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Bioprocess design to scale-up adventitious root biomass and associated key flavouring N-alkylamides from *Spilanthes paniculata* Wall. ex DC. in a modified photobioreactor

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ABSTRACT

A novel photo-bioreactor configuration is developed for root biomass production from *Spilanthes paniculata*. The plant is widely used in food industry for food flavouring and food preservation, and is a rich source of N-alkylamides that has uses in cosmetics and medicines too. Further, roots are genetically and phytochemically very stable over successive generations but being a filamentous plant organ, it requires special attention during scaling-up process. Therefore, this study is aimed to provide optimized culture parameters for large-scale production of adventitious root biomass and N-alkylamides in modified stirred tank photobioreactor (MSTR). Bioreactor parameters, like inoculum density (6.0 g/L, FW), aeration (0.5 vvm) and agitation (150 rpm) were successfully optimized in MSTR. It yielded highest roots biomass of 73.34 ± 2.20 g/L (FW) and 10.01 ± 0.29 g/L (DW) on ½ MS (only major salts reduced to half strength) liquid basal medium with 5 % sucrose. It also resulted in increased production of two prominent N-alkylamides, spilanthol (720.44 ± 27.45 μg/L) and DTAI (3608.03 ± ± 92.81 μg/L). Additionally, logistic equation was employed for kinetic modelling of root biomass growth in MSTR. Metabolite productivity data obtained from MSTR was fitted to Ludeking-Piret and modified Gompertz equation, which showed partially growth-associated phenomenon for metabolite production. Maximum metabolite concentration was 963.76 μg/L for spilanthol and 3914.71 μg/L for DTAI. Outcomes of this study may overcome limitations of plant biomass availability due to seasonal and geographical restrictions. This study is a pioneering step towards fulfilling a wide gap between laboratory and industrial-scale plant biomass and metabolites production.

Key Message

A novel and efficient bioreactor configuration was developed for large-scale adventitious root biomass and N-alkylamides production, along with kinetic modelling studies using different mathematical models to further scale-up the process to pilot or commercial bioreactors.

1. Introduction

Spilanthes species plants are widely used as condiments, appetite stimulants, flavouring and seasoning agents in various traditional cuisines. These plants are also used as leafy vegetables in the preparation of soups (tacacá), sauces (tacupi) and stews in the local cuisines of Brazil, Japan, Africa and the West Indies (Paulraj et al., 2013; Lagnika et al., 2016). *Spilanthes* species are extensively used as vegetables by ethnic communities and tribes in the eastern Himalayan regions of India (Prakash Arya et al., 2017). A tincture of freshly harvested *Spilanthes* leaves is currently considered a botanical food supplement to alleviate thrush and nail infections. The utilisation of these plants, particularly *Spilanthes* spp., as food supplements is primarily due to the presence of N-alkylamides (NAAs). These compounds exhibit a characteristic tingling, numbing, and cooling effect, making them valuable in novel culinary applications such as chewing gums, mouth-refreshing candies, and gourmet cuisine. Several NAAs containing products as food supplements are available in the market. A Japanese firm has developed a series of organoleptic products, one of which contains spilanthol as an ingredient to stimulate a tingling sensation (Sharma & Arumugam, 2021). Apart from their characteristic pungency, NAAs exhibit

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analgesic, antimicrobial, antimalarial, insecticidal, anti-inflammatory, anti-viral and immunomodulatory properties (Paulraj et al., 2013; Hossain et al., 2014; Rajendran et al., 2017). However, most N-alkylamide containing plants are marketed as food supplements and are occasionally used for medicinal purposes (Wynendaele et al., 2018). Besides these food and pharmacological applications, *Spilanthes* spp. plants are also added to cosmetic products due to their wrinkle-smoothing and anti-ageing properties (Veryser et al., 2014).

The commercial interest in N-alkylamides has enormously increased in the last few decades due to their multifaceted applications in the food, pharmaceutical, and cosmetic industries. Structurally, these compounds are amides containing polyunsaturated aliphatic acid chains on one side and a comparatively short amine on the other, connected through an amide bond. These compounds have been reported in several plants of *Spilanthes* spp. from the Asteraceae family. The genus *Spilanthes* has more than 60 species found in tropical and subtropical regions of America, Africa, and Asia (Prachayasittikul et al., 2013). There are six prominent members in India namely *Spilanthes calva* DC., *S. paniculata* DC., *S. radicans* Jacq., *S. ciliata* Kunth, *S. uliginosa* Sw. and *S. oleracea.* Typically, these plants are half a meter tall, short-lived perennials with simple, ovate opposite leaves and prostrate or ascending cylindrical hairy stems (Paulraj et al., 2013).

Spilanthes paniculata Wall. ex DC. commonly known as a toothache plant, is a multi-utility vital folklore widely used for various household applications, including food, traditional medicine and cosmetic applications. The plant is a great source of NAAs, and these compounds have been isolated from all parts of the plant. The principal NAAs reported from this plant are (2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide (spilanthol) and dodeca-2E,4E,8Z,10E,Z-tetraenoic acid isobutylamides (DTAI). *S. paniculata* is conventionally propagated through seeds, which quickly lose their viability. Seasonal dependence and slow germination are other major limiting factors in conventional propagation. Moreover, propagation by seeds is also undesirable due to highly heterozygous nature of the plant resulting from protandry, which prevents self-pollination (Reddy, 2007). Many small, bright yellow flowers are aggregated into the capitulum (flower head), making them attractive to insects, which in turn facilitates cross-pollination, resulting in high heterogeneity in the quality and quantity of chemical profiles of the plant. The extensive utilisation of these plants for medicinal, food, and cosmetic purposes has prompted researchers to explore alternative methods for large-scale biomass and metabolite production, thereby preserving the diminishing natural flora. Plant tissue culture offers sustainable methods for the large-scale production of in vitro biomass and metabolites.

Previous reports mention the in vitro plant studies, including callus cultures, leaf-disc cultures and nodal segment cultures for large-sale biomass and metabolite production (Singh & Chaturvedi, 2012; Rajendran & Chaturvedi, 2017). The low, unstable metabolite yields and labour-intensive processes are significant bottlenecks for large-scale and commercial production. Therefore, to meet the increasing industrial demand for these metabolites, researchers have employed bioreactor technology as a promising alternative to conventional plant tissue culture methods (Rahmat et al., 2021). The excellent property of adventitious roots growing in a suspension medium provides an opportunity to scale-up the process. The suitable bioreactor configuration for plant biomass generation remains a challenge to industrial production. Several studies have been conducted on key medicinal plants regarding biomass cultures in bioreactors; however, most of these studies have focused on hairy root cultures for metabolite production (Hao et al., 2021). However, compared to the transformed hairy roots, adventitious roots are considered genetically more stable and maintain metabolic profiles similar to those of natural plants (Murthy et al., 2016).

Several structural and non-structural metabolites are produced in plant systems throughout their life cycle. Under in vitro conditions, these metabolites are affected by various internal and external parameters, and their productivity can be altered by modulating these parameters. Mathematical models are advantageous in understanding the effect of these parameters in controlling the productivity of a specific plant system under in vitro conditions (Chattopadhyay et al., 2002). Several mathematical models like logistics, Leudeking-Piret (L-P) and the modified Gompertz model have been commonly used for biomass and product formation in various microbial and plant systems. The logistics model provides information on initial and final biomass production, as well as the maximum specific growth rate (μ_{max}). The L-P model is generally used for product formation and calculates two important empirical parameters, α and β, called growth coupling and non-growth coupling constants (Sivarathnakumar et al., 2019). The value of α would be non-zero if the product formation is associated with biomass growth; however, β will be zero. Whereas the α will be zero if the product formation is non-growth associated, and if the α and β both are non-zero, show that the product formation is partially growth associated. The modified Gompertz equation is also used to predict the maximum product concentration (P_m), maximum rate of production ($r_{p,m}$), and lag phase time (t_L). Sometimes, multimathematical modelling can better illustrate the results and yield fruitful outcomes because a single equation may not be sufficient to explain the experimental data (Pan et al., 2020).

The present study represents a novel effort to establish adventitious root culture of *Spilanthes paniculata* and develop suitable bioreactor configurations for the large-scale root biomass and NAAs production. Different configurations of bioreactors, including balloon-type bubble bioreactor (BTBB), stirred tank bioreactor (STR), and modified stirred tank bioreactor (MSTR) were used in the study. N-alkylamides were identified and validated using High-performance liquid chromatography (HPLC) and mass spectrometry (MS) based detection and quantification methods. Additionally, the study provides an appropriate kinetic assessment and modelling of biomass growth and metabolite accumulation in the adventitious roots of *S. paniculata* at a 3-L bioreactor level.

