity of 9.5 mg l^{-1} day^{-1}.

Keywords Bio-pesticide · Azadirachtin · Continuous cultivation · Mathematical modeling · Cell retention

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Nomenclature

dX/dt	Rate of biomass formation (g/day) in Eq. 6
dS/dt	Rate of substrate consumption (g/day) in Eqs. 7–9
dP/dt	Rate of product formation (g/day) in Eq. 10
F	Total flow rate (l/day) in Eqs. 7–9
F_1	Glucose feed rate (l/day) in Eq. 7
F_2	Nitrate feed rate (l/day) in Eq. 8
F_3	Phosphate feed rate (l/day) in Eq. 9
K_{S_1}	Saturation constant for glucose (g/l) in Eq. 1
K_{S_2}	Saturation constant for nitrate (g/l) in Eq. 1
K_{S_3}	Saturation constant for phosphate (g/l) in Eq. 1
K_{I_1}	Inhibition constant for glucose (g/l) in Eq. 1
K_{I_2}	Inhibition constant for nitrate (g/l) in Eq. 1
K_{I_3}	Inhibition constant for phosphate (g/l) in Eq. 1
K_1	Growth associated product formation constant (g/g) in Eq. 5
K_2	Non-growth associated product formation constant (g/g) in Eq. 5
S_{0_1}	Initial glucose concentration (g/l) in Eq. 7
S_{0_2}	Initial nitrate concentration (g/l) in Eq. 8
S_{0_3}	Initial phosphate concentration (g/l) in Eq. 9
m_{S_1}	Maintenance coefficient for glucose (g g^{-1} day^{-1}) in Eq. 7
m_{S_2}	Maintenance coefficient for nitrate (g g^{-1} day^{-1}) in Eq. 8
m_{S_3}	Maintenance coefficient for phosphate (g g^{-1} day^{-1}) in Eq. 9
n	Exponent in Sigmoidal kinetics for phosphate uptake (dimensionless) in Eq. 1
V	Volume (L) in Eq. 7–9
X	Biomass (g/l) in Eq. 6–10
Y_{X/S_1}	Yield of biomass (g/g) with respect to carbon in Eq. 7
Y_{X/S_2}	Yield of biomass (g/g) with respect to nitrate in Eq. 8
Y_{X/S_3}	Yield of biomass (g/g) with respect to phosphate in Eq. 9

Greek Letters:

μ	Specific growth rate (day^{-1}) in Eq. 1
μ_m	Maximum specific growth rate (day^{-1}) in Eq. 1

Introduction

Azadirachta indica (neem) is an Indian medicinal plant, which grows well in arid regions of Asian and African subcontinent. The seed of this plant species are well known as a source of broad-spectrum biopesticides (azadirachtin and related limonoids) in agriculture [1]. Azadirachtin is the major limonoid present in the seed kernels and is presently the most effective, non-toxic, and environmentally safe pesticide from plant origin worldwide [2]. The insecticidal properties of azadirachtin are based on its varying modes of action against a wide range of agricultural insects/pests. It acts as an antifeedant, insect growth regulator and sterilant [3].

Azadirachtin is presently being isolated by solvent extraction from the seed kernels of *A. indica*. The azadirachtin bio-pesticides are selling for US$ 125/l, which based on

the formulations containing 3% azadirachtin. This eventually equates to a price of US$ 2.60/g to the end user. According to an estimated data for the neem pesticides, to compete with others like pyrethroids or *Bacillus thuringienesis*, it would require production of the formulated azadirachtin at 25–38% reduced production cost [4]. However, distribution of neem tree is limited to arid zone of tropical regions due to its susceptibility to excessive frost. Azadirachtin production from seeds is also associated with several disadvantages, e.g., the seeds are produced once in a year and a major fraction of seeds gets deteriorated during storage [5]. Seeds also have large variations with respect to azadirachtin content mainly due to diverse genotype and wider geographical distribution of neem trees [6]. It is also observed that mature seeds get adulterated by fungus upon falling and may lead to deadly production of aflatoxin. Due to all these constraints, the supply of seeds kernel vis a vis azadirachtin extraction has been variable and inadequate with respect to its huge demand in agriculture sector. Plant cell cultivation technology can provide a viable alternative to whole plant cultivation for the production of secondary metabolites. The production of azadirachtin by cell cultures of *A. indica* has received significant attention from different investigators as being a promising alternative protocol [7].

