Comparison of Various Strategies Designed to Optimize Indole Alkaloid Accumulation of a Cell Suspension Culture of *Catharanthus roseus*

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The cell line CP-3 of *Catharanthus roseus* produces low levels of indole alkaloids during stationary phase of growth, but increased levels when transferred to growth-limiting production media. Highest specific yields of alkaloids were usually achieved 8–15 days after transfer of the cells to the production medium. We attempted to reduce the time required for alkaloid production by altering preculture conditions, and by adding an elicitor preparation known to stimulate alkaloid accumulation in other cells. Reduction of 2,4-D in the last growth medium before transfer to production medium and a high initial inoculum load into the production medium resulted in rapid and greatest alkaloid accumulation (up to 2 mg ajmalicine/g dry mass or 40 mg/l within 7 days). The addition of elicitor to the cell suspension stimulated high levels of tryptamine biosynthesis and accumulation under all culture conditions. Additionally, two cell lines derived from CP-3 cells and maintained on NAA/kinetin containing medium were selected for rapid growth in the absence of 2,4-D and were characterized with respect to their ajmalicine production. At a high inoculum load these cells produced up to 1.3 mg/g or 20 mg ajmalicine/l when cultivated on the growth medium. The biotechnological utility of such cell lines is discussed.

Introduction

Cell cultures of *Catharanthus roseus* produce a variety of indole alkaloids, often with ajmalicine, serpentine or catharanthine being the major constituents [1–3]. Higher levels of these alkaloids, however, accumulate only when cells are transferred to production media [4–6]. Accumulation of indole alkaloids normally starts 4–5 days after cell transfer to the production medium and greatest specific yields are reached after 8–15 days [7]. Additionally, the induction of indole alkaloid biosynthesis seems to depend upon the physiological state of the cells at the time of their transfer [8]. It has recently been shown that the patterns of activities of two likely regulatory enzymes (tryptophan decarboxylase and geraniol-10-hydroxylase) of the two precursor pathways of monoterpene indole alkaloids were distinct [9, 10]. A timely coordination of these pathways may be required to shorten the pre-induction period of alkaloid biosynthesis, and optimize productivity. Therefore different approaches were used in efforts to reach these goals.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthylacetic acid.

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An obvious technique allowing for increased alkaloid accumulation might be the use of suitable elicitors. For example, cultured cells may respond sometimes with rapid and increased accumulation of secondary metabolites when exposed to microbial elicitor preparations [11–14]. Here we used a culture filtrate of *Micromucor isabellina* known to stimulate alkaloid accumulation in a low-yielding strain of *C. roseus* [15]. Another way of improving yield is to optimize the culture conditions in such way that the cells enter the production medium in the "best" inducible or physiologically responsive state. We and others, have shown that phosphate and 2,4-D are two media constituents which may negatively affect the ability of CP-3 cells to accumulate indole alkaloids [4, 5, 16, 17]. While a decrease of the phosphate concentration in the growth medium decreases growth, reduction of the 2,4-D concentration in the last medium before the production medium has only a minor effect on growth and would allow for reduction of the time required for greatest alkaloid formation. Deletion of 2,4-D alone from the growth medium resulted in a low level of indole alkaloid formation [5]. A third alternative for improving the alkaloid formation of an established cell line might be the biochemical selection of high-yielding sublines. Since the presence of 2,4-D has a negative effect on alkaloid formation, new CP-3 derived sublines which

grow rapidly in the absence of 2,4-D were selected and their alkaloid productivity was assessed.