2. Materials and methods

2.1. Establishment of adventitious root cultures in suspension medium

S. paniculata plants (Fig. 1A) were in vitro established according to the tissue culture protocol developed by Singh and Chaturvedi (2010). The nodal segment (Fig. 1B) was inoculated in a test tube (Borosil, 25 × 150 mm) for root and shoot development on semi-solid basal medium consisting of ½ MS (Murashige & Skoog, 1962; only macronutrients reduced to half strength) with 5 % sucrose and 0.8 % agar (Fig. 1C). The inoculated nodal segments were incubated at a 16/8 h light/dark photoperiod at 25 ± 2 °C for 28 days. The nodal segment along with adventitious roots induced at its base (Fig. 1D) was detached from the newly developed axillary shoot and washed thoroughly with sterile distilled water to remove the traces of agar. To establish root suspension cultures, these adventitious roots along with the nodal segment explant was transferred to 250 ml shake flasks containing 100 ml of liquid MS basal medium with 3 % sucrose (control) (Fig. 1E). The medium pH was maintained at 5.8 throughout the experiments. In another experiment, to see the effect of auxins on adventitious root proliferation, the liquid basal medium was variously supplemented with 1, 2.5, 5, 10 μM concentrations of auxins, like IBA/IAA/NAA and 3 % sucrose. The root biomass growth was monitored for FW and DW after 21 days of culture. Additionally, the shoots that proliferated from the axillary-bud present on the nodal segment explant was also detached and transferred to a semi-solid basal medium (½ MS + 5 % sucrose) for further shoot development; the nodal segment explants from these in vitro shoots were utilized for additional experimental cycles.

2.2. Effect of carbon source on adventitious root proliferation

A carbohydrate supply in growth medium is required to fulfil the necessary energy demands and to maintain the medium osmotic balance

during biomass growth (Paiva Neto et al., 2003). This experiment analysed the influence of different carbon sources, including disaccharides (maltose and sucrose), monosaccharides (glucose and fructose) and sugar alcohol (mannitol) on root biomass growth during suspension culture. Different carbon source was utilized at 3 % concentration to liquid MS basal medium (100 mL) in a shake flasks of 250 mL capacity. MS Basal suspension medium with 3 % sucrose served as control. To this an inoculum size of 0.9 g fresh weight (FW) of *S. paniculata* adventitious roots were inoculated. The cultures were incubated in the above-mentioned conditions for 3 weeks and monitored regularly. Three samples of each carbon source were harvested after the culture period to analyse the effect of these carbon sources on root biomass growth in terms of fresh and dry weights.

2.3. Effect of medium salt strength and sucrose concentrations on adventitious root proliferation in liquid medium

Medium macronutrient salt strength and sucrose concentrations are two critical parameters responsible for root proliferation in *S. paniculata* (Singh & Chaturvedi, 2010). The effect of these parameters was assessed by inoculating 0.9 g roots (FW) of *S. paniculata* in 100 mL MS basal suspension medium in a 250 mL shake-flask containing 0.25x, 0.5x, 1.0x, 1.5x and 2.0x MS macronutrient salt concentrations, supplemented with 3 % or 5 % sucrose in each. MS Basal suspension medium with 3 % sucrose served as control. The experimente were repeated three times under similar conditions to verify the reproducibility of the results. After 3 weeks of inoculation, the cultures were harvested, and biomass growth was recorded by measuring the fresh and dry weights. The NAAs identification and quantification were performed through HPLC analysis, and metabolite productivity was calculated using Eq. (1).

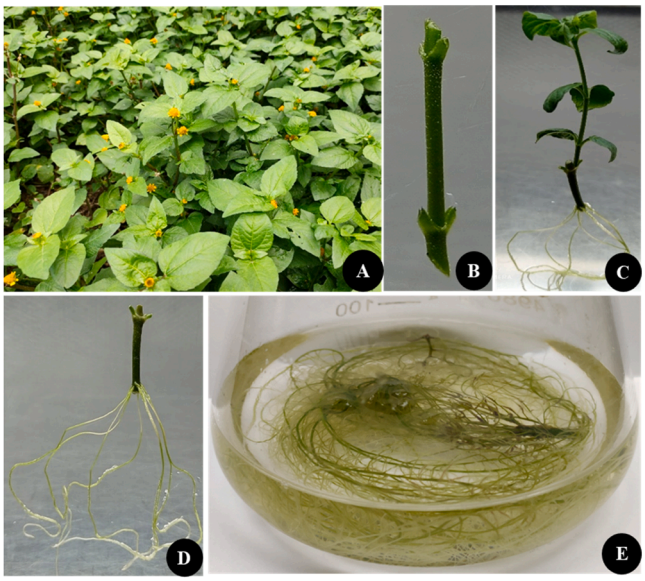

Fig. 1. Process of establishing adventitious roots from in vitro nodal segment cultures of *S. paniculata*, (A) Mother plant of *Spilanthes paniculata*, **(B)** A nodal segment containing 2 nodes inoculated on ½ MS + 5 % sucrose medium, **(C)** Same as B, showing shoot proliferation from axillary buds present at the node and root development from the base of the nodal explant, after 21 days of culture, **(D)** Adventitious roots to be inoculated in liquid medium of similar composition, **(E)** Adventitious roots grown in shake-flask after 21 days of inoculation.

$$\text{N}-\text{alkylamide productivity}(\mu\text{g/L}) = \text{Dry weight}(\text{g/L}) \times \text{N}-\text{alkylamide content}(\mu\text{g/gDW}) \quad (1)$$

2.4. N-alkylamide analysis of adventitious roots

2.4.1. Preparation of reference standards

Reference standard of the isomeric mixture, dodeca-2E,4E,8Z,10E, Z tetraenoic acid-isobutylamides (DTAI) and (2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide (spilanthol) were purchased from Phytolab GmbH & Co. KG (Germany) and Chromadex (USA), respectively. These commercially available reference standards were dissolved in methanol (HPLC grade) at various concentrations ranging from 0.5 mg/mL to 0.03125 mg/mL, followed by filtration using 0.22 µm syringe filters (Axiva, India). The standards were analysed in triplicate by HPLC for each concentration to check the repeatability and precision of the results. The linearity was determined by running different standard concentrations, and calibration curves were plotted separately for each reference standard, with the peak area (y-axis) plotted against concentration (x-axis) (Fig. S1). The straight-line equations and a correlation coefficient value (R^2) were generated for each reference standard. The R^2 value was used to assess the goodness of data fitting into the equation (Table S1).

2.4.2. Preparation of root extract and HPLC analysis

The HPLC sample preparation and analysis of N-alkylamides were performed according to the protocol established by Singh and Chaturvedi (2012), after slightly modifying the column temperature. The dried and powdered roots were mixed with hexane in the solvent to biomass ratio of 1:10 and kept on a shaker for 24 h at 25 °C. The solvent extract was collected, filtered (0.22 µm syringe filter), dried and redissolved in HPLC grade methanol to prepare the samples for HPLC analysis. The NAAs were detected and quantified using an HPLC (Shimadzu, Japan) system equipped with a 4.6 mm × 250 mm BDS Hypersil RP-18 column, having a pore size of 5.0 µm. The samples were filtered through 0.22 µm nylon syringe filters (Axiva, India) before being injected into the HPLC system. The mobile phase used for NAAs detection was a mixture of acetonitrile (Merck, India) and water (Merck Millipore) (90:10) at a flow rate of 0.5 mL/min and a column temperature of 60 °C. An aliquot of 10 µl of each sample was injected into the HPLC system with a 100 µl injection loop, and the metabolites were detected by a UV–Vis detector at 230 nm wavelength. The amount of spilanthol and DTAI was calculated through linear equations obtained from the calibration curves for each reference standard and reported as µg/g DW.

2.4.3. Validation of N-alkylamides using high resolution mass spectrometry (HRMS)

Mass spectrometry of NAAs was performed using an Agilent quadrupole mass analyser (UHPLC–QTOF-MS, Agilent Technologies, USA) with an ESI probe in positive ion mode. All prominent peaks obtained in the HPLC chromatogram were collected, concentrated, and re-dissolved in methanol for analysis. The presence of spilanthol and DTAI was confirmed by comparing the sample mass spectra with commercially available reference standards. The mass spectrometry data of reference standards and root samples were obtained in the scan mode range of 0–600 m/z. Mass Hunter Qualitative Analysis software (B.08.00 version, Agilent Technologies) was used for chromatogram processing and result analysis.

2.5. Adventitious roots culture in various bioreactor configurations

The culture of plant biomass in a bioreactor is crucial for scaling up the technology developed at the shake-flask level. Attempts have been made to reduce production costs by maximising plant biomass generation through bioreactor technology. However, the use of bioreactors for plants remains limited due to their distinct morphological and physiological characteristics. Special attention to bioreactor configuration is required to scale up the biomass and metabolites production. Sometimes, minor modifications are necessary to counteract the increasing shear stress and decreasing mass transfer rates (nutrients and oxygen) during biomass growth (Eibl & Eibl, 2008). In this experiment, roots of *S. paniculata* were cultured in three different bioreactors to check the suitable configuration for scaling up biomass production. Balloon type bubble bioreactor (BTBB), stirred tank reactor (STR) and modified stirred tank reactor (MSTR), were employed for root biomass generation. A minor modification to the STR was made to reduce shear stress and enhance root biomass growth (Fig. S2). All bioreactor systems, selected for the study, were of 3 L capacity with 50 % (1.5 L) working volume (Fig. 2A-I). The agitation and mixing in STR and MSTR were performed by rotating marine-type impellers, fitted into the bioreactors, at a fixed rpm. Since BTBB was free from any internal mechanical agitator, the nutrient mixing was accomplished by passing the air through the medium at a flow rate of 0.5 vvm. Each bioreactor containing 1.5 L of optimised liquid medium (pH - 5.8) consisting of ½ MS (only major salts reduced to half strength) basal medium with 5 % sucrose. The medium pH was set at 5.8 by 0.1 N HCl/NaOH. The root biomass of 6.0 g/L (FW) was inoculated into the medium to start the culture. The bioreactors were provided with 16/8 h photoperiod by LED lights (Philips, India) and the medium temperature was maintained at 25 ± 2 °C using Instech water chiller.