The commercial application of plant cell cultivation has been mainly hampered by the low product formation and productivities. The product formation is particularly low in batch cultivation. This can, however, be overcome to a large extent by adopting different modes of cultivation, process optimization, and elicitation, etc.

Continuous cultivations are known to be one of the tools to improve productivity, but higher productivities are invariably observed only if the reactor is operated at high dilution rate (= specific growth rate). However, there is a risk of washout when reactors are operated continuously at high dilution rates. Retention of cells from a continuous reactor operating at high dilution rate could prevent washout and significantly enhance the cell concentration at high dilution rates. Spin filter has been very commonly used to retain the cells and improve productivity in microbial cultivation [8]. The application of most appropriate dilution rate, inlet substrate concentration, and selection of right pore size of spin filter is particularly important for deriving improvement in productivity by spin filter continuous cultivation without losing significant unconverted nutrients in the exit stream. The effect of selected process variables on overall process response can be facilitated by the development of mathematical model. Mathematical model can eventually be used to simulate the different process operating strategies to ensure non-limiting and non-inhibitory concentrations of key limiting nutrients in the bioreactor. Offline optimized selected cultivation can be then implemented experimentally to achieve higher productivity.

In this investigation, *A. indica* suspension culture was cultivated in a 3-l stirred tank bioreactor, and batch growth/inhibitory kinetics was studied to identify limitation and inhibition of key substrates (carbon, nitrate, phosphate). Based on the batch kinetics/inhibitory data, an unstructured mathematical model was proposed. The model was extrapolated to simulate continuous cultivation of *A. indica* with *in situ* cell retention by spin filter device for higher biomass and azadirachtin production. One such simulated nutrient feed/withdrawal strategy was adopted to enhance biomass and azadirachtin accumulation in the bioreactor. It was anticipated that the continuous cultivation with cell retention would be particularly advantageous for slow-growing plant cells as it could result in high-density cultures growing at significantly high growth rates (= high dilution rates). This reactor-operating strategy was also expected to feature enhanced cell-to-cell contact; therefore, it might possibly result in high product accumulation inside the cells.

Materials and Methods

Development of Callus Culture

Neem seeds obtained from different parts of India were analyzed for azadirachtin content. For callus culture development, the seeds were depulped and surface sterilized in 70% *v*/*v* ethanol for 1 min followed by 3–4 times rinsing with sterile distilled water (SDW). The seeds were thereafter sterilized in 0.1% *w*/*v* $HgCl_2$ for 8 min and rinsed with SDW 4-5 times. This was followed by dissection of seeds. Seed kernel (along with embryo) was, thereafter, excised and used as an explant for callus induction on Murashige & Skoog medium (MS) [9]. A high-azadirachtin-containing callus culture was developed from higher-azadirachtin-containing seed kernels (which originated from Trivandrum, Kerala, India). MS medium supplemented with naphthalene acetic acid (10 μM), benzyladenine (2.5 μM) with 8 g/l agar (Hi-media) was found to be suitable for callus development. Callus culture was maintained by regular subculturing and incubations at 25 °C for 16/8 h light/dark period on the same medium.

Suspension Culture of *A. indica*

The suspension culture was initiated by transferring the friable callus in 50 ml liquid MS medium. Growing cultures were then transferred to modified Murashige & Skoog medium (statistically optimized) containing glucose (25.0 g/l), nitrate (5.7 g/l), phosphate (0.094 g/l), inoculum (5 g/l; dry cell weight basis), 8 mg/l indole butyric acid, and 4 mg/l benzyladenine (with rest of the constituents same as that of MS media) [10]. The cultures were incubated in gyratory shaker rotating at 125 rpm and maintained at 25 °C under dark conditions.

