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ORIGINAL PAPER

Manipulation of culture strategies to enhance capsaicin biosynthesis in suspension and immobilized cell cultures of *Capsicum chinense* Jacq. cv. Naga King Chili

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Abstract Manipulation of culture strategies was adopted to study the influence of nutrient stress, pH stress and precursor feeding on the biosynthesis of capsaicin in suspension and immobilized cell cultures of *C. chinense*. Cells cultured in the absence of one of the four nutrients (ammonium and potassium nitrate for nitrate and potassium stress, potassium dihydrogen orthophosphate for phosphorus stress, and sucrose for sugar stress) influenced the accumulation of capsaicin. Among the stress factors studied, nitrate stress showed maximal capsaicin production on day 20 (505.9 ± 2.8 μg g^{-1} f.wt) in immobilized cell, whereas in suspension cultures the maximum accumulation (345.5 ± 2.9 μg g^{-1} f.wt) was obtained on day 10. Different pH affected capsaicin accumulation; enhanced accumulation of capsaicin (261.6 ± 3.4 μg g^{-1} f.wt) was observed in suspension cultures at pH 6 on day 15, whereas in case of immobilized cultures the highest capsaicin content (433.3 ± 3.3 μg g^{-1} f.wt) was obtained at pH 5 on day 10. Addition of capsaicin precursors and intermediates significantly enhanced the biosynthesis of capsaicin, incorporation of vanillin at 100 μM in both suspension and immobilized cell cultures resulted in maximum capsaicin content with 499.1 ± 5.5 μg g^{-1} f.wt on day 20 and $1{,}315.3 \pm 10$ μg g^{-1} f.wt on day 10, respectively. Among the different culture strategies adopted to enhance capsaicin biosynthesis in cell cultures of *C. chinense*, cells fed with vanillin resulted in the maximum capsaicin accumulation. The rate of capsaicin production was significantly higher in immobilized cells as compared to freely suspended cells.

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Introduction

The genus *Capsicum* consists of approximately 25 wild and 5 domesticated species. The five domesticated species are *Capsicum annuum, C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*. Of the domesticated species, *C. chinense* is the most pungent fruit type. *C. chinense* Jacq.cv. Naga King Chili is an important spice crop of India belonging to the family Solanaceae [1]. It has been acknowledged as the hottest chili in the world measuring 1,001,304 Scoville Heat Units (SHU) [2]. Nagaland government has patented this chili and registered as the proprietor with the Government of India, under Geographical Indication Registry. The Naga King Chili has received the attention of world scientific community due to its extremely high pungency and unique aroma [3]. The pungent principle of chili fruit are capsaicinoids. In nature, capsaicin and dihydrocapsaicin account for 90 % of the total capsaicinoids content in chili fruits [4]. These compounds are synthesized from L-phenylalanine through the phenylpropanoid biosynthetic pathway to render vanillylamine, which is ultimately linked to the branched fatty acid residues that are synthesized from L-valine or L-leucine to give the five capsaicinoid analogs (Fig. 1): capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin [5].

Capsaicin has a wide application in the food, medicine and pharmaceutical industries [6–9]. In recent years, various strategies to obtain high yields suitable for commercial exploitation have been investigated in plant cell cultures, efforts have focused on isolating the biosynthetic activities