2.5.1. Optimisation of culture parameters in MSTR

Maintaining optimal growth parameters such as inoculum size, agitation rate and aeration is necessary for maximum root biomass proliferation and metabolite production in bioreactors (Arias et al., 2021; Lee et al., 2011; Pillai et al., 2015). To optimise these parameters, three experiments were conducted in 3 L MSTR containing 1.5 L of optimised autoclaved medium, ½ MS (only major salt concentrations reduced to half strength) basal medium with 5 % sucrose. Medium pH of 5.8 was maintained by the addition of 0.1 N HCl/NaOH. The cultures were provided with a 16/8 h photoperiod, and the temperature was maintained at 25 ± 2 °C. **(i) Inoculum size optimisation:** An inoculum size was optimised by inoculating adventitious root biomass (FW) of *S. paniculata* at different biomass concentrations, measured as 1.5, 3.0, 4.5, 6.0, 7.5 or 9.0 g/L, in 1.5 L sterilised medium in a 3 L bioreactor. The aeration and agitation rates were maintained at 0.5 vvm and 150 rpm, respectively. After 25 days of culture, fresh and dry weights were measured in each treatment, and N-alkylamides content and productivities were analysed. The growth index of different inoculum sizes was calculated using Eq. (2) given below. **(ii) Rate of aeration:** The bioreactor medium inoculated with 6.0 g/L (FW) (result obtained from the previous experiment) inoculum was aerated at different aeration rates as 0.25, 0.5, 0.75 or 1.0 vvm for 25 days. After the culture period, dry and fresh weights of the harvested biomass were measured, and N-alkylamides content and productivities were analysed. **(ii) Rate of**

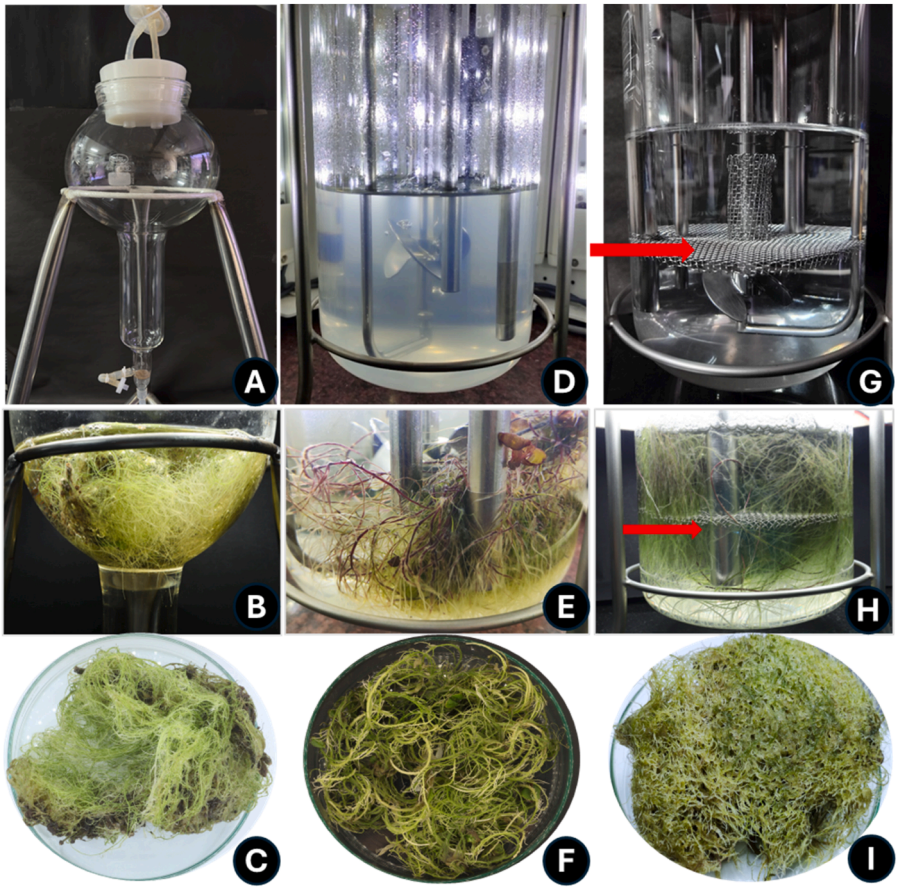

Fig. 2. Adventitious root culture in different configurations of bioreactors, (A) Balloon type bubble bioreactor (BTBB), **(B, C)** Biomass cultured and harvested from balloon type bubble bioreactor (BTBB), **(D)** Stirred tank bioreactor (STR), **(E, F)** Biomass cultured and harvested from stirred tank reactor (STR), **(G)** Modified stirred tank reactor (MSTR), (**H, I**) Biomass cultured and harvested from modified stirred tank reactor (MSTR) *The red arrow indicates the position of stainless-steel mesh in MSTR.

agitation: Optimisation of impeller rotation speed is also a crucial factor in managing the proper nutrient and oxygen distribution in the medium and the resulting shear stress in the bioreactor. The bioreactor containing 1.5 L of sterilised medium inoculated with 6.0 g/L (FW) as an inoculum was maintained at various agitation speeds of 90, 120, 150, 180 or 210 rpm. The culture was aerated at a rate of 0.5 vvm for 25 days. After the culture period, dry and fresh weights of root biomass were measured from each treatment, and N-alkylamides content and productivities were determined.

$$\text{Growth Index } = \frac{\textit{Final DW } (g/l) \ - \textit{Initial DW } (g/l)}{\textit{Initial DW } (g/l)} \tag{2}$$

2.6. Measurement of volumetric gas/liquid mass transfer coefficient (k_La) of STR and MSTR

The volumetric gas-liquid mass transfer coefficient (k_La) is a crucial factor influencing the oxygen transfer rate (OTR) in many aerated biochemical processes. In the last five decades, several reliable physical and chemical methods have been developed for k_La determination (Poughon et al., 2003). In the current study, the static gassing-out method was employed to investigate the effect of impeller speed (90, 120, 150, 180, and 210 rpm) on the volumetric mass transfer coefficient (k_La). The experiment was performed on 3 L capacity STR and MSTR, containing 1.5 L of optimised medium with pH set at 5.8. The bioreactors were maintained at 25 ^oC temperature and supplied with 0.5 vvm aeration. This method is based on monitoring the dissolved oxygen concentration in the medium after initial gassing-out with nitrogen (De Ory et al., 1999). Experiments were performed in triplicate for each impeller speed, in both configurations. The dissolved oxygen concentration was monitored every 90 s, during the aeration phase, using a dissolved oxygen sensor installed in the bioreactor system.

The following equation can express the oxygen transfer rate (OTR) in the liquid phase.

$$\frac{dC_L}{dt} = k_La\ (C^* - C_L) \tag{3}$$

Where - k_L: Mass transfer coefficient (L/h), a: specific surface of the interface (/L) C*: Saturated dissolved oxygen concentration or maximum possible dissolved oxygen concentration, C_L: Dissolved oxygen concentration at a time 't'.

On integrating the Eq. (3):

$$\ln(C^* - C_L) = -k_La.t \tag{4}$$

Eq. (4) suggests that negative slope of a plot between logarithmic value of (C*- C_L) and time will be k_La of the reactor (De Ory et al., 1999).

2.7. Kinetics of root biomass growth and N-alkylamide accumulation in MSTR

To describe the growth kinetics of biomass and metabolite production, 6.0 g (FW) of adventitious roots were inoculated in MSTR containing 1.5 L of ½ MS liquid medium with 5 % sucrose. The pH of the medium was adjusted to 5.8 before autoclaving, and the inoculated cultures were maintained under a 16/8 h light/dark photoperiod with aeration and agitation of 0.5 vvm and 150 rpm, respectively. The culture temperature was maintained at 25 ± 2 °C. During the 25-day culture period, roots were harvested at 3-day intervals to analyse their fresh and dry weights and N-alkylamides content. The metabolite productivity of each sample was also calculated according to Eq. (1).