Substrate Inhibition Studies

The effect of varying initial substrate concentrations [carbon (C), nitrate (N) and phosphate (P)] on the specific growth rate (μ) of *A. indica* was investigated in shake flask using optimized media and culture conditions as described above. The carbon concentration was varied from 25 to 150 g/l, nitrate from 5.7 to 21.0 g/l, and phosphate from 0.094 to 0.4 g/l to study the limiting and inhibitory effects of these nutrients on specific cell growth. Initially, 5 g/l cells were inoculated in medium containing various concentrations of substrates (C, N, P), and biomass concentration (X) was estimated by measuring the dry cell weight of the cells at an interval of 12 h during the log phase of culture. Specific growth rate (μ) was calculated from $\ln(X) - \ln(X_0)$ versus time (t) plot where, X_0=initial biomass concentration at the time of inoculation.

Batch Cultivation of *A. indica* in 3 l Stirred Tank Bioreactor

A. indica cells were cultivated in 3 l stirred tank bioreactor (Applicon, Dependable Instruments, The Netherlands) with 1.5 l working volume. The statistically optimized medium recipe (as described above) was used for the study of growth and product formation in batch cultivation. Mixing was achieved by low shear setric impeller [11] at an agitation rate of 125 rpm. Dissolve oxygen was maintained above 30% in the reactor by manually varying the air-flow rate. The temperature and pH was maintained at 27 °C and 5.8, respectively, by biocontroller (ADI 1030, Applicon, Dependable Instruments, The

Netherlands). Samples (25 ml) were collected every second day and analyzed for dry cell weight, azadirachtin content, residual glucose, and nitrate and phosphate concentrations. Batch kinetic/inhibitory data were used to identify and estimate the parameters of mathematical model of the system.

Continuous Cultivation of *A. indica* in 3 l Stirred Tank Bioreactor with Cell Retention

The batch kinetic model identified above was extrapolated to simulate different cell retention continuous cultivations for identification of right nutrient (C, N, P) feeding strategies. Continuous cultivation of *A. indica* was carried out in 3 l bioreactor (1.5 l working volume) equipped with an internal cell separator (spin filter, 10 μm pore size) on the agitator shaft of the bioreactor above impeller. The medium composition and culture conditions were kept same as that of batch cultivation. The cultivation was started as a batch and continued for initial 8 days. Model simulated nutrient feeding strategies for C, N, P were initiated [MS salts with glucose (75 g/l), nitrate (10 g/l) and phosphate (0.5 g/l)] after eighth day at a feed rate of 500 ml/day and continued till 40th day of cultivation in the annular space of the bioreactor outside the spin filter. The duly filtered spent medium was constantly withdrawn at the same flow rate of 500 ml/day from inside of the spin filter by means of a peristaltic pump.

Analytical Procedures

For dry cell weight estimation, cells were harvested and collected by centrifugation at 3,000 rpm for 15 min and washed with distilled water. The fresh cells were dried at 28 °C± 2 °C to a constant dry cell weight. Glucose, nitrate and phosphate were measured spectophotometrically [12-14]. Extraction of azadirachtin was done as described earlier [15]. Final dry cell mass was redissolved in methanol prior to analysis by high performance liquid chromatography (HP1100, Agilent technologies, USA) to avoid the degradation or chemical conversion of azadirachtin to some other analogue in organic solvent. A Novapack C-18 column (250 mm length×4.6 mm diameter) maintained at 45 °C was used as stationary phase, while acetonitrile/water (10:90) was employed as mobile phase with a flow rate of 0.5 ml/min. Absorbance of azadirachtin was measured at 214 nm by Diode Array Detector, which was eluted at a retention time of 3.0 min. Azadirachtin peak in sample was compared with standard azadirachtin (Sigma, USA; catalogue no. A-7430) and confirmed for spiking of pure azadirachtin in sample.

Model Development

Following assumptions were made for the development of mathematical model

1. Carbon, nitrate, and phosphate were assumed to be the major limiting nutrients while rest of the minor nutrients were always available in excess in the culture broth.
2. Temperature (27 °C) and pH (5.8) were maintained constant throughout the cultivation.