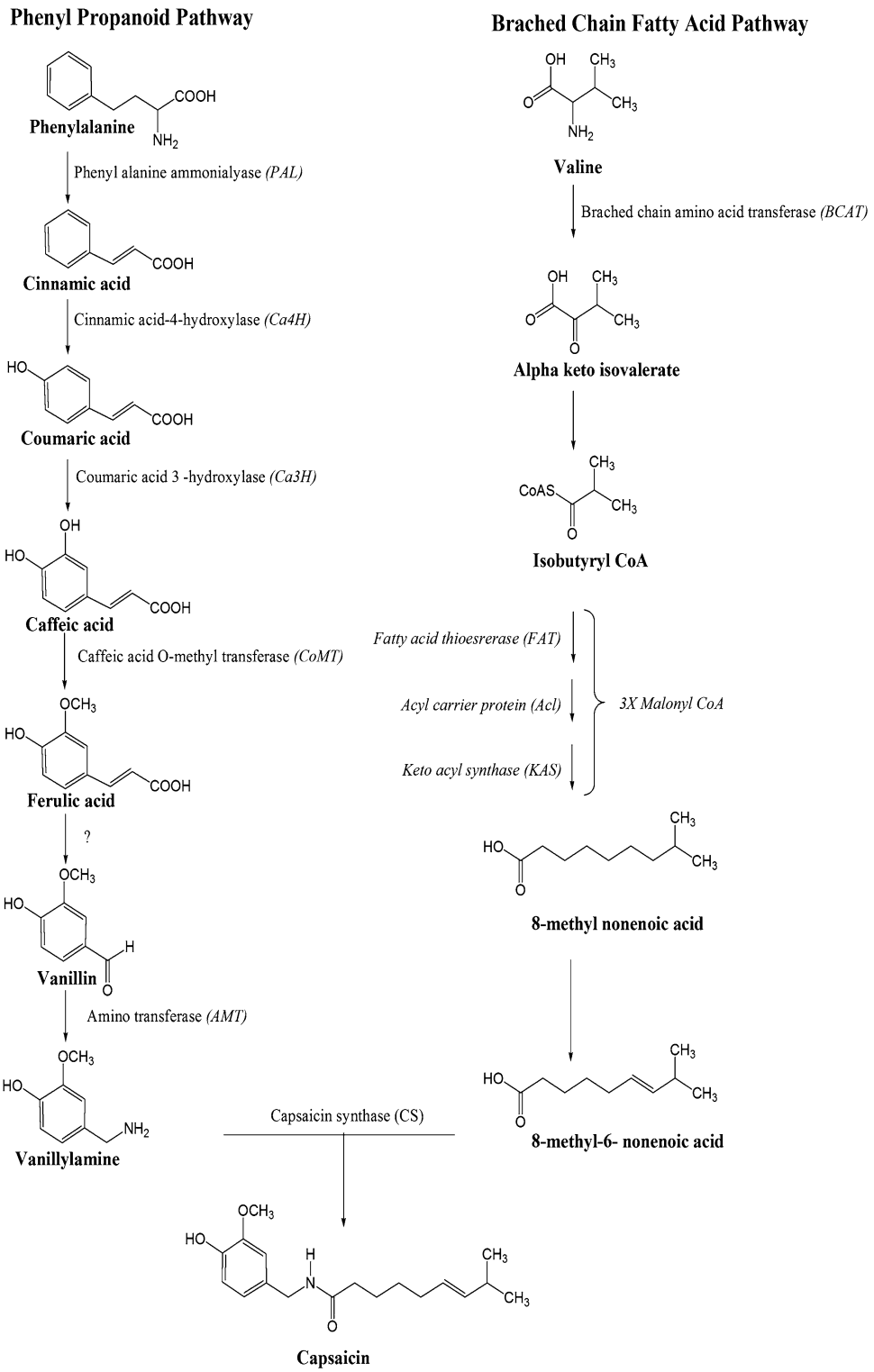

Fig. 1 Capsaicin biosynthetic pathway in *Capsicum* showing the principal enzymes and intermediates. *PAL* phenylalanine ammonia lyase, *Ca4H* cinnamic acid 4-hydroxylase, *Ca3H* coumaric acid 3-hydroxylase, *CoMT* caffeic acid O-methyltransferase, *AMT* aminotransferase, *BCAT* branched chain amino acid transferase, *FAT* fatty acid thioesterase, *AcL* acyl carrier protein, *KAS* β-ketoacyl synthase, *CS* capsaicin synthase

of cultured cells, achieved by optimizing the cultural conditions, selecting high-producing strains, and employing precursor feeding, transformation methods, and immobilization techniques [10]. More recently, genetic engineering and metabolic engineering have opened a new promising perspective for improved production in a plant or plant cell cultures [11, 12]. The biosynthetic capacity of in vitro cultured cells and tissues to produce capsaicinoids has been investigated by different workers using immobilized cell cultures, nutrient limitation, osmotic stress,

precursors, and elicitors [9, 13–16]. A decrease in the major nutrients results in increase in secondary metabolites [17]. The synthesis of secondary products such as alkaloids and phenolics is enhanced in post-exponential-phase cultures, when nutrient limitation of cell division becomes evident [18]. Lindsey proposed that the effect of nutrients such as nitrate or phosphate on secondary metabolic activity may act to a large extent by altering the activity of primary metabolic pathways. The stress factors such as nitrate and phosphate in the medium increase the capsaicin production in immobilized cells [13]. Addition of phenylalanine and isocapric acid (8-methylnonanoic acid) has been shown to increase significantly the yield of capsaicin in both immobilized and freely suspended cells of *C. frutescens* [19]. According to Lindsey [18], capsaicin accumulation in chili cell cultures is largely limited by precursor availability. Considerable increase in the amount of capsaicin was observed by supplementing the medium with Phenylalanine and Phenylpropanoids in suspension cell cultures of *C. annum* [20]. The optimal medium pH for plant cell cultures is usually adjusted between 5 and 6 and higher pH affects the solubility of the salt in the medium and thus their availability to the cells. It has been reported that lowering the medium pH, and thus changing the pH gradient between the cells and the medium, induced the release of alkaloids and their entrapment as ions in the extracellular milieu [21].

However, till date no attempt has been made on *C. chinense,* which is the most pungent species. Therefore, the aim of the present study was to investigate the influence of nutrient stress, pH stress and precursor feeding on the biosynthesis of capsaicin in suspension and immobilized cell cultures of *C. chinense*.