Materials and Methods

Cell cultures

Initiation and maintenance of cell line CP-3 (*Catharanthus roseus* (L.) G. Don) as well as some characteristics have been described previously [5, 7, 10, 18, 19]. The cells were subcultured every 14 days (fresh mass 23–26 g/70 ml) using a ratio of 1:10 (inoculum:fresh medium v/v) with MS-medium plus 2 μM 2,4-D. The cell lines CP-3NK1 and CP-3NK2 were selected by growing CP-3 cells on liquid MS-medium containing 10 μM NAA and 1 μM kinetin. After 4–5 passages of 21 days with greatly reduced growth on this 2,4-D-free medium, large, slightly yellowish coloured aggregates of cells had formed. During the next 3–4 passages white, small aggregates developed and growth rates increased. After 7 months one finely dispersed cell line (CP-3NK1) and one lumpy line (CP-3NK2) were recovered. These cells had growth rates similar to the parent line. Both cell lines were maintained for a further 6 months on the NAA/kinetin medium before analysis. The production medium used was IM 2 [20].

Elicitor preparation

The preparation of culture filtrates of *Micromucor isabellina* has been described previously in detail [15].

Analytical methods

Harvesting of cells, determination of fresh and dry mass, extraction of the cells for indole alkaloids and tryptamine have been described [5, 7, 10]. The extracts were either chromatographed on silica TLC-plates using the systems, xylol:2-butanone:diethylamine 20:10:0.6 and $CHCl_3$:MeOH:25% NH_4OH 10:4:1 or by HPLC using a RP-18-7μ column with CH_3CN:triethylamine formiate 50:50 pH 8.5. Ajmalicine and tryptamine were quantified by their absorbances at 280 nm. TLC-plates were directly measured using a Shimadzu-TLC-scanner. In the case of feeding experiments with [2-^{14}C]tryptamine or [3-^{14}C]tryptophan TLC-plates were scanned with a Silena linear radioactivity scanner or by scintillation counting of scrapped off spots.

Results

Elicitation of CP-3 cells coming from the growth medium

Cell line CP-3 was transferred every 14 days when the fresh mass has reached ca. 25 g/70 ml medium. At the end of the culture cycle cells have accumulated 0–40 μg ajmalicine/g dry mass. Ajmalicine accounts for 70–80% of the total indole alkaloids at this stage. A culture filtrate of *Micromucor isabellina*, which induced alkaloid formation from 0 to 250 μg ajmalicine and 375 μg catharanthine/g dry mass in FD/ST-84/07 cells within 72 h [15], was added to 10–12-day-old suspensions of CP-3 cells. We expected to see an increased yield with CP-3 cells in response to elicitor since this line normally produces 0.8–1.5 mg ajmalicine/g on production media.

The effect of different amounts of the elicitor on the productivity is shown in Table I. Within the first 24 h the cells became grey and the medium was brownish coloured. The dry mass of the treated cells was slightly reduced, indicating that some constituents may have been released into the medium. Indeed the levels of phenolics were increased 8-fold according to the Folin-test. Under these conditions the medium remained brownish for the 72 h experimental period. [^{14}C]tryptophan or tryptamine were rapidly taken-up during this time which indicated cell viability. Overall, tryptamine and alkaloid levels in the medium represented less than 10% of those found within the cells. While tryptamine levels of the cells were increased 3-fold, ajmalicine levels were either unaltered or even lower (Table I). TLC or HPLC analyses of the CH_2Cl_2-extracts did not indicate increased accumulation of any other indole alkaloids (*e.g.* catharanthine, serpentine). Thus, in our

Table I. Effect of the elicitor concentration on growth, tryptamine and ajmalicine levels. The elicitor was added to 10 ml suspensions of 10-day-old cells for 72 h. Initial values at day 11: fresh mass 2.2 g; dry mass 117 mg; tryptamine 810 μg and 60 μg ajmalicine/g dry mass.