The batch kinetics at pH 5.8 and temperature 27 °C was used for the development of unstructured mathematical model. To account for any possible inhibition during feeding, different concentrations of major nutrients (C, N, P) were tested for their inhibition level. The complete inhibition for carbon, nitrate, and phosphate (at which μ becomes zero) was found to be at 162.27, 26.35, and 0.63 g, respectively.

The specific growth rate (μ) was described by incorporating limitation and inhibition terms for batch cultivation as follows.

$$\mu = \mu_m \left[\frac{S_1}{S_1 + K_{S_1}}\right]\left[\frac{S_2}{S_2 + K_{S_2}}\right]\left[\frac{(S_3)^n}{(S_3)^n + (K_{S_3})^n}\right]\left[\frac{K_{I_1}}{K_{I_1} + S_1}\right]\left[\frac{K_{I_2}}{K_{I_2} + S_2}\right]\left[\frac{K_{I_3}}{K_{I_3} + S_3}\right] \quad (1)$$

The specific substrate consumption rate (q_S) was assumed to be mainly due to growth of cells, and for maintenance. "m" features substrate consumption for non-growth-related activities of the plant cells, e.g., transport of nutrient to the cells, maintenance of intracellular pH. etc. The specific substrate consumption rates for S_1 (glucose), S_2 (nitrate), and S_3 (phosphate) were represented by the following equations:

$$q_{S_1} = -\left[\frac{1}{Y_{X/S_1}}\mu + m_{S_1}\right] \quad (2)$$

$$q_{S_2} = -\left[\frac{1}{Y_{X/S_2}}\mu + m_{S_2}\right] \quad (3)$$

$$q_{S_3} = -\left[\frac{1}{Y_{X/S_3}}\mu + m_{S_3}\right] \quad (4)$$

The following equation was used to describe the specific rate of azadirachtin formation, which summarizes the product formation due to growth, and non-growth related culture activities:

$$q_p = \frac{1}{X}\frac{dP}{dt} = K_1\mu + K_2 \quad (5)$$

For optimal estimation of model parameters, a non-linear regression technique [16] assisted by a computer program [17, 18] was used to minimize the deviation between the model predictions and batch experimental data. For the calculation of the model predictions, the system of differential equations of the model was solved using an integration program based on the Runge-Kutta Method of fourth order. The optimization program for the direct search of the minimum of a multivariable function (sum of the square of weighed residues) [17] was based on the original method of Rosenbrock [19].

Batch kinetic model was extrapolated for continuous cultivation with cell retention by incorporating the dilution term for substrates (C, N, P) as shown below (Eqs. 7–9). Dilution term was not applied for cell growth (as shown in Eq. 6) as no cells were withdrawn from the bioreactor. Similarly dilution term was not applied for product formation as the product was intracellular in nature (Eq. 10).

$$\frac{dX}{dt} = \mu X \quad (6)$$

$$\frac{dS_1}{dt} = -\left[\frac{\mu X}{Y_{X/S_1}} + m_{s_1}X\right] + \left[\frac{F_1 S_{0_1}}{V}\right] - \left[\frac{F S_1}{V}\right] \quad (7)$$

$$\frac{dS_2}{dt} = -\left[\frac{\mu X}{Yx_{/s_2}} m_{s_2} X\right] + \left[\frac{F_2 S_{0_2}}{V}\right] - \left[\frac{F S_2}{V}\right] \tag{8}$$

$$\frac{dS_3}{dt} = -\left[\frac{\mu X}{Yx_{/s_3}} m_{s_3} X\right] + \left[\frac{F_3 S_{0_3}}{V}\right] - \left[\frac{F S_3}{V}\right] \tag{9}$$