Materials and methods

Callus cultures

Seeds of *C. chinense* were obtained from Rüziephema village, Nagaland, India. The seeds were thoroughly washed in running tap water, then treated with 2 % Labolene (v/v) for 10 min and finally rinsed five times with distilled water. These were then surface sterilized with 0.1 % $HgCl_2$ for 5 min followed by several washes with sterile distilled water. The sterilized seeds were cultured in MS medium [22] containing 3 % (w/v) sucrose and 0.8 % (w/v) agar, the pH of the medium was adjusted to 5.8 before autoclaving. Friable callus was obtained from in vitro germinated seedlings in MS medium fortified with 2,4-dichlorophenoxyacetic acid (2,4-D; 2 mg/l) and N^6-furfuryladenine (Kinetin; 0.5 mg/l) as reported earlier [9].

Cell suspension

Cell suspension cultures were initiated from the callus and maintained in the same medium without agar. Cultures were agitated on the rotatory shaker (125 rpm) at temperature of 25 ± 2 °C; 14/10 h photoperiods with an irradiance of 62.2 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes and were routinely subcultured every 15 day.

Cell immobilization

Cells from 15-day-old suspension cultures were separated from the medium through filtration and suspended in a solution of 2.5 % sodium alginate. This was then extruded into calcium chloride (0.9 %) through sterilized pipette. The resulting beads were thoroughly washed several times with sterilized distilled water before transferring to the nutrient medium as described by Ravishankar et al. [13]. Cultures were agitated on the rotatory shaker under the same culture condition as described above.

Nutrient stress treatment

Both suspension and immobilized cells were subjected to nutrient stress by eliminating one of the essential nutrients of MS medium such as ammonium and potassium nitrate for nitrate and potassium stress, potassium dihydrogen orthophosphate for phosphorus stress, and sucrose for sugar stress.

pH stress treatment

To investigate the effect of medium pH on the production of capsaicin in *C. chinense* cell cultures, the culture medium was adjusted to different pH (3–8) using 1 N NaOH or 1 N HCl before autoclaving. Cultures were incubated on rotary shaker (125 rpm) under the same culture conditions of temperature and light described above. Capsaicin content was determined at 5-day interval in the 25-day culture cycle.

Treatment with precursors and intermediates

Freshly prepared solution of precursors and intermediates were added in the culture medium. Phenylalanine, vanillin and vanillylamine were dissolved in sterile distilled water, whereas cinnamic, *p*-coumaric, caffeic, and ferulic acids were first dissolved in a small volume of 1 M KOH, adjusted to pH 5.8 with 0.1 N HCl, and then sterile distilled water was added to make the desired volume. All solutions were prepared as 10 mM stocks, sterilized by filtration through Millipore membrane (0.22 μm), and

added to autoclaved culture medium to give a final concentration of 100 μM, as described by Nunez-Palenius and Ochoa-Alejo [20]. All experiments were carried out in 100-ml Erlenmeyer flasks containing 25 ml MS liquid medium/flask inoculated with 2 g callus (f.wt) of 15-day-old cell suspension cultures under the same culture conditions above. Cells were collected from the suspensions by filtration, at 5-day interval for 25 days and used for determination of capsaicin content.

Extraction, separation and quantification of capsaicin

Capsaicin was extracted from either cell or liquid medium (both for suspension and immobilized cell) following the method described by Nunez-Palenius and Ochoa-Alejo [20]. Thin layer chromatography (TLC) was carried out for the separation of capsaicin from other impurities. The TLC purified capsaicin was measured at 280 nm using Perkin Elmer Lambda 35 UV/Vis Spectrometer as reported earlier [9]. Capsaicin identity was further confirmed by High Performance Liquid Chromatography using Perkin Elmer series 200 equipped with a μBondapak C_{18} column (10 μm particle size, 300 × 3.9 mm), The mobile phase consisted of a binary mixture of methanol in water at 60:40 ratio as reported by Cooper [23]. Detection was at 280 nm, and the flow rate was maintained at 1 ml/min.

Statistical analysis

All experiments were carried out with three replicates each and data were analyzed using one-way analysis of variance (ANOVA, $P < 0.05$; Tukey (HSD) comparison of means) in JMP® version 7.0.1(SAS Institute, Cary NC). The significant differences among the means were assessed by Tukey's honestly significant difference (HSD) test used post hoc on significant findings.