Elicitor [μl]	Fresh mass [g/10 ml]	Dry mass [mg/10 ml]	Tryptamine [μg/g dry mass]	Ajmalicine [μg/g dry mass]
0	3.0	120	1207	61
50	2.4	96	3350	55
100	2.3	98	4260	37
200	2.3	97	3590	29
400	2.5	108	2950	32
800	2.4	102	3460	41

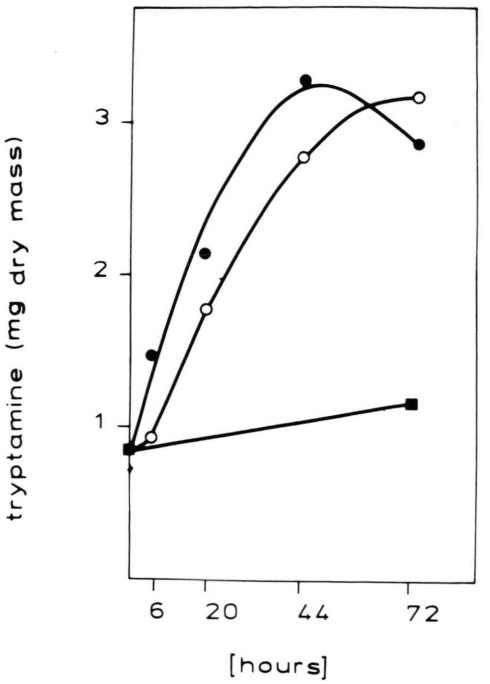

Fig. 1. Accumulation pattern of tryptamine in response to the addition of 100 µl (○) or 300 µl (●) elicitor to 10 ml suspensions of 11-day-old CP-3 cells. Controls (■).

experimental system the elicitor enhanced tryptamine levels, in addition to the visible changes indicating increased activities of other metabolic pathways (phenylpropanoids, phenolics). The time-course of tryptamine accumulation in response to the elicitor is given in Fig. 1. The highest specific yields are achieved within 24–48 h and are elicitor concentration-dependent.

We treated the cell suspension for various periods of time with the elicitor (Table II) in efforts to determine the most efficient alkaloid-stimulating dosage. The presence of elicitor for 1 h was sufficient to cause the same increase of tryptamine levels as found when cells were treated for 72 h with the elicitor. Again, however, we observed no increases in the indole alkaloid accumulation.

Elicitation of CP-3 cells on the production medium IM 2

Since cells coming directly from the growth medium did not respond in the desired way (*i.e.* with increased alkaloid production), CP-3 cells determined for indole alkaloid production were also tested. Table III shows the accumulation of ajmalicine and tryptamine in cells grown for one cycle on 1 µM 2,4-D before being treated with the elicitor and transferred to the production medium. In the presence of the elicitor the cells accumulated 10-times less ajmalicine on the production medium than controls (Table III). On the other hand, the levels of tryptamine found in cells cultured in the production medium were 3–8 times greater after elicitor treatment. In general, tryptamine levels were lower in cells producing alkaloids. Thus the levels of tryptamine accumulation varied with the culture conditions, but a 2–3-fold increase over controls was always observed after elicitor treatment. When cells were labelled with [3-^{14}C]tryptophan in the presence of the elicitor, the radioactivity of the alkaloid fraction was mainly found in tryptamine, while in control cells most of the radioactivity was found in ajmalicine and other indole alkaloids (data not shown).

Table II. Formation of tryptamine by CP-3 cells treated for the time indicated with the elicitor. All cells were harvested 72 h after initiation of the experiment. Conditions as in Table I. Control cells had at 0 h 762 µg and at 72 h 1207 µg tryptamine/g. After removal of the elicitor the cells were transferred into conditioned medium of the same age. Elicitor concentrations were 100 and 300 µl/10 ml suspension.

Elicitor presence [hours]	µg Tryptamine/g 100 µl	300 µl
1	2566	3995
2	3489	3927
4	3472	3442
8	2579	4651
72	3227	–

Table III. Effect of the elicitor on tryptamine and ajmalicine pools of cells grown for one cycle on the reduced 2,4-D level (1 µM). A) addition of 150 µl elicitor/10 ml suspension; B) 1:8 dilution of the suspension with IM 2; C) B plus 300 µl elicitor/35 ml culture. Initial and final dry masses/flask: A = 170 ± 10% mg; B from 75 to 420 mg; C from 75 to 340 mg.