$$\frac{dP}{dt} = (K_1 \mu + K_2)X \tag{10}$$

Results and Discussion

The batch kinetic profile of dry cell weight, azadirachtin, residual glucose, nitrate, and phosphate at optimal medium and culture conditions is shown in Fig. 1. The highest cell density in the bioreactor reached 15.5 g/l (dry cell weight basis) in 10 days of cultivation period. The production of azadirachtin mainly followed the growth-associated kinetics and reached a final concentration of 0.05 g/l on the tenth day of cultivation (volumetric productivity of 5 mg l^{-1} day^{-1}). Glucose and phosphate were consumed at a faster rate during the initial lag phase (up to 4 days), and by the tenth day, both phosphate and glucose were fully consumed. This is generally observed in plant cells that phosphate is taken up fast by the cells during the lag phase and is stored as an internal phosphate pool. Nitrate uptake occurred at a slower rate and approximately 32% nitrate (1.82 g/l) remained unutilized in the medium by tenth day. The batch kinetic profile revealed the reduction in dry biomass and azadirachtin when culture featured complete consumption of glucose and phosphate (on tenth day of cultivation). This clearly indicated the possible limitation by these nutrients during the batch cultivation. It was therefore concluded that the feeding of essential nutrients (from day 8) could result in enhancement of biomass and azadirachtin production.

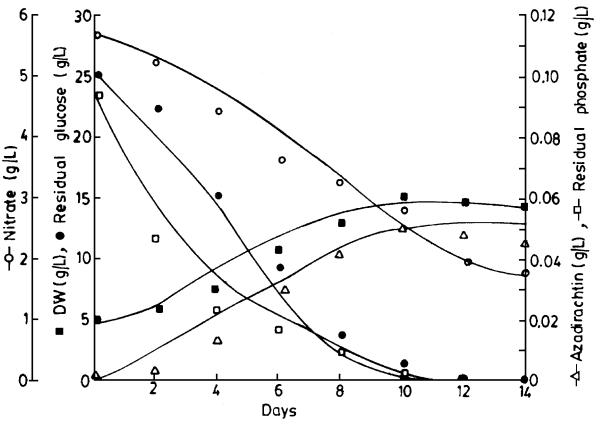

Fig. 1 Comparison of the experimental data (points) and model simulated values (*smooth lines*) for batch kinetics of *A. indica*. *Filled square*, dry cell weight; *open triangle*, azadirachtin; *filled circle*, residual glucose; *open circle*, residual nitrate; *open square*, residual phosphate

From the batch kinetics of *A. indica*, it was also very clear that increase in biomass and azadirachtin production was restricted due to substrate (C, N, P) limitations, and glucose was not the only limiting nutrient. To describe the *A. indica* suspension kinetics efficiently a simple mathematical model Eqs. 1–5 was proposed based on in-depth batch kinetic studies and studies of limiting and inhibiting effects of glucose, phosphate, and nitrate. The values of model parameters (summarized in Table 1) were obtained (by the procedure described before). The model equations described above in Eqs. 1 to 5 were simulated on computer by using the optimal values of the model parameters. A comparison between the model fitted (smooth line) and experimental data (points) is shown in Fig. 1. As shown in the figure, the kinetic profiles of cell growth, azadirachtin formation, and substrate consumption were well described by the developed unstructured kinetic model.

Selection of Operating Conditions with Respect to Substrate and Feed Rate (C, N, P) in Continuous Cultivation

In order to overcome the different substrate limitations, the batch model was extrapolated to simulate continuous cultivation under cell retention conditions. Various offline computer simulations (by numerically integrating Eqs. 6–10) were done, and different concentrations of feed substrates (glucose, nitrate and phosphate) and feeding rate were tested to maximize the biomass/azadirachtin accumulation. Some of the representative simulations are shown in Table 2. Offline simulation indicated that increasing the initial nitrate concentration from 5.7 to 10 g/l resulted in corresponding increase in biomass and azadirachtin accumulation (trial 5–6) at same D=0.2 days^{-1}. However, further increase in nitrate concentration (keeping all other parameters similar to trial 6) led to decreased biomass and azadirachtin accumulation (trial 7). Therefore, nitrate concentration was kept constant at 10 g/l, and further simulations were attempted to identify right glucose and phosphate concentrations and their respective feed rates. From trials 7–9, it was observed that increase in phosphate concentration (from 1.0 to 2.0 g/l) with D and inlet glucose, nitrate concentration constant

Table 1 Optimized model parameters for batch cultivation.