The root dry weight biomass and N-alkylamides productivity data were subjected to construct the unstructured kinetic models for adventitious root growth and metabolite production from MSTR. The logistic equation was used to predict the root biomass growth according to the procedure followed by Sivarathnakumar et al. (2019). The logistic function was obtained from the following equation –

$$\frac{dX}{dt} = \mu_{max}X\left(1 - \frac{X}{X_{max}}\right) \tag{5}$$

Where – X = root biomass at time t, $\frac{dX}{dt}$ = the rate of change of root biomass (X) with time (t), μ_{max} = maximum specific growth rate of root biomass, X_{max} = carrying capacity of the environment, representing the maximum root biomass the environment can sustain indefinitely without deteriorating,

On integrating Eq. (5):

$$X\ (t) = \frac{X_0 * e^{\mu_{max}t}}{\left(1 - \left(\frac{X_0}{X_{max}}\right) * (1 - e^{\mu_{max}t})\right)} \tag{6}$$

Where – X_0 = initial root biomass at the time of inoculation

The kinetic model parameters for N-alkylamides production were obtained by constructing the unstructured model by fitting the experimental data in the Luedeking-Piret plot and modified Gompertz equations. The Luedeking-Piret equation was primarily developed to model the lactic acid production from *Lactobacillus delbrueckii* and applied to describe the polysaccharide production from *Xanthomonas campestris* B-1459. The N-alkylamides production here was simulated according to the procedure followed by Zhang et al. (2018).

$$\text{Ludeking} - \text{Piret equation}: \frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \tag{7}$$

Where – P is the N-alkylamide productivity (μg/L), α (μg/μg) and β (μg/μg/day) are empirical parameters representing the growth-associated and non-growth-associated components of product formation, respectively.

The logistic equation was substituted into the Eq. (7):

$$\frac{dP}{dt} = \alpha\mu_{max}X\left(1 - \frac{X}{X_{max}}\right) + \beta X \tag{8}$$

On integrating the Eq. (8):

$$P_t = P_0 + \alpha X_0\left(\frac{e^{\mu_{max}t}}{1 - \left(\frac{X_0}{X_{max}}\right) * (1 - e^{\mu_{max}t})} - 1\right) + \beta\frac{X_{max}}{\mu_{max}}\ln\left[1 - \left(\frac{X_0}{X_{max}}\right) * (1 - e^{\mu_{max}t})\right] \tag{9}$$

Where – P_t is the metabolite productivity at time t (μg/L), P_0 is the initial productivity (μg/L), t is the culture period (days), X_0 and X_{max} are the initial and the final root biomass DW (g/L), respectively, μ_{max} is the maximum specific growth rate (d^{-1}).

The Modified Gompertz model is also widely used to collect information on crucial parameters like initial lag time, maximum rate of product formation, and potential maximum product concentration (Dodić et al., 2012). The modified Gompertz equations were derived from the Gompertz equation, expressed as follows:

$$Y = a.\exp\{-\exp(b - ct)\} \tag{10}$$

Where – Y is the product produced (μg/L), 'a' is the maximum product concentration (μg/L), t is the culture period (day), b (no unit), and c (day^{-1}) are constants.

The Gompertz equation was modified to obtain the modified Gompertz equation to simulate the metabolite production, expressed as follows (Lo et al., 2010):

$$P = Pm * \exp\left\{-\exp\left[\left(\frac{r_{p,m} * \exp(1)}{P_m}\right) * (t_L - t) + 1\right]\right\} \tag{11}$$

Where: P is the product concentration (μg/L), P_m is the maximum product concentration (μg/L), $r_{p,m}$ is the maximum rate of product

formation (μg/L/day), and t_L is the lag phase period (days).

The commercially available OriginPro 2024b (OriginLab Corporation) software was used to fit the experimental data to various kinetic models using non-linear regression analysis, and the obtained statistical indicator (R^2) value directly from the software was used to assess the goodness of the curve fitting.

2.8. Statistical analysis

Each experiment was performed in triplicate to ensure the reproducibility of the results. The results analysis and graphical representation were performed using OriginPro 2024b (OriginLab Corporation) software. The mean ± standard deviation of the triplicate values is presented as the result. A Tukey test was performed to determine the statistical significance of the different treatments, with $p < 0.05$ considered statistically significant.

3. Results and discussion

3.1. Effect of IAA, IBA and NAA concentrations on adventitious root proliferation

The adventitious roots developed on semi-solid medium (½ MS + 5 % sucrose, only major salt concentrations reduced to half strength) were further proliferated in liquid MS basal medium supplemented with various concentrations (1.0, 2.5, 5.0 and 10 μM) of different auxins (IBA, IAA and NAA). The medium without any growth regulator (basal medium) was treated as a control for the experiment. The experimental results showed that root biomass decreased compared to the control when IAA and NAA were added to the medium. But, in IBA supplemented medium, the root biomass increased with increasing the auxin concentration upto 2.5 μM, where maximum root biomass (7.71 ± 0.44 g/L, DW) was produced (Fig. S3). The biomass yield declined on further increasing the IBA concentration beyond 2.5 μM. In all three auxin-supplemented media, callus proliferation from roots was observed later after 10 days of inoculation (Fig. S4). Whereas, control medium (basal medium) effectively supported almost equivalent amount of root biomass growth and development, compared to the auxin medium, and also did not cause root biomass to form calluses. Therefore, the basal medium (medium without auxin) was selected for further experiments to ensure phytochemical stability and reproducibility of the results.

3.2. Effect of carbon sources on root proliferation

Root induction and proliferation are high-energy processes that can only occur at the expense of available energy sources, primarily the carbohydrates. The nature of supplemented carbohydrates is also crucial in influencing the root biomass (Yaseen et al., 2013). This study investigated the effect of different carbon sources, including disaccharides (maltose and sucrose), monosaccharides (glucose and fructose) and sugar alcohol (mannitol) on adventitious root biomass. Among various carbon sources tested, sucrose proved to be an ideal energy source, yielding the highest biomass accumulation of 58.86 ± 4.91 g/L (FW) and 7.25± 0.44 g/L (DW). The medium supplemented with fructose (36.15 ± 1.89 g/L, FW and 4.97 ± 0.26 g/L, DW) and maltose (35.19 ± 3.62 g/L, FW and 4.26 ± 0.89 g/L, DW) yielded approximately similar amount of root biomass followed by glucose (27.49 ± 2.32 g/L, FW and 3.64 ± 0.35 g/L, DW) and mannitol (14.64 ± 3.52 g/L, FW and 1.84 ± 0.30 g/L, DW) (Fig. S5). The difference in root biomass generation is probably due to the nature of sugar molecules supplemented in the medium. Monosaccharides supplemented to the medium were readily available for growing biomass and depleted earlier, resulting in early maturation and reduced culture cycle. However, the disaccharides, mainly sucrose, consist of glucose and fructose. The glucose unit supported the initial biomass growth, and fructose was utilised in the later stage of the culture cycle, leading to a prolonged culture cycle and carbon source availability. Apart from working as an energy source, these carbohydrates also participate in molecular signalling. Sucrose is also the most common carbohydrate in the phloem sap of many plants and is often considered the preferred sugar for in vitro plant cell culture (Yaseen et al., 2013).

3.3. Medium salt strength and sucrose concentration

The optimal nutrient strength is crucial in regulating the growth of adventitious roots and secondary metabolite production. The adventitious roots of *S. paniculata* were cultured in liquid MS basal medium containing varying macronutrient strengths (¼ MS, ½ MS, 1 MS, 1.5 MS, and 2 MS) supplemented with 3 % or 5 % sucrose. The cultures were harvested after 3 weeks of incubation, and data on the dry weight yield of root biomass and production of N-alkylamides were collected (Table S2). The results indicated that root growth and metabolite accumulation were increased by reducing the salt strength to half. However, further reduction of salt strength to a quarter decreases the biomass yield and N-alkylamides production. Higher salt strengths, particularly 1.5 and 2 MS, also show a decline in biomass growth, likely due to changes in water potential and the inhibition of water and nutrient absorption from the medium. Unlike high salt concentration, the roots in ¼ MS medium though grew well but accomplished rapid maturity probably due to the lack of nutrients. The 5 % sucrose concentration was superior to 3 % for root biomass growth in each salt treatment medium. The highest biomass (8.64 ± 0.38 g/L, DW), spilanthol (615.58 ± 70.75) and DTAI (3034.56 ± 348.81) productivity was obtained in ½ MS basal medium supplemented with 5 % sucrose. Therefore, ½ MS (only major salt concentrations reduced to half strength) + 5 % sucrose medium composition was preferred for further experiments to achieve the maximum root biomass and N-alkylamide production.

The medium salt strength and sucrose concentration significantly influenced root biomass and metabolite production in *S. paniculata.* Similar results were observed in *Allamanda cathartica* L., *Hypericum perforatum* L., *Panax notoginseng, Raphanus sativus* and *Withania somnifera,* where the medium salts were reduced to half for increasing the root biomass production (Cui et al., 2010a; Fumie Betsui et al., 2004; Gao et al., 2005; Khanam et al., 2018; Sivanandhan et al., 2012). As our findings show, in some cases, a higher sucrose concentration also enhanced root biomass production. The higher sucrose concentrations of 5 % in *Eurycoma longifolia* and 6 % in *Podophyllum hexandrum* Royle were superior for biomass and metabolite production in suspension medium (Hussein et al., 2012; Rajesh et al., 2014).