	A		B		C	
Time [h]	Trypt	Ajmal	Trypt	Ajmal	Trypt	Ajmal
	µg/g dry mass					
0	336	250				
24	807	210	150	260	992	352
72	1475	204	137	307	1023	201
96	875	135	131	813	861	206
144	985	120	180	1208	678	131

The negative effect of the elicitor on alkaloid accumulation was even more pronounced on cells kept for 4 days on the production medium before elicitation (Table IV). As chromatographic analyses revealed no other alteration of the alkaloid pattern in response to the elicitor, it was evident that the alkaloid formation of CP-3 cells could not be further improved, but rather was severely hampered by our treatments.

Table IV. Reaction of CP-3 cells grown for 4 days on the production medium IM 2 before addition of different amounts of the elicitor for 48 h.

Elicitor [µl]	Biomass mg dry mass/35 ml	Tryptamine µg/g dry mass	Ajmalicine µg/g dry mass
0	607	320	805
50	482	817	704
100	513	941	544
200	536	918	446
400	555	886	274
1600	492	1113	260

Experiments to optimize alkaloid production of CP-3 cells on the production medium IM 2

When CP-3 cells are transferred to the production medium IM 2 levels of at least 0.5–1.0 mg ajmalicine/g are achieved. Without the elicitor the cells had produced 0.8 mg ajmalicine/g or 16 mg/l within 6 days on the production medium (Table IV). This typical result indicates that the highest specific yields may be achieved after 10–14 days in our system. This rather delayed response is a problem which calls for further attention. Therefore the preculture conditions were altered and the initial inoculum was varied to see whether or not this shortens the time required for alkaloid production and improves yield. Cells were grown for the last cycle before the production medium on growth medium with 50% of the original 2,4-D concentration (1 µM). This decrease in 2,4-D concentration causes a more granular appearance of the suspension. Fig. 2 compares the ajmalicine accumulation pattern in the production medium of cells coming from growth medium, or the 2,4-D-reduced medium. The cells coming from the lower 2,4-D medium contained a higher level of ajmalicine which varied between 100–300 µg/g dry mass in independent experiments. Thus, at the time of transfer to the production medium the cells contained a 5-6-fold increased ajmalicine level compared to cells originating from the normal maintenance medium. Under these conditions the highest specific yield of 1.6 mg/g was achieved after 5–6 days while the cells coming from the normal growth medium had just achieved 40% the maximum highest value of 1.2 mg/g (Fig. 2). The lag phase before alkaloid accumulation can be effectively shortened by this simple alteration of the culture conditions. When the highest specific yields of ajmalicine in various independent experiments were compared it was evident that preculture on the low 2,4-D-medium resulted in usually higher specific yields with an average of 1.6 mg (1.4–2.1 mg) compared to 1.1 mg (0.8–1.4 mg) of cells coming from the normal growth medium.

In order to achieve the highest total yields the specific alkaloid content must be seen in relation to biomass production. Therefore the influence of the initial inoculum on alkaloid induction and biomass production was followed. Cells coming from the low 2,4-D containing medium were diluted 1 + 2, 1 + 5 and 1 + 11 with IM 2. The highest specific yields were achieved at a dilution 1:6 (Fig. 3). This response also occurred in cells coming from the normal growth medium (data not shown). On a biomass basis, however, high initial inoculum gave the high-