Parameters	Units	Value
μ_m	(day^{-1})	0.65
K_{S_1}	(g/L)	17.66
K_{S_2}	(g/l)	1.04
K_{S_3}	(g/l)	0.14E-5
K_{I_1}	(g/l)	162.27
K_{I_2}	(g/l)	26.35
K_{I_3}	(g/l)	0.63
K_1	(g/g)	0.003695
K_2	(g/g)	0.000107
K_2	(g/g)	0.000107
Y_{X/S_1}	(g/g)	2.22
Y_{X/S_2}	(g/g)	0.0588
Y_{X/S_3}	(g/g)	0.0079
m_{S_1}	(g g^{-1} day^{-1})	0.0272
m_{S_2}	(g g^{-1} day^{-1})	0.023
m_{S_3}	(g g^{-1} day^{-1})	0.00011
n	(dimensionless)	0.3611

Table 2 Offline computer simulated trials for feeding of glucose, nitrate and phosphate in spin filter continuous cultivation.

No.	D, day^{-1}	Feed rate l/day	Feed glucose, g/l	Residual glucose, g/l	Feed nitrate, g/l	Residual nitrate, g/l	Feed phosphate, g/l	Residual phosphate, g/l	Final DCW, g/l	Final product, g/l	No. of days	Aza prod., mg l^{-1} day^{-1}
1.	0.06	100	25	4.97	5.7	0.29	1.0	0.82	27.84	0.15	30	5
2.	0.06	100	50	11.39	5.7	0.14	1.0	0.66	32.19	0.15	22	6.8
3.	0.10	150	50	16.46	5.7	0.11	1.0	0.80	42.78	0.20	24	8.3
4.	0.13	200	50	20.53	5.7	0.08	1.0	0.87	53.56	0.25	26	9.6
5.	0.2	300	50	20.71	5.7	0.27	1.0	0.89	73.29	0.35	28	12.5
6.	0.2	300	50	8.49	10.0	3.82	1.0	0.85	84.09	0.39	28	13.9
7.	0.2	300	50	9.95	15.0	9.14	1.0	0.85	79.63	0.37	28	13.2
8.	0.2	300	50	13.0	10.0	4.0	1.5	1.36	81.32	0.40	32	12.5
9.	0.2	300	50	15.49	10.0	3.67	2.0	1.87	85.43	0.45	38	11.8
10.	0.2	300	75	12.22	10.0	1.75	1.0	0.77	113.14	0.52	28	18.5
11.	0.2	300	75	9.06	10.0	3.10	0.6	0.36	94.98	0.41	22	18.6
12.	0.2	300	75	2.92	10.0	2.63	0.5	0.25	101.36	0.44	22	20.0
13.	0.2	300	75	6.92	10.0	2.63	0.4	0.20	100.01	0.40	22	18.18
14.	0.2	300	100	9.65	10.0	1.0	0.5	0.17	124.79	0.54	24	22.5
15.	0.2	300	125	22.0	10.0	0.18	0.5	0.13	136.71	0.58	24	24.16
16	0.26	400	75	62.64	10.0	0.74	0.5	0.34	173.91	0.99	36	26.05
17.	0.33	500	75	62.17	10.0	1.12	0.5	0.45	217.23	1.24	40	31.5
18.	0.33	500	100	87.69	10.0	1.12	0.5	0.45	217.31	1.26	40	31.5
19.	0.33	500	125	112.72	10.0	1.12	0.5	0.45	217.32	1.26	40	31.5