Results and discussions

Effect of nutrient stress on capsaicin accumulation

In a series of preliminary experiment, the effect of nutrient stress on capsaicin production was investigated. The accumulation of capsaicin in the cells grown in the absent of one of the four nutrients viz. ammonium and potassium nitrate for nitrate and potassium stress, potassium dihydrogen orthophosphate for phosphorus stress, and sucrose for sugar stress, indicated that capsaicin synthesis proceeded at a relatively high rate in the medium lacking major nutrients as compared to the control. Production of secondary metabolites in cell cultures was found to be affected by nutrient limitation. Several workers have attempted to enhance capsaicin in *Capsicum* cell cultures by nutrient limitation [13, 18]. In general, a decrease in major nutrients increases secondary products but decrease biomass growth [17]. In the present investigation, nitrate stress showed maximal capsaicin production (Table 1). Maximum capsaicin content was obtained when the cells were grown in the medium devoid of ammonium nitrate. Capsaicin production increased twofold over the control with a total production of 345.5 ± 2.9 μg g^{-1} f.wt with biomass productivity of 0.25 ± 0.006 g/culture d.wt on day 10 in suspension cultures (Table 1) (Fig. 2). Further incubation of cells under stress treatment resulted in reduction of biomass productivity and capsaicin content

Table 1 Effect of nutritional limitation on capsaicin production (μg g^{-1} f.wt) in cell cultures of *C. chinense* during the growth cycle

Treatment	Days of culture				
	5	10	15	20	25
Suspension cultures					
Control	90.1 ± 5.7^{e}	137.5 ± 4.6^{g}	142.6 ± 4.2^{g}	152.6 ± 3.1^{g}	165.8 ± 2.8^{e}
MS minus KNO_3	132.9 ± 6.7^{d}	260.6 ± 5.8^{e}	310.1 ± 5.7^{c}	217.7 ± 5.8^{e}	120 ± 2.9^{f}
MS minus NH_4NO_3	129.5 ± 4.3^{d}	345.5 ± 2.9^{c}	222.8 ± 3.1^{e}	168 ± 3.4^{f}	102.8 ± 3.7^{g}
MS minus KH_2PO_4	156.3 ± 5.9^{c}	245.1 ± 2.8^{f}	184.9 ± 2.8^{f}	145.2 ± 5.7^{g}	110.5 ± 5.7^{gf}
MS minus sucrose	80.7 ± 5.7^{e}	95.4 ± 2.8^{h}	110.6 ± 2.9^{h}	85.3 ± 3.1^{h}	79.6 ± 5.7^{i}
Immobilized cultures					
Control	279.3 ± 11.5^{ab}	420.1 ± 8.7^{a}	388.5 ± 7.2^{b}	340.6 ± 5.7^{c}	296.3 ± 8.4^{d}
MS minus KNO_3	288.3 ± 7.2^{a}	297.3 ± 6.0^{d}	302.3 ± 3.9^{c}	385.6 ± 2.9^{b}	355.3 ± 2.9^{b}
MS minus NH_4NO_3	271.6 ± 4.4^{b}	362 ± 4.1^{b}	473.6 ± 4.1^{a}	505.9 ± 2.8^{a}	405.1 ± 2^{a}
MS minus KH_2PO_4	275.6 ± 2.9^{ab}	301.5 ± 5.7^{d}	270.3 ± 5.4^{d}	380.9 ± 5.7^{b}	320.1 ± 5.8^{c}
MS minus sucrose	159.3 ± 9.2^{c}	295.3 ± 8.4^{d}	317.3 ± 9.3^{c}	281.3 ± 4.6^{d}	285.3 ± 7.8^{d}

Data scored at 5-day interval between 5 and 25 day of culture, mean ± SE. HSD comparisons are significant when letters are different within columns (ANOVA, $P < 0.05$)

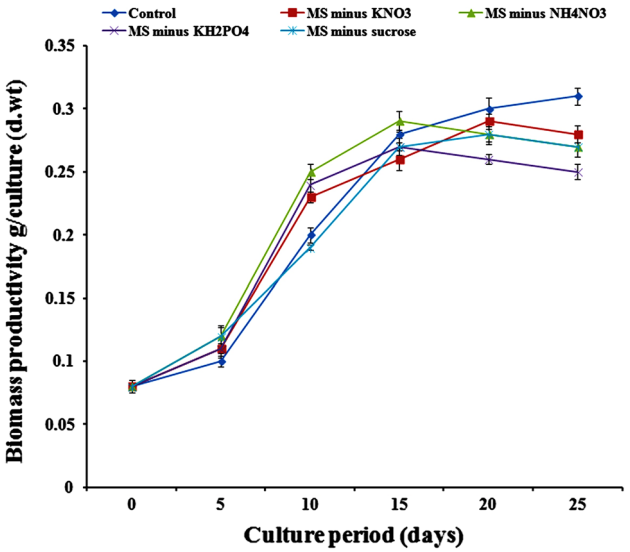

Fig. 2 Time profile of cell biomass productivity in suspension culture of *C. chinense* under nutrient stress conditions