3.4. N-alkylamide analysis through HPLC and mass spectrometry

The N-alkylamides (spilanthol and DTAI) in the adventitious roots of *S. paniculata* were analysed using HPLC and mass spectrometry (Fig. 3A-F). The mobile phase ratio of 90:10 acetonitrile: water, with a flow rate of 0.5 mL/min was appropriate for satisfactory detection and peak separation of metabolites. The HPLC chromatogram obtained two prominent peaks at retention times of 6.6 and 6.9 min (Fig. 3E). The peak at 6.6 min was found to be consistent with the retention time of commercially available spilanthol standard (Fig. 3A), confirming the presence of spilanthol in the root sample. Similarly, the prominent peak at 6.9 min was also found to be consistent with the retention time of commercially purchased DTAI standard (Fig. 3C). The HPLC peaks were collected, concentrated, and further analysed using high resolution mass spectrometry (HRMS-ESI) in positive ion mode. The characteristic mass spectrum of both the standards and in vitro root samples was obtained. The *m/z* values of both the metabolites and their dimers are mentioned in Table S3. The *m/z* values of 222.185 and 248.202 obtained in the spectrum of spilanthol (Fig. 3B) and DTAI standards (Fig. 3D) are due to the formation of a protonated molecule. However, the *m/z* values of 443.363 and 496.394 are due to the protonation of dimers of both the

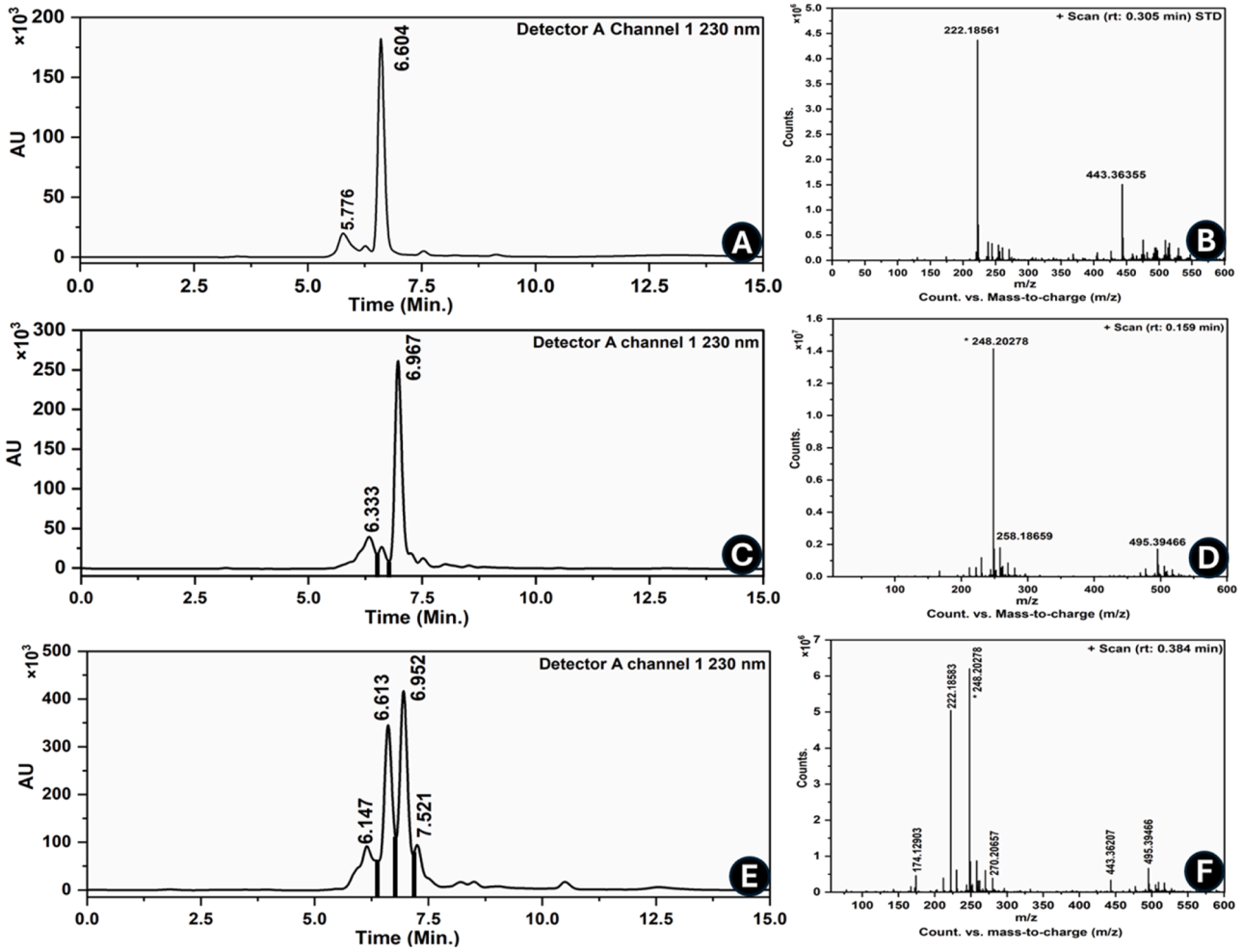

Fig. 3. HPLC and mass spectrometry analysis showing the presence of spilanthol at a retention time of 6.6 min and DTAI at 6.9 min along with their respective *m/z* values, (A, B) Spilanthol standard, (C, D) DTAI standard and, (E, F) In vitro generated adventitious roots.

reference standards. The identical *m/z* values corresponding to the protonated monomer and dimer of both compounds were obtained in the mass spectrum of root sample, confirming the presence of these molecules in the in vitro generated adventitious roots of *S. paniculata* (Fig. 3F). Like our findings, similar *m/z* values of spilanthol and DTAI monomer have been reported by Rajendran and Chaturvedi (2017) and Goey et al. (2012), in their studies. The quantification of these metabolites from in vitro root samples was performed using equations obtained through the linear curve plotted between known concentrations of standards and their respective peak areas.

3.5. *In vitro culture of adventitious roots of S. paniculata in bioreactor*

Owing to unique root morphology and physiology, special attention is required during their culture in a bioreactor. In this experiment, adventitious roots of *S. paniculata* were cultured in three different bioreactor configurations to promote their mass proliferation. The results indicate that the modified stirred tank reactor (MSTR) was the most suitable bioreactor configuration, producing the highest fresh weight (FW) root biomass of 73.34 ± 2.20 g/L and dry weight (DW) root biomass of 10.01 ± 0.29 g/L (DW). The second most effective bioreactor configuration was the stirred tank reactor (STR), producing 64.66 ± 1.6 g/L (FW) and 8.13 ± 0.31 g/L (DW) of root biomass, followed by balloon type bubble bioreactor (BTBB) with a biomass yield of 57.72 ± 2.13 g/L (FW) and 6.93 ± 0.45 g/L (DW) (Fig. S6). Across all the bioreactor configurations, the spilanthol and DTAI contents were 72.43 ± 2.58 μg/g DW and 362.75 ± 8.84 μg/g DW of root biomass. However, the overall productivity of these metabolites varies. The highest productivities of spilanthol and DTAI per litre of the medium were 720.44 ± 27.45 μg/L and 3608.03 ± 92.81 μg/L, respectively, from dry root biomass obtained in MSTR.

Out of the three bioreactor configurations tested, the modified stirred tank reactor (MSTR) was found to be the most efficient design for culturing and proliferating the adventitious roots of *S. paniculata.* The metabolite productivity was also highest in this reactor design. The superiority of MSTR over other configurations is due to the additional stainless-steel mesh fitted inside the glass vessel. The mesh reduces the shear stress and root entanglement by providing support to the growing roots and separating them from continuously rotating shaft and impellers. The inherent advantage of using stainless steel to modify the STR is that it not only reduces the shear stress but also improves the hydrodynamics of the bioreactor by increasing the volumetric mass transfer coefficient (k_La) value from 14.49 to 15.64 at 150 rpm (Fig. 4A, B). The values of k_La at different rpm in MSTR and STR also follow similar trends (Fig. 4C, D). In one published report, the bioreactors were modified using polyurethane foam (PUF) discs for hairy root cultures. However, the mass transfer hindrance and stability of the foam were the major concerns for the large-scale production of hairy root biomass. The lower biomass growth in BTBB is probably due to the lack of an efficient mass transfer system. Thus, modifying stirred tank reactors, using stainless steel mesh inside the glass vessel to provide support to the growing root, has proved advantageous in overcoming the problems of low biomass