resulted in higher biomass and azadirachtin accumulation, but azadirachtin productivity decreased. Therefore, phosphate concentration was reduced up to 0.5 g/l (keeping all other parameters D, inlet glucose, nitrate same), which resulted in an increase of azadirachtin productivity (trials 9–12). Trial 13, simulation demonstrated that decreasing the phosphate concentrations lower than this (0.5 g/l) would lead to a decrease in the final biomass content and productivity. Therefore, based on these simulations, nitrate and phosphate concentrations were frozen at 10 and 0.5 g/l, respectively, and glucose concentrations and/or its feed rate were then changed to study their influence on biomass, azadirachtin accumulation and productivity. Biomass and azadirachtin production increased by increasing the glucose concentration from 75 to 125 g/l (at constant D=0.2 $days^{-1}$, feed rate 300 ml/day; trials 13–15); however, much significant increase in biomass and azadirachtin production was observed when feed rate was also increased from 300 to 500 ml/day at 75 g/l glucose concentration at D=0.33 $days^{-1}$ (trials 16–17). Trials 17–19, simulations exhibited that at 500 ml/day feed rate (D=0.33 $days^{-1}$), further increase in glucose concentration (from 75 to 125 g/l) was not leading to increase in biomass and azadirachtin accumulation. Besides high-unconverted glucose was left in bioreactor (trials 18–19) which was not desirable. However, for model simulation 19, model had predicted a total biomass of 217.23 g/l with an azadirachtin content of 1.24 g/l in 40 days of cultivation (significantly higher than batch); therefore, this strategy was experimentally implemented. The effect of feeding of glucose (75 g/l), nitrate (10 g/l), and phosphate (0.5 g/l) at 500 ml/day feed rate (at D=0.33 $days^{-1}$) on biomass and azadirachtin production is shown in Fig. 2. The continuous cultivation data demonstrate that in 40 days of cultivation, biomass content of 95.8 g/l and azadirachtin concentration of 0.38 g/l was accumulated. This leads to an overall azadirachtin productivity of 9.5 mg l^{-1} day^{-1} at the end of the cultivation (40 day). This was significantly higher than that obtained in batch cultivation (5 mg l^{-1} day^{-1}). The residual concentration of

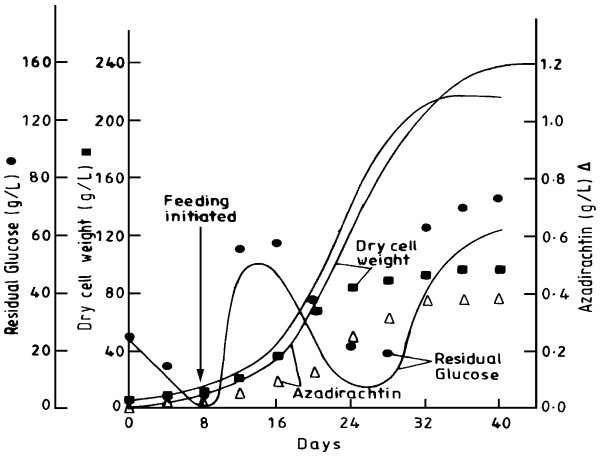

Fig. 2 Comparison of experimental data (points) and model simulated values (*smooth lines*) for model based continuous cultivation with cell retention. *Filled square*, dry cell weight; *open triangle*, Azadirachtin; *filled circle*, residual glucose

glucose, nitrate, and phosphate was extraordinarily high at the end of 40 days cultivation as compared to the batch system; however, as azadirachtin is a high-value product and on the contrary substrates are of reasonably low values, substantial increase in the product concentration would overcome the marginal cost increase resulting from higher residual substrate concentrations.

As shown in Figs. 2 and 3, the model suitably described the trends obtained by all the process variables (biomass, glucose, nitrate, phosphate, and azadirachtin) at least during the entire batch cultivation period (0–8 days) and initial part of the continuous cultivation (8–16 days). However, the experimental deviation from model simulation during the later part of cultivation was observed for all process variables (biomass, substrates and product), which could possibly be due to relatively higher biomass concentration in bioreactor leading to less nutrient/oxygen availability and/or increased medium viscosity. Decreased cell activity and reduced growth rate could possibly be due to increased accumulation of