(102.8 ± 3.7 μg g^{-1} f.wt). Prolonged exposure of cells to nutrient stress reduced cell biomass (0.28 ± 0.007 g/culture d.wt) compared to control cells (0.31 ± 0.007 g/culture d.wt) as observed in Fig. 2 on day 25 in suspension cultures. In the case of immobilized cultures, threefold increases with a total production of 505.9 ± 2.8 μg g^{-1} f.wt on day 20 were recorded (Table 1). In both suspension and immobilized cultures, nitrate stress enhanced capsaicin production; however, a delayed capsaicin production with higher capsaicin accumulation was obtained in immobilized cultures. Complete elimination of nitrate in cultures of *Chrysanthemum cinerariaefolium* induced twofold increases in pyrethrin accumulation in the second phase of culture [24]. Reduction in capsaicin content was also observed in both suspension and immobilized cells after certain optimal level is attained. This may be due to prolonged exposure of cells in nutrient deficient medium. Nitrogen concentration has been found to affect the level of proteinaceous or amino acid products in cell cultures. The ratio of ammonium to nitrate and total nitrogen has shown to markedly affect the production of secondary metabolites. Increased alkaloid production was obtained with an ammonium:nitrate ratio of 5:1 and 60 mM total nitrogen [25]. Ammonium ion as the sole source is usually undesirable, probably because under such situation, the pH of the medium has a tendency to fall below 5 during cultures. This drop in pH may restrict the availability of nitrogen [26, 27]. The general plant tissue culture medium has both nitrate and ammonium as source of nitrogen. Limitation of potassium by eliminating either potassium nitrate or potassium dihydrogen orthophosphate affected capsaicin accumulation. Potassium nitrate elimination showed slightly higher capsaicin accumulation as compared to potassium dihydrogen orthophosphate elimination in both suspension and immobilized cells (Table 1). Potassium plays an important role in cell metabolism. Potassium ions inhibit the enzymes such as the glycolysis enzyme pyruvate kinase [28]. Elimination of phosphate in suspension cultures slightly enhanced capsaicin production on day 10 (245.1 ± 2.8 μg g^{-1} f.wt), whereas a decrease in capsaicin biosynthesis was observed in immobilized cultures as compared to the control. Phosphate concentration in the medium can have a major effect on the production of secondary metabolites in plant cell cultures. Higher levels of phosphate were found to enhance the cell growth, but showed negative influence on secondary product accumulation [29]. Reduced phosphate levels induced the production of ajmalicine and phenolics in *C. roseus* cell cultures of caffeoyl putrescines in *Nicotiana tabacum* and Harman alkaloids in *Peganum harmala* [30]. It is apparent from the results presented in Table 1 that, sucrose elimination decreased capsaicin production over the control, respectively. Sucrose availability in the medium affects capsaicin biosynthesis in both suspension and immobilized cultures. Production of secondary metabolites in plant cell cultures was found to be affected by sugar concentration. The yields of benzophenanthridine alkaloids from suspension cultures of *Eschscholtzia californica* was increased to tenfold by increasing the sucrose concentration to 8 % (w/v) [31]. However, higher concentration of sucrose at 5 % (w/v) reduced the anthocyanin production in cell suspension cultures of *Aralia cardata* [32]. The level of sucrose has been shown to affect the production of secondary metabolites in cell cultures [29, 33]. A dual role of sucrose as carbon source and osmotic agents was observed in *Solanum melongena* [34].

In the present investigation with *C. chinense* cell cultures, nitrate stress resulted in the highest capsaicin production followed by phosphorus and sucrose stress. Figure 2 demonstrated the effect of nutrient stress on cell biomass productivity. With elimination of one of the four nutrients viz. ammonium nitrate, potassium nitrate, potassium dihydrogen orthophosphate and sucrose, all treatments resulted in the reduction of cell biomass productivity as compared to the control cells. In the case of control cells, cell biomass gradually increased from day 5 to 25, similarly slight increased in biomass was also observed from cells under stress treatments from day 5 to 15. However, from day 20 to 25, reduction of biomass was observed in all the cells under stress as compared to control cells. This is consistent with the earlier findings of Ravishankar et al. [13] where the authors proposed that nitrate stress enhanced the capsaicin production in suspension and immobilized cells cultures probably by channeling the precursor to capsaicin synthesis than being utilized for growth processes. Accumulation of capsaicin increased

Table 2 Effect of pH stress on capsaicin production in cell cultures of *C. chinense* during the growth cycle