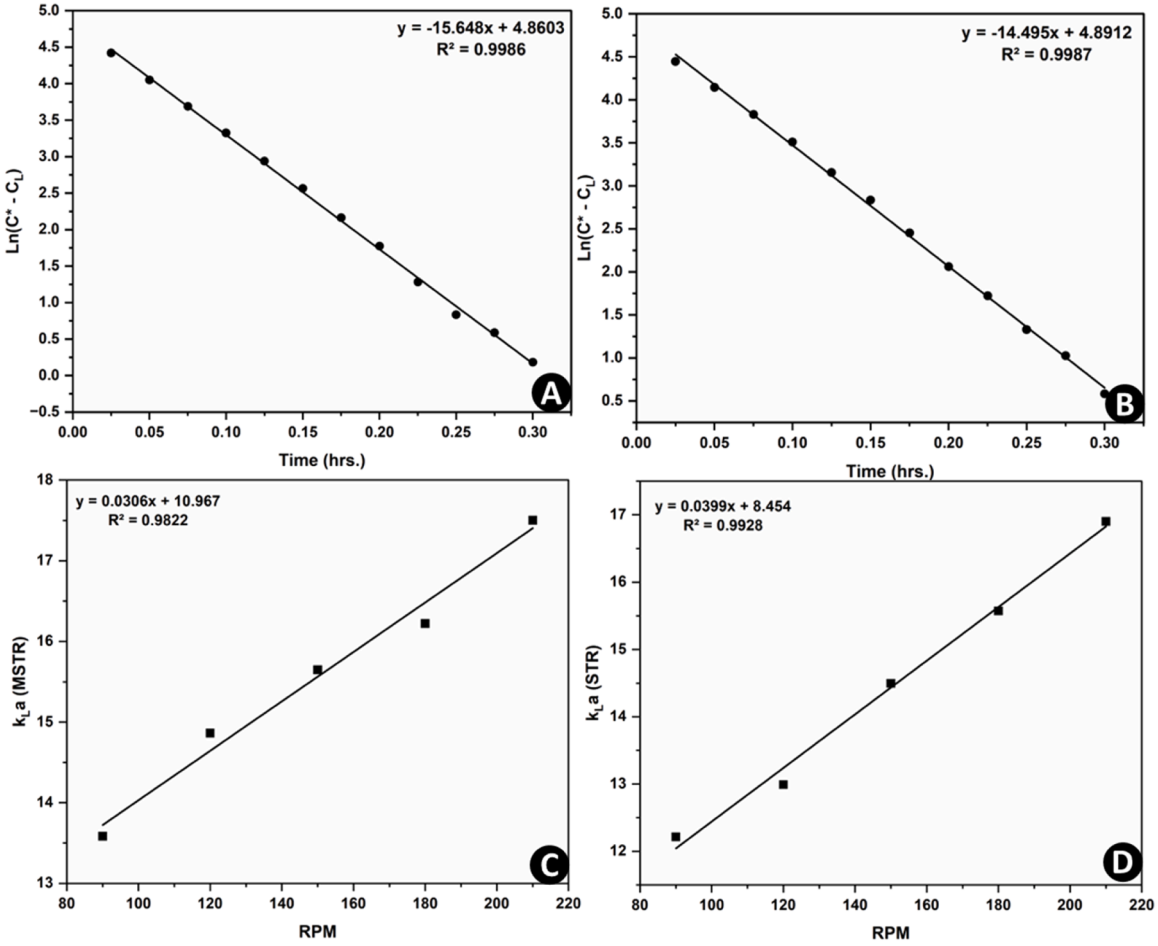

Fig. 4. Measurement of volumetric gas/liquid mass transfer coefficient (k_La) of STR and MSTR, (A) Plot between Ln (C^*-C_L) vs. time in modified stirred tank reactor at 150 rpm, **(B)** Plot between Ln (C^*-C_L) vs. time in stirred tank reactor at 150 rpm, **(C)** Relationship between k_La value with RPM in modified stirred tank reactor, **(D)** Relationship between k_La value with RPM in stirred tank reactor.

productivity, limited mass transfer and high shear stress (root entanglement) existing in conventional bioreactors. Although MSTR resolves the central issue of root entanglement, a uniform transfer of nutrients and oxygen but still there is a challenge during vigourous root biomass growth that restrict its growth in some regions of the bioreactor.

3.5.1. Optimisation of process parameters for MSTR

Three important process parameters, inoculum size, aeration rate and agitation speed, were optimised for MSTR to maximise the root biomass growth of *S. paniculata.* The inoculum density significantly improves the root biomass and metabolite production in the bioreactor. The biomass concentrations, measured at 1.5, 3.0, 4.5, 6.0, 7.5 or 9.0 g/L, were inoculated in 3 L MSTR containing 1.5 L optimised rooting medium. Fresh and dry weights of root biomass increased with an increase in inoculum size. The maximum root biomass yield obtained was 73.34 ± 2.20 g/L (FW) and 10.01 ± 0.29 g/L (DW) at the inoculum size of 6.0 g/L (FW). The highest growth index of 11.22 ± 0.37 was also obtained at this inoculum size. The biomass growth was negatively affected by further increase in inoculum size. The higher inoculum size generally results in a shorter lag phase but an early onset of decline and senescence, thereby reducing the overall biomass and metabolite production. The biomass growth rate per day also followed a similar trend, reaching a maximum of 2.93 ± 0.07 g/L/d at an inoculum size of 6.0 g/L (FW) and decreasing further on increasing inoculum density (Table S4). The productivity of spilanthol (715.98 ± 44.29 μg/L) and DTAI (3509.69 ± 261.23 μg/L) per litre of medium was also observed to be maximum at an inoculum size of 6.0 g/L (FW). A similar result was obtained by Lee et al. (2011) during adventitious root culture of *Eleutherococcus koreanum* Nakai in bioreactor. They received the highest percentage of dry weight at an inoculum size of 5.0 g/L (FW). Cui et al. (2010b) also reported that an inoculum density of 6.0 g/L (FW) is suitable for biomass production, and accumulation of phenolics and flavonoids during bioreactor culture of *Hypericum perforatum* L.

The optimisation of aeration rate is crucial to control the effect of gaseous composition on root biomass growth. At various aeration rates of 0.25, 0.5, 0.75 or 1.0 vvm, although there were no significant differences in the dry weights of root biomass, the fresh weights showed a considerable change in root biomass on increasing aeration. The maximum root biomass yield of 72.03 ± 4.30 g/L (FW), and productivities of Spilanthol (697.35± 10.25 μg/L) and DTAI (3501.53 ± 57.15 μg/L) were obtained at 0.5 vvm. The fresh weight yield and productivity decreased on further increasing the aeration rate (Table S5). The increase in aeration beyond 0.5 vvm causes high foaming and shear stress, which likely reduces the biomass and N-alkylamide production due to low oxygen transfer and induction of early senescence in the culture system. These findings suggest that an aeration rate of 0.5 vvm was suitable for maximum biomass and N-alkylamides production during adventitious root culture of *S. paniculata* in bioreactor. Wang et al. (2015) reported that 0.5 vvm is the most effective aeration rate to produce maximum ginsenosides and polysaccharides from adventitious roots of *Panax quinquefolium* L in bioreactor. In another study, 0.5 vvm was found to be an ideal aeration rate for producing maximum saponins and polysaccharides from adventitious root cultures of *Panax ginseng* (J. Wang et al., 2015).

The rate of agitation is also one of the most vital parameters for root culture in stirred tank bioreactors since it controls the oxygen, heat and nutrient movement between the medium and cell's surface, air fragmentation into tiny bubbles to increase the gas-liquid contact area and overall homogeneity of the medium (Abdella et al., 2020). Various agitation speeds of 90, 120, 150, 180 or 210 rpm have been investigated for maximum root biomass growth and N-alkylamides production in *S. paniculata*. The results indicated that biomass and metabolite production increases with an increase in agitation speed. The maximum root biomass yield of 72.74 ± 6.14 g/L (FW), and productivities of spilanthol (720.44 ± 27.45 μg/L) and DTAI (3608.03 ± 92.81 μg/L) were obtained at 150 rpm (Table S6). The biomass growth and metabolites productivities were reduced at a higher agitation rate due to higher shear stress exerted by impeller rotation. Therefore, an optimum agitation speed of 150 rpm was used for further experiments in MSTR.

3.6. Kinetics of adventitious root growth and N-alkylamides production

The kinetic studies were performed to determine the relationship between biomass growth and metabolite production during adventitious root cultures of *S. paniculata* in MSTR. The changes in root biomass growth and N-alkylamide production were monitored at every 3-day intervals. As shown in Fig. 5A, the growth of adventitious root biomass in MSTR typically follows a sigmoid curve. Minimal growth was observed during the first 3 days of culture due to an initial lag phase. The root cultures in MSTR enter the exponential phase on 5th day of inoculation and maximum biomass growth reached on 24th day of culture, and afterwards, it decreases. The specific growth rate (μ) for root biomass is a straightforward method to calculate the growth period. It was determined from the slope of a curve between ln (X_t/X_0) vs time (days), where X_t (g/L) is the root biomass (DW) per litre at different time intervals, and