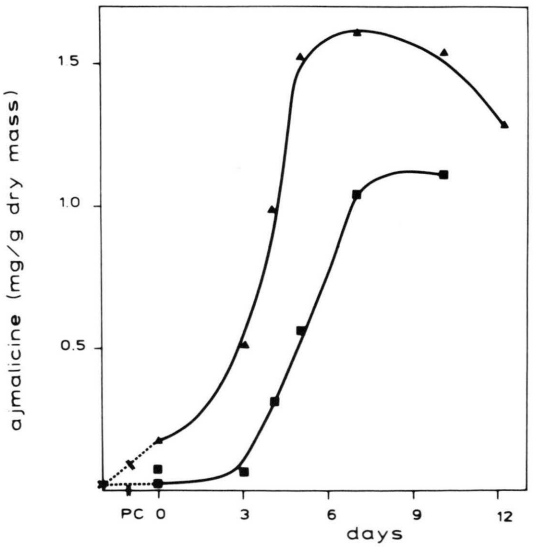

Fig. 2. Inducibility of ajmalicine formation by CP-3 cells on the production medium IM 2, when have been maintained for the last growth period (PC) on MX-medium (2 µM 2,-D) (■) or on reduced MX-medium (1 µM 2,4-D) (▲).

est total yields/l (Fig. 3). At dilutions higher than 1:8 alkaloid and biomass production were reduced. This may indicate that the stress induced by the production medium was too severe for a portion of cells and depressed their growth. The highest total yields were achieved at the 1:3 dilution with 45 mg/l (Fig. 3). Although the highest specific yield was achieved after 4–5 days; the greatest ajmalicine levels/l were realized 10–12 days later in accordance with biomass production.

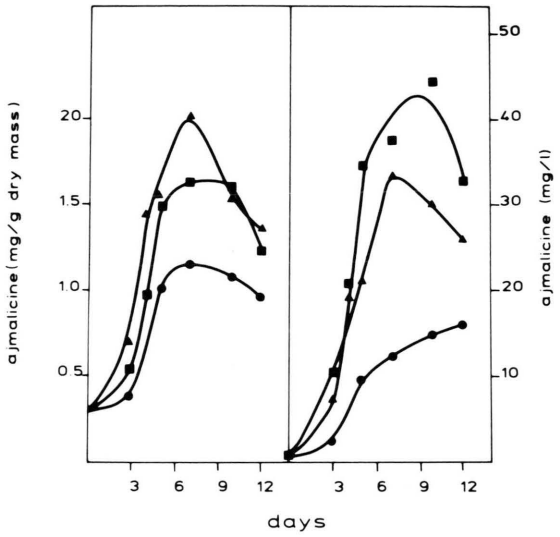

Fig. 3. Specific and total production of ajmalicine in production medium IM 2 at various dilutions. (■) 1:3 (▲) 1:6 (●) 1:12. The cells were precultured on 1 μM 2,4-D medium for 14 days.

Alkaloid production of CP-3 daughter lines selected for 2,4-D independence

As the negative effect of 2,4-D on the alkaloid production of *C. roseus* cells is known, we tried to maintain CP-3 cells in the absence of 2,4-D. When CP-3 cells were grown for several passages on MS-medium with NAA and kinetin large, porous cell aggregates of up to 2 cm in length formed. Growth decreased significantly and was nearly arrested after 5 passages. However, growth resumed slowly, and two lines, a pipettable suspension CP-3NK1, and a lumpy culture CP-3NK2, were recovered. These cell lines were maintained on the NAA/kinetin medium for more than 12 months before analysis. The present growth rates of CP-3NK1 and CP-3NK2 are similar to those of the parent line.

These two cell lines and their parent line were compared with respect to their tryptamine and ajmalicine production capacity. While ajmalicine levels of the parent line varied between 0–40 μg/g dry mass at the end of the 14-day growth cycle, the selected, derived lines accumulated regularly between 300 and 500 μg ajmalicine on their 2,4-D free maintenance medium; 3–5 mg ajmalicine/l were produced during the usual growth cycle. Although this was quite substantial compared to the parent line, it was clear that this level had to be improved. Therefore CP-3NK cells were grown on phytohormone-free medium. However, growth ceased immediately and the indole alkaloid accumulation was rather low (data not shown). The response of the CP-3NK cells to the IM 2-production medium was delayed and the specific yields of ajmalicine after 14 days were 1 mg/g lower than observed with determined CP-3 cells. Thus adaptation of culture conditions and development of a new production medium may be required for greatest alkaloid production by the new lines. In Fig. 4 the growth and production pattern of CP-3NK1 is illustrated when a very high initial inoculum was chosen. During the growth phase the specific yields of ajmalicine were nearly as low as those of CP-3 cells, but during later growth stages alkaloid accumulation increased dramatically. The CP-3NK cells expressed high biosynthetic activity for