Medium pH	Days of cultures				
	5 days	10 days	15 days	20 days	25 days
Suspension cultures					
Control (pH 5.8)	90.1 ± 5.7^{h}	137.8 ± 4.6^{h}	142.6 ± 4.2^{f}	152.6 ± 3.1^{e}	165.1 ± 2.8^{e}
pH 3	183.8 ± 2.8^{d}	246.1 ± 3.5^{c}	110.3 ± 3^{g}	121.8 ± 3.5^{g}	112.5 ± 6^{f}
pH 4	139.8 ± 5.7^{f}	226.3 ± 3.1^{d}	184.6 ± 4.1^{e}	127.1 ± 3.6^{g}	96.4 ± 8.7^{fg}
pH 5	119.5 ± 5.7^{g}	160.4 ± 6.5^{g}	196.6 ± 3.1^{e}	132.8 ± 9.4^{f}	108.3 ± 4.4^{f}
pH 6	126.7 ± 3.1^{f}	196.6 ± 3.8^{e}	261.6 ± 3.4^{d}	162.6 ± 4^{e}	133.6 ± 3.1^{b}
pH 7	202.2 ± 5.3^{c}	230.4 ± 5.7^{d}	141.3 ± 4.3^{f}	134.9 ± 3^{f}	90.6 ± 5.5^{g}
pH 8	170.3 ± 5.7^{e}	173.1 ± 3.4^{fg}	121.1 ± 5.8^{g}	125.7 ± 8.6^{f}	77 ± 4.3^{f}
Immobilized cultures					
Control (pH 5.8)	279.3 ± 11.5^{a}	420.4 ± 8.7^{a}	388.4 ± 7.2^{a}	340.1 ± 5.7^{a}	296.3 ± 8.4^{b}
pH 3	104 ± 13.1g^{h}	156.4 ± 7.3^{h}	123.4 ± 5.7^{g}	107.4 ± 5.3^{h}	101.2 ± 5.2^{f}
pH 4	110.4 ± 5.2^{g}	183.2 ± 3^{f}	197.3 ± 5.8^{e}	210 ± 5.7^{d}	200 ± 4.6^{d}
pH 5	264 ± 3.7^{b}	433.3 ± 3.3^{a}	355.3 ± 2.6^{b}	301.6 ± 2.7^{b}	357.6 ± 3.7^{a}
pH 6	293.2 ± 6.5^{a}	300.5 ± 6.5^{b}	310.7 ± 4.4^{c}	280.4 ± 5.8^{c}	268 ± 5.7^{c}
pH 7	280.6 ± 6.4^{a}	300.5 ± 5.7^{b}	310.7 ± 5.7^{c}	280.4 ± 8.5^{c}	267 ± 6.3^{c}
pH 8	108 ± 5.7^{g}	134.2 ± 7.8^{i}	154.9 ± 7.8^{f}	124 ± 6.2^{g}	102.6 ± 6.1^{f}

Data scored at 5-day interval between 5 and 25 day of culture, mean ± SE. HSD comparisons are significant when letters are different within columns (ANOVA, $P < 0.05$)

gradually from day 5 to day 25 in the control cells; however, cells under nutrient stress, maximum capsaicin biosynthesis was observed on day 10–15 (Suspension cultures) and day 15–20 (Immobilized cell cultures). This may be due to the fact that, stress affected cell growth cycle by shortening the growth phase of the cells leading to early product accumulation in the cultures.

Effect of pH stress on capsaicin biosynthesis

The effect of pH stress was investigated in a range of 3–8 on capsaicin production. Higher pH affected the solubility of the salt in the medium. In suspension cultures, highest capsaicin accumulation was 261.6 ± 3.4 μg g^{-1} f.wt at pH 6 on day 15 (Table 2), whereas in immobilized cell cultures, significantly higher capsaicin accumulation (433.3 ± 3.3 μg g^{-1} f.wt) was recorded at pH 5 on day 10 (Table 2). Further increase or decrease of the medium pH resulted in the lowering of capsaicin accumulation. The pH of the medium is determined by the contents of ammonium, nitrate and phosphate as well as the concentrations of excreted acids in the medium. Since these salts are also essential nutrients, their concentration decreases as the cells grow so that the buffering capacity of the medium is reduced [35]. The change in medium pH affects the production of secondary metabolites. Medium pH influenced cell biomass and alkaloids production in cell suspension culture of *Eurycoma longifolia* [36]. Different medium pH (5.25–6.25) affected the yield of anthocyanin accumulation in *Melastoma malabathricum* [37]. The effect of pH (4.8–9.8) on the production of pilosine and pilocarpine and on their partition between cell and medium in two lineages (P and PP) of *Pilocarpus microphyllus* cell suspension cultures has been reported [38].

In our study, pH 5 and 6 was found to favor capsaicin production in cell cultures. The change in the medium pH below 5 or above 6 reduced capsaicin accumulation in *C. chinense* cell cultures. This may be due to the fact that extreme shift in medium pH either low or high may restrict nutrient availability to the cells. The observation that significant effect of pH at 5.8 and pH 6 on capsaicin production could be due to the fact that capsaicin biosynthesis involved a complex network and several enzymes participate in the biosynthetic pathway (Fig. 1) which may have a slightly different optima for activity and/or secretion. Further work on identifying key regulatory factors affecting capsaicin biosynthesis will bring more insights into the understanding of these enzymes.

Influence of precursors and intermediate on capsaicin biosynthesis

To enhance the synthesis of secondary metabolites, several organic compounds can be added to the culture medium [39]. The concept is based on the idea that any compound, which is an intermediate in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product [29].