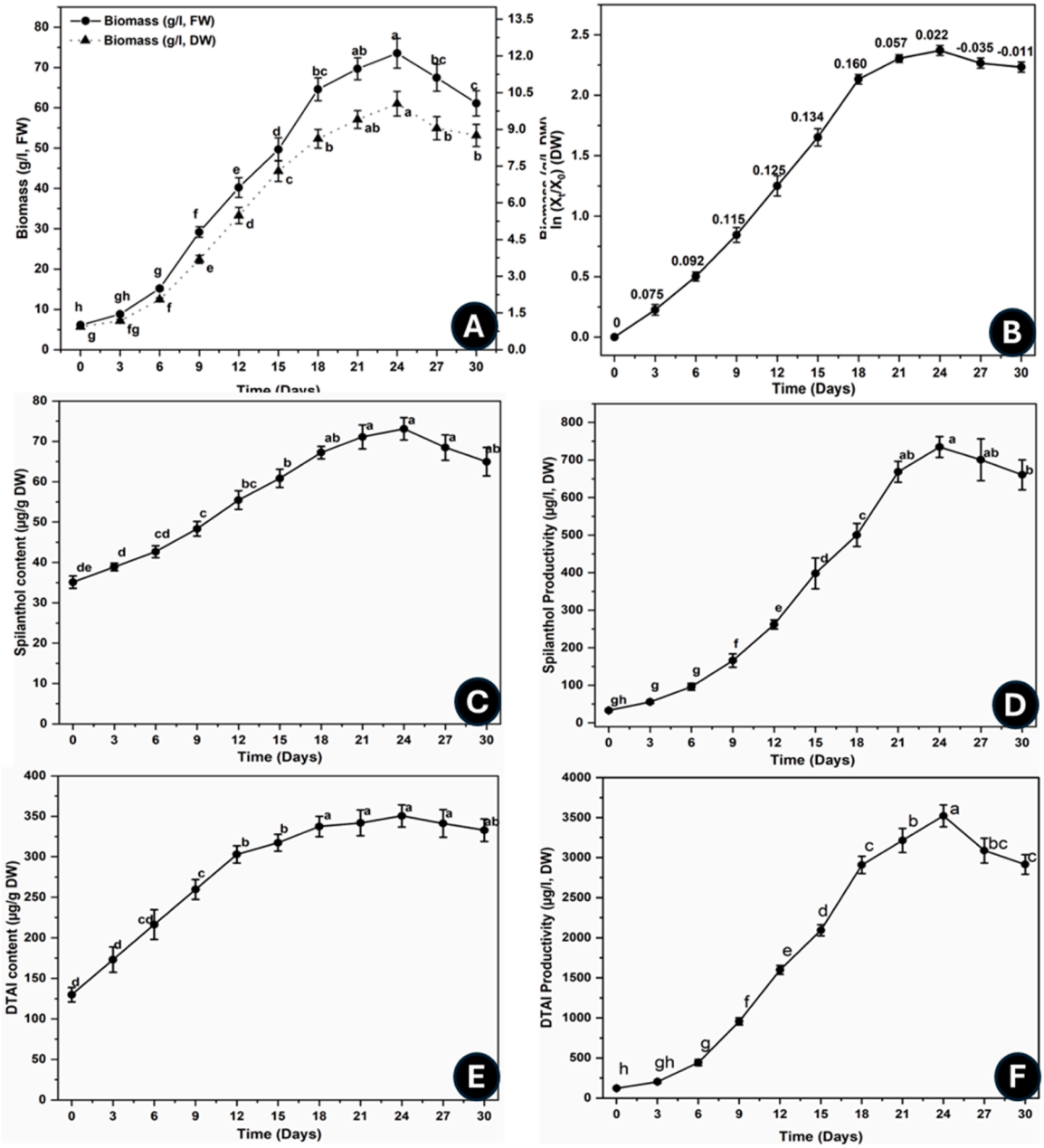

Fig. 5. Change in root biomass, N-alkylamides content and productivity during adventitious root suspension culture of *S. paniculata* in MSTR, (A) Fresh weight and dry weight harvested at a 3-day interval, (B) A graph between ln (X_t/X_0) of dry weight and time (days), (C) Total spilanthol content, (D) Total spilanthol productivity, (E) Total DTAI content and, (F) Total DTAI productivity.

X_0 (g/L) is the initial root (DW) concentration (Pachauri & Chaturvedi, 2025; Reiniati et al., 2017). The lowest μ of 0.075 /day was observed between 0 and 3 days, representing the initial lag phase of the culture. Subsequently, the μ increases rapidly and reaches the maximum of 0.160 /day on the 18th day of culture, indicating the exponential growth rate of root biomass. From 19th day onwards, μ decreases, indicating that biomass growth is reduced due to less nutrient availability and other internal factors. The specific growth rate is negative (- 0.035 /day) from 25th day onwards, indicating that root culture has entered the senescence phase of biomass (Fig. 5B). The results suggested that adventitious roots actively grew during 15th to 18th days after inoculation, and the maximum root biomass was achieved on 24th day of culture and then there was a decline.

The logistic equation is frequently used to describe the growth pattern of many biological systems. This model does not include any nutrient consumption parameter, considering that sufficient nutrient is available for the cells to grow in a limited space (Dodić et al., 2012). The dry weight data from the biomass growth curve (Fig. 5A) was used to fit into Eq. (6) to construct the adventitious root growth kinetic model in MSTR. OriginPro. (2024b) software was employed to calculate the maximum specific growth rate (μ_{max}) and root biomass concentration (X_{max}), which were 0.162/day and 11.52 g/L, respectively. A fitted curve was generated, and calculated values are consistent with the experimental values (Fig. 6A). The calculated parameter values were substituted into Eq. (6) to derive a new Eq. (12) for estimating the biomass concentration at different time intervals. The value of R^2, as a measurement of goodness of fitting for the proposed model, was 0.948. After carefully examining the experimental and predicted values of root biomass, the logistic equation was found to be appropriate for describing the batch culture cycle of adventitious roots of *S. paniculata* in MSTR.

$$X\ (t) = \frac{0.753\ e^{0.162t}}{((0.934 + \ 0.065e^{0.162t}))} \tag{12}$$

Where - *X* is the adventitious root biomass concentration (g/L) at time t.

The production of N-alkylamides from adventitious root cultures of *S. paniculata* was modelled by Leudeking-Piret (L-P) and modified Gompertz equations. The changes in metabolite productivity over time were investigated to understand the characteristics of spilanthol and DTAI production. As shown in Fig. 5C, total spilanthol content was not much significant during the initial 6 days of culture, followed by a gradual increase and reached a maximum on 24th day of culture. On the other hand, DTAI content rapidly increases immediately after inoculation and reaches a maximum on 24th day of culture (Fig. 5E). The productivity of spilanthol (Fig. 5D) and DTAI (Fig. 5F) increases with the culture period, and maximum productivity was on 24th day of culture. The maximum productivity calculated for spilanthol and DTAI was 734.61 ± 27.86 μg/L and 3522.24 ± 137.36 μg/L, respectively.

The L-P regression equation is often used to determine the relationship between biomass growth and metabolite production. The metabolite productivity data was fitted into Eq. (9) to construct the kinetic model for spilanthol and DTAI productivity from adventitious roots of *S. paniculata*. Various kinetic parameters (P_0, α and β) were calculated and fitted curves were generated for spilanthol (Fig. 6B) and DTAI productivity (Fig. 6C). The value of R^2 as a measurement of goodness of fitting for the proposed model was 0.929 for spilanthol and 0.956 for DTAI. The regression analysis indicated that the constructed kinetic models and their derived equations can predict the synthesis of spilanthol and DTAI during MSTR culture of adventitious roots of *S. paniculata*. The L-P model also indicated whether the metabolite production is growth or non-growth associated by calculating the values of α and β. The value of α would be non-zero, and that of β would be zero for growth-associated or vice-versa in non-growth associated metabolite production. In our study, the value of α is 58.80 for spilanthol and 345.83 for DTAI production. However, the value of β is 1.04 for spilanthol and −0.50 for DTAI production. These values suggest that production of spilanthol and DTAI is partially growth-associated phenomenon during the adventitious root cultures of *S. paniculata*. Initial metabolite productivity (P_0) was also calculated for spilanthol (27.403 μg/L) and DTAI (102.219 μg/L) through the L-P equation. The estimated values of various kinetic parameters (P_0, α and β) were