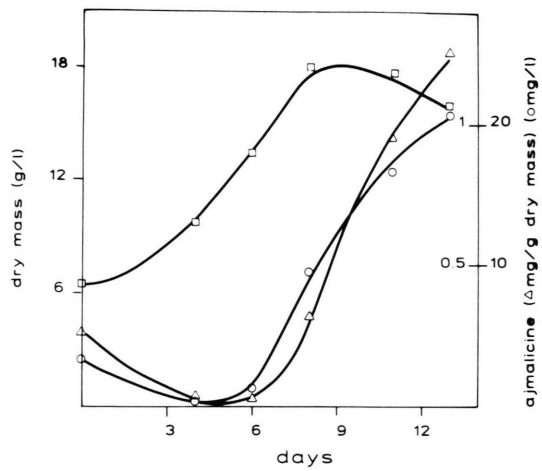

Fig. 4. Accumulation of ajmalicine in CP-3NK1 cells on their growth medium when inoculated at high cell densities.

indole alkaloids only in the stationary phase. In this they resemble the parent CP-3 cells, but the new cell lines grown on NAA/kinetin as the sole growth regulators express this pathway much better on their growth medium than CP-3 cells which require 2,4-D for growth. Due to the high inoculum (Fig. 4) media constituents were depleted earlier, stationary phase was reached after 7–9 days and onset of high product formation occurred during the usual period. Under these conditions CP-3NK cells produced 20 mg ajmalicine within 12–14 days on their growth medium. We expect that this productivity can be further improved.

The capacity of the new cell lines CP-3NK1 and CP-3NK2 to produce high levels of ajmalicine is an encouraging aspect. At the time of ajmalicine accumulation (Fig. 4) a parallel increase in the accumulation of tryptamine to 1–1.3 mg/g dry mass was observed. Additionally, the ratio of the alkaloids differed from that of CP-3 line. The original CP-3 derived ratio of ajmalicine : catharanthine or serpentine was not significantly altered in the new lines, however, the ratio of ajmalicine to one unidentified ceric ammonium sulfate positive spot of the alkaloidal fraction was changed from 20:1 in CP-3 cells to 4:1 or less. Other minor alkaloids were also present in higher amounts than in CP-3 cells. All these compounds were produced in cells grown in the NAA/kinetin growth medium, and increased in stationary phase cells from day 10 on (Fig. 4). A complete analysis of these lines may be required for evaluation of the real indole alkaloid production capacity. Thus, the new lines should not only be seen under the quantitative aspect of improving yields of a distinct constituent, but must also be seen as an opportunity to find new or altered product patterns.

Discussion

The cell line CP-3 may be regarded as having average stable productivity for indole alkaloid biosynthesis which has been maintained for ca. 10 years. During this period the levels of its main alkaloid ajmalicine, which accounts for 60–80% of the indole alkaloid spectrum, fluctuated between 0.6–2.0 mg/g dry mass or 15–40 mg/l on the IM 2 production medium. The lag phase before induction of alkaloid biosynthesis varied in response to the preculture conditions and the physiological state of the cells [8, 19]. We suggest that the activities of two likely regulatory enzymes (tryptophan decarboxylase, geraniol-10-hydroxylase) of the two precursor pathways are not coordinated in our systems [10]. If these enzymes can function in coordinated fashion then we believe a) that the pre-induction period could be shortened, b) that the fluctuations in alkaloid accumulation can be minimized, and c) that the total yield might be further improved by optimized experimental alterations.