Table 3 Accumulation of capsaicin (μg g^{-1} f.wt) under various treatments of precursors and intermediates in cell cultures *C. chinense* during the growth cycle

Treatment (100 μM)	Days of cultures				
	5	10	15	20	25
Suspensions cultures					
Control	90.1 ± 5.7^{l}	137.8 ± 4.6^{k}	142.6 ± 4.2^{l}	152.6 ± 3.1^{h}	165.1 ± 2.8^{g}
Phenylalanine	255.8 ± 2.9^{g}	301.3 ± 5.9^{i}	389.8 ± 5.7^{e}	412.9 ± 6.4^{c}	375.7 ± 8.6^{a}
Cinnamic acid	149.6 ± 5.8^{k}	181.9 ± 3^{k}	188.6 ± 4.3^{k}	234 ± 5.7^{h}	127.1 ± 4^{i}
p-coumaric acid	181.1 ± 5.8^{j}	320.6 ± 5.8^{h}	340 ± 5.9^{f}	378 ± 6.3^{d}	321 ± 5.7^{b}
Caffeic acid	212.2 ± 6.1^{i}	220 ± 8.7^{j}	240 ± 7.2^{i}	250 ± 4.3^{h}	234.7 ± 11.5^{e}
Ferulic acid	255.5 ± 8.6^{g}	302 ± 4.4^{i}	333.1 ± 4.4^{f}	271.5 ± 2.1^{g}	260 ± 3.1^{d}
Vanillin	351.7 ± 11^{d}	380 ± 10^{g}	420.5 ± 8.4^{d}	499.1 ± 5.5^{a}	390 ± 2.6^{a}
Vanillylamine	341.5 ± 5.8^{d}	401 ± 4.9^{f}	412 ± 4.9^{d}	450.5 ± 3.8^{b}	376.8 ± 5.2^{a}
Immobilized cultures					
Control	279.3 ± 11.5^{f}	420.1 ± 8.7^{e}	388.5 ± 7.2^{e}	340.6 ± 5.7^{e}	296.3 ± 8.4^{c}
Phenylalanine	480.7 ± 5.7^{b}	520.1 ± 2.8^{d}	326 ± 3.0^{f}	144.9 ± 2.8^{i}	152 ± 4^{gh}
Cinnamic acid	231 ± 5.9^{h}	322 ± 5.8^{h}	280 ± 5.7^{h}	347.5 ± 4.6^{e}	212 ± 13^{f}
p-coumaric acid	390.2 ± 12.7^{c}	806.2 ± 9.1^{c}	720.1 ± 4.6^{b}	220.2 ± 5.7^{h}	146.3 ± 8.7^{h}
Caffeic acid	200 ± 5.1^{i}	218.1 ± 4.6^{f}	300.6 ± 5.2^{g}	371.4 ± 4^{d}	169.8 ± 5.7^{g}
Ferulic acid	298.3 ± 10^{e}	423.7 ± 4.9^{e}	216.8 ± 3.7^{j}	245.1 ± 7.9^{h}	125.2 ± 2.8^{i}
Vanillin	855.6 ± 2.9^{a}	1,315.3 ± 10^{a}	800.9 ± 4.9^{a}	467 ± 4.9^{b}	125.2 ± 7.9^{i}
Vanillylamine	494.1 ± 8.7^{b}	1,075.1 ± 8.3^{b}	600 ± 5.7^{c}	300 ± 5.7^{f}	253.3 ± 3.3^{d}

Data scored at 5-day interval between 5 and 25 day of culture, mean ± SE. HSD comparisons are significant when letters are different within columns (ANOVA, $P < 0.05$)

C. chinense cell cultures were exposed to precursors and intermediate i.e., Phenylalanine, cinnamic, *p*-coumaric, caffeic, ferulic acids, vanillin, vanillylamine, and their influences on capsaicin biosynthesis were analyzed. Both suspensions and immobilized cell cultures were employed to study the influence of phenylalanine and phenylpropanoids on the production of capsaicin. Variation in the production of capsaicin was obtained in the growth cycle. The highest capsaicin content (499.1 ± 5.5 μg g^{-1} f.wt) in suspension cultures was recorded in the cells treated with 100 μM vanillin on day 20 (Table 3). This concentration of precursors was chosen because it did not cause significant growth alterations when added to the culture medium [20]. In the case of immobilized cultures, enhanced capsaicin production was obtained as compared to the suspension cultures. The maximum capsaicin content (1,315.3 ± 10 μg g^{-1} f.wt) on day 10 was recorded in the cells grown in the presence of vanillin (Table 3). Addition of 100 μM vanillin to the culture medium enhanced capsaicin biosynthesis in both suspension and immobilized cultures. In our study, we observed that precursors feeding slightly increased cell biomass productivity as compared to the control cells; however, the difference was minimal (Fig. 3). The cultures fed with vanillin resulted in maximum (0.35 ± 00.6 g/culture d.wt) cell biomass productivity as compared to other precursors. Although addition of precursors enhanced capsaicin biosynthesis significantly, however, cell biomass productivity was not significantly affected by precursor-feeding strategy. The effect of precursor feeding (phenylalanine) on the production of isoflavones in *Psoralea corylifolia* has been reported. Daidzein and genistein levels were greatly affected by concentrations of phenylalanine [40]. Treatment with