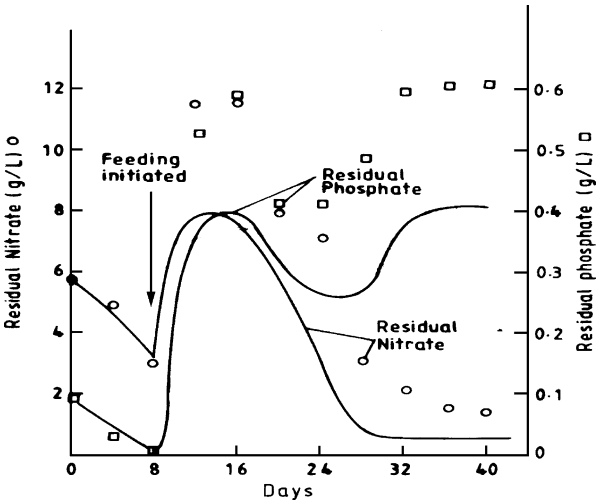

Fig. 3 Comparison of experimental data (points) and model simulated values (*smooth lines*) for model based substrate(s) consumption with respect to C, N, P for model based continuous cultivation with cell retention. *Open circle*, residual nitrate, *open square*, residual phosphate

product (azadirachtin) in the cells, the result of which may be the decreased consumption of substrates and hence deviation between the model simulation and experimental data. However the success of the model can be gauged by its capability to describe the trends observed in the experiment for all process variables. Similar decreased podophyllotoxin production in cell retention continuous cultivation by *Podophyllum hexandrum* was also observed by Chattopadhyay et al. [20]. In that study also it was shown that relatively higher biomass concentration leads to less substrate transport to the cells thereby decreasing the cell activity and growth rate.

The mathematical model for continuous cultivation was a rather simple extrapolation of unstructured batch model; therefore, point to point matching of model simulation with experiment data points was not expected also for continuous cultivation. The model, however, provided the suitable guidance for highly productive non-limiting, non-inhibitory continuous cultivation. Model-based continuous cultivation significantly reduced the number of experiments for predicting the appropriate nutrient feedings, their times and rates during spin filter continuous cultivation which led to significant improvement in productivity as compared to batch cultivation.

Cell retention systems have distinct advantages with respect to achieving high cell density in continuous cultivations. In situ cell retention systems are particularly successful in improving the productivity of plant cell cultivation mainly because the plant cells are slow growing; therefore, dilution rate cannot be kept higher than the specific growth rate in the normal continuous cultivation. With cell retention system, the reactor can be operated at high dilution rates, and at the same time, the cells can be retained by the spin filter device. The use of a spin filter for somatic embryogenesis in plant suspension culture for overcoming the technical hurdles for its application to industrial plant propagation had been reported by Wheat et al. [21]. Production of recombinant human granulocyte-macrophage colony-stimulating factor by continuous cultivation with an internal cell separator was also attempted by Lee et al. [22]. The present investigation, however, demonstrates the productivity enhancement of biopesticide by implementing model-based cultivation that significantly reduced the numerous experimental trials.

Conclusions

The batch cultivation of *A. indica* in bioreactor resulted in biomass accumulation of 15.5 g/l (dry cell weight basis) and azadirachtin production of 0.05 g/l after 10 days with a volumetric productivity of 5 mg l^{-1} day^{-1}. The complete inhibition for carbon, nitrate, and phosphate (at which μ becomes zero) was found to be at 162.27, 26.35, and 0.63 g, respectively. A simple unstructured mathematical model was developed, and its parameters were identified using the batch kinetic/inhibitory data. The model elucidated the C, N, and P depletion under different bioreactor operating strategies. The model was extrapolated to simulate spin filter continuous cultivation and was used for the development of suitable nutrient feeding strategy with respect to C, N, and P. Feeding of MS salts with glucose (75 g/l), nitrate (10 g/l), and phosphate (0.5 g/l) at a feed rate of 500 ml/day between 8th and 40th day of cultivation resulted in production of 95.8 g/l biomass and 0.38 g/l of azadirachtin with an overall productivity of 9.5 mg l^{-1} day^{-1}. Approximately twofold increase in productivity (9.5 mg l^{-1} day^{-1}) was observed in continuous cultivation as compared to batch (5 mg l^{-1} day^{-1}). The model identified in the present study is system independent and may be adopted and used for process development and optimization of other plant cell suspension cultures as well.

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