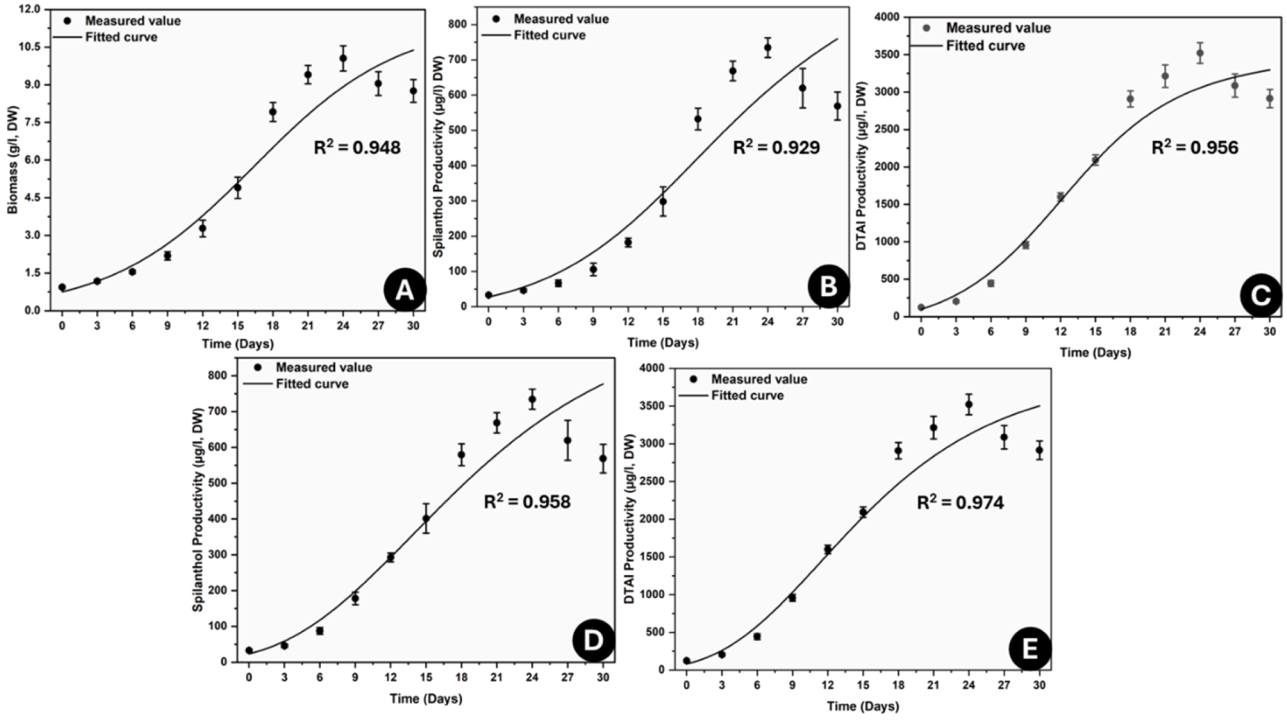

Fig. 6. Fitted curves generated for root biomass and N-alkylamides productivity data from MSTR culture, (A) root biomass data fitted to the Logistic equation, **(B, C)** Spilanthol and DTAI productivity data fitted to the Leudeking-Piret (L-P) equation, **(D, E)** Spilanthol and DTAI productivity data fitted to the modified Gompertz equation.

substituted into Eq. (9) and two new equations were derived for spilanthol (Eq. (13)) and DTAI (Eq. (14)) productivity calculations at different time intervals.

Ludeking-Piret model derived equation for spilanthol productivity –

$$P_t = 27.404 + \left(\frac{44.31\ e^{0.162t}}{(0.930 + 0.069e^{0.2620t})} - 44.31\right) + 74.25\ \ln\left[(0.934 + 0.065e^{0.162t})\right] \quad (13)$$

Where - P_t is the productivity of spilanthol at time t.

Ludeking-Piret model derived equation for DTAI productivity –

$$P_t = 102.21 + \left(\frac{260.65\ e^{0.162t}}{(0.9346 + 0.065e^{0.162})} - 260.65\right) - 35.88\ln\left[(0.9346 + 0.065e^{0.162t})\right] \quad (14)$$

Where - P_t is the productivity of DTAI at time t.

The L-P model has been widely explored to construct the metabolite production kinetic models in plant cell/tissue cultures. Xu ZhiRong et al. (2019) reported the kinetics of paclitaxel production from the cell suspension culture of *Taxus chinensis* var. mairer using the L-P equation. In another study, production of triterpenoid saponins and polysaccharides from adventitious root cultures of *Lessertia frutescens* was successfully modelled using this equation (Wang et al., 2024). Our present study was also consistent with previous reports on modelling plant metabolite production. It can reasonably predict the spilanthol and DTAI production from adventitious root cultures of *S. paniculata*.

The modified Gompertz equation was also applied to model the spilanthol and DTAI production from adventitious root cultures of *S. paniculata*. According to this model, an initial lag phase (t_L) of 3.3 ± 1.0 days for spilanthol and 2.9 ± 0.6 days for DTAI production was observed, and the maximum rate of metabolites production ($r_{p,m}$) was calculated as 33.66 ± 3.15 μg/L/d and 170.20 ± 13.05 μg/L/d for spilanthol and DTAI, respectively. Maximum metabolite concentration (P_m) was also calculated for spilanthol (963.760 μg/L) and DTAI (3914.709 μg/L) through the modified Gompertz equation. The experimental data were well fitted to Eq. (11), and the fitted curves were generated for spilanthol (Fig. 6D) and DTAI (Fig. 6E) productivity. The correlation coefficients obtained during non-linear regression analysis were 0.958 for spilanthol and 0.974 for DTAI production, indicating that the modified Gompertz equation can describe well the production of these metabolites during batch culture of adventitious roots of *S. paniculata*. Obtained kinetic parameters (t_L, $r_{p,m}$ and P_m) values were substituted in Eq. (11) and two new equations were derived for spilanthol (Eq. (15)) and DTAI (Eq. (16)) productivity calculations at different time intervals.

Modified Gompertz model derived equation for spilanthol productivity –

$$P = 963.760 * \{\exp[(-exp(0.094 * (3.302 - t) + 1)]\} \quad (15)$$

Where - P_t is the productivity of spilanthol at time t.

Modified Gompertz model derived equation for spilanthol productivity –

$$P = 3914.709 * \{\exp[(-exp(0.118 * (2.979 - t) + 1)]\} \quad (16)$$

Where - P_t is the productivity of DTAI at time t.

These models were validated by comparing the experimental data with the values predicted by logistic, Ludeking-Piret and modified Gompertz equations. The plot of these values follows the $y = x$ form. The statistical indicator (R^2) value was 0.929 for the logistic equation (Fig. S7A) used to model biomass production. The R^2 values for the Ludeking-Piret equation were 0.874 for spilanthol (Fig. S7B) and 0.924 for DTAI productivity (Fig. S7C). The modified Gompertz model also predicted the production of these compounds, and the statistical indicator values of the plot between experimental and predicted values were 0.893 for spilanthol (Fig. S7D) and 0.928 for DTAI productivity data (Fig. S7E). Since most of the values lie within the 95 % confidence band, concluding that these models can predict the behaviour of biomass and metabolite production from adventitious roots of *S. paniculata* under defined conditions. The study provides a comprehensive details about the optimized protocol for large-scale production of N-alkylamides from adventitious root cultures in bioreactor system. However, uniform transfer of nutrients and oxygen may still be a challenge during vigourous root biomass growth in pilot scale bioreactor, mainly due to filamentous morphology of these roots.

4. Conclusions

S. paniculata is widely used as a food supplement and food preservative in various traditional cuisines. The plant is used directly as a leafy vegetable and as a nutrient supplement in healthcare products by isolating its N-alkylamides (NAAs). Due to their diverse applications in food and pharmaceuticals, the plants are exploited on an industrial scale. The development of in vitro root biomass production in liquid medium provides an excellent opportunity to scale-up the product and process at large-scale. The outcome of the current study demonstrates the development of a suitable bioreactor configuration for large-scale production of root biomass and N-alkylamides. A reduced macronutrient concentration (½ MS, with only major salts reduced to half strength) in basal medium (devoid of plant growth regulators) containing higher sucrose (5 % w/v) concentration was found to be the best for root proliferation as well as metabolite production. The HPLC analysis of in vitro generated adventitious root biomass sample shows a peak for spilanthol at a retention time of 6.6 min and for DTAI at 6.9 min. The *m/z* values of 222.185 and 248.202 obtained during HRMS analysis correspond to the formation of the protonated molecules of spilanthol and DTAI. However, the *m/z* values of 443.363 and 495.394 are due to the protonation of the dimers of both metabolites. During scale-up studies, maximum root biomass of 10.01 ± 0.29 g/L (DW) was obtained in MSTR as compared to that obtained in STR (8.13 ± 0.31 g/L, DW) and BTBB (6.93 ± 0.45 g/L, DW). The optimisation of MSTR process parameters resulted in inoculum density of 6.0 g/L (FW), agitation speed of 150 rpm and aeration rate of 0.5 vvm were the best. The highest biomass yield of 73.34 ± 2.20 g/L (FW) and 10.01 ± 0.29 g/L (DW), and productivities of spilanthol and DTAI as 720.44 ± 27.45 μg/L and 3608.03 ± 92.81 μg/L, respectively, were obtained in MSTR. The kinetic analysis of root biomass production showed that maximum specific growth rate was achieved between 15–18 days of culture. However, the highest root biomass and metabolite productivity was obtained on 24th day of culture in MSTR. The logistic model for root biomass production provided X_{max} and μ_{max} values of 11.53 g/L and 0.162 /day, respectively. Unstructured models (Ludeking-Piret and modified Gompertz) were successfully applied for the mathematical explanation of spilanthol and DTAI production during batch operation in MSTR. The experimental data of metabolite productivity was fitted better (regarding R^2 value) in the modified Gompertz equation than the Ludeking-Piret equation. The kinetic models also showed that metabolite production in the adventitious root cultures of *S. paniculata* is a partially growth-associated phenomenon. The conclusions of this research will provide a reference for in vitro root biomass production of *S. paniculata* to overcome the seasonal and geographical restrictions and further scaling up of the medicinally important metabolite production process from adventitious root cultures of *S. paniculata* in pilot-scale or commercial-scale bioreactors.

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Data availability

The data generated in this study will be available on request. The supporting data of this study are available in this article's supplementary material.

Ethical Statement- studies in humans and animals

The authors declare that the work reported in this paper is **NOT** conducted on humans or animal systems. The study in this report is conducted on plant system only.

CRediT authorship contribution statement

Krishna Kant Pachauri: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Rakhi Chaturvedi:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have NO known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2025.101567.

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