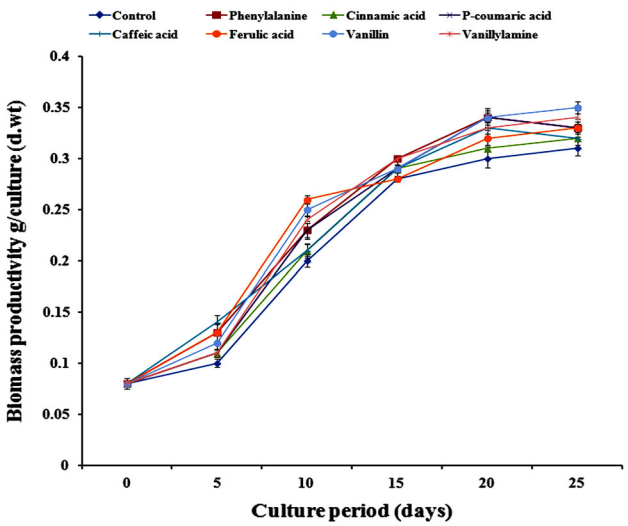

Fig. 3 Time profile of cell biomass productivity in cell suspension cultures of *C. chinense* under precursors and intermediates feeding

L-threonine, as precursor, stimulated the production of adhyperforin in *H. perforatum* shoot cultures [41].

The present study showed that among all the precursors and intermediates studied, addition of 100 μM vanillin to the culture medium resulted in the maximum capsaicin biosynthesis followed by vanillylamine. Since vanillin and vanillylamine were the intermediates that more measurably stimulated the content of capsaicinoids in our study, this effect might be due to the fact that these metabolites participate at the last steps of the capsaicin biosynthetic pathway, and thus a low diversion would be expected, or alternatively, they are not important substrates for the synthesis of a great variety of secondary metabolites [20]. Statistically significant increases in capsaicin contents in both suspension and immobilized cell cultures were also obtained in the presence of phenylalanine, cinnamic, *p*-coumaric, caffeic, and ferulic acids as compared to control cells. However, capsaicin content was found to be significantly lower statistically as compared to cells fed with vanillin and vanillylamine. The low stimulation rate of these metabolites may be due to their early participation in the capsaicin synthetic pathway thereby high diversion of these metabolites in the formation of other secondary metabolites. Hahlbrock and Scheel [42] reported that phenylpropanoids are also the source of intermediates for the biosynthesis of a wide variety of secondary metabolites (lignin, coumarins, benzoic acids, flavonoids, etc.); therefore, a diversion of capsaicinoid precursors would be expected.

Influence of immobilization matrix on capsaicin biosynthesis

The rate of capsaicin production is significantly higher in immobilized cells as compared to freely suspended cells. Immobilization in calcium alginate enhanced the production of plumbagin in *Plumbago rosea* by three, two and one folds compared to that of control, un-crosslinked alginate and $CaCl_2$ treated cells, respectively [12, 43]. In the present investigation with *C. chinense,* immobilized cell cultures resulted in higher accumulation of capsaicin as compared to freely suspended cells.

This may be due to the probable reason that the immobilization matrix may act as an inducer for certain metabolic process. The matrix can also act as physical barrier for the formation of plasmodesmata between cells and cause an alteration of the natural age distribution of cells, which exhibits a radial distribution of new cells on the periphery [44]. Because of the physical organization of cell aggregates, there would be expected to be gradients in the concentrations of nutrients between the external medium-cell interface and the centre of the cell mass [18]. Immobilization facilitates the importance of cellular crosstalk, which can establish inter-cellular communication by the action of signaling molecules. This should enhance the biosynthesis of plant cells [45, 46]. The interaction of all these factors could potentially influence the cellular metabolism of the cells. This potential changes in the cellular metabolism lead to an increase in capsaicin accumulation in immobilized cells.

The present investigation may throw more light on the accumulation of capsaicin in cell cultures of *C. chinense* under various treatments such as nutrient stress, pH stress and precursor feeding. Among the different culture strategies adopted to enhance capsaicin biosynthesis in cell cultures of *C. chinense*, cells fed with either vanillin or vanillylamine resulted in the maximum capsaicin accumulation, hence, these metabolites may be considered as a choice for further research. Since the present investigation is a first report on *C. chinense,* a more in-depth understanding of the underlying biological mechanisms will enable researchers to fully harness the potential of *C. chinense* cell immobilization to realize enhanced product yields on an industrial scale. The activity of regulatory enzymes of capsaicin biosynthesis might be useful in further elucidating the mechanism of capsaicin production from *C. chinense* cells upon immobilization.

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