We attempted to optimize alkaloid using various methods. First we used a microbial elicitor preparation which was known to rapidly cause increased indole alkaloid accumulation in a low yielding *C. roseus* line [15]. It was expected that the effect of this elicitor would be even more pronounced on CP-3 cells. However, the results clearly showed that the effects of such elicitor preparations might be different for different cell lines of one species. The response of the *C. roseus* cells to an elicitor preparation can not be deduced on the basis of whether a cell line is able to produce high levels of the desired compounds or not. This suggests that an empirical screening program is required to discover potent alkaloid elicitors.

The negative effect of one microbial elicitor preparation on our cell line CP-3 is not necessarily an indication that alkaloid formation can not be stimulated by this method in this line. There is increasing evidence which indicates that cultured plant cells are responsive to elicitor and other alkaloid stimulating agents at specific times during the growth cycle [15, 25, 34]. In our experiments cells of early stationary phase were treated with elicitor preparations and may have been unresponsive. Preliminary tests with cells of other growth phases showed, however, also no indication for increased alkaloid production (Berlin, unpublished). Thus, the question arises as whether or not an extensive screening of randomly chosen microbial elicitor preparations would be a worthwhile expenditure for improving the productivity of CP-3 cells. At present we restrain from this approach for two reasons. Eilert *et al.* [14] tested 8 different microbial extracts on 5 different *C. roseus* lines. Only one line reacted with increased alkaloid accumulation and, this was achieved with several of the elicitor preparations. The induction period for highest specific yields was reduced by the elicitor treatments, but due to the need for biomass production the overall production process could not be streamlined. The total yields were also not increased.

Thus, more specific microbial elicitors may be required to stimulate monoterpene indole alkaloid formation.

The culture filtrate of *Micromucor isabellina* had a stimulative effect on biosynthetic capacity of CP-3 cells. However, the biosynthetic stimulation was toward the initial portion of indole alkaloid biosynthesis and caused increased tryptamine levels. The high accumulation of tryptamine in the presence of the elicitor indicates either an induction, or increase of tryptophan decarboxylase (TDC) activity. This enzyme is easily induced under various culture conditions [18, 19, 21]. A close correlation between indole alkaloid formation and TDC induction had been proposed [18]. Later, however, it was shown that TDC can be induced without concomitant alkaloid accumulation [19, 22]. The high accumulation of tryptamine and the decrease of ajmalicine production in response to elicitor treatment is another example that this enzyme is indeed not a good indicator of the capacity of cultured cells to produce alkaloids.

Interestingly, Eilert *et al.* [14] observed tryptamine accumulation with all lines and all elicitor preparations. Although, the enzymes phenylalanine ammonia lyase [23–25], or tryptophan decarboxylase can be easily to induced, this does not necessarily mean that all following steps or branches of biosynthetic pathways are co-induced. For example mainly phytoalexins, such as certain isoflavonoids and furanocumarins accumulate when the phenylpropanoid pathway is induced by microbial elicitors [23–28]. Other species-specific characteristic phytoalexins were also produced in response to different microbial insults on cultured plant cells [29–31]. The rare, and quantitatively low stimulative effect of elicitors on indole alkaloid biosynthesis suggest that productivity improvements can perhaps be better achieved by other techniques. Thus, searches for *specific* biotic or abiotic effectors is evidently needed to improve indole alkaloid formation distinctly [13, 34].

Consequently, alternative methods designed to shorten and improve the alkaloid production of CP-3 cells seem to be more promising at present. This can be achieved by altering the growth medium, or the ratio of medium : inoculum. For example, lowering of 2,4-D in the last growth medium has a positive effect on alkaloid accumulation. After 5–6 days (when we often first observed distinct alkaloid accumulation of cells originating from the normal growth medium) highest specific product levels were reached. Due to this alteration the cells had accumulated lower levels of tryptamine, but showed increased levels of indole alkaloids (*e.g.* ajmalicine, catharanthine and serpentine) when to be transferred to the production medium. During the phase of highest alkaloid formation tryptamine was detectable at low or trace levels in cell extracts of CP-3 cells; thus, part of the tryptamine formed can evidently be used directly for indole alkaloid biosynthesis. Therefore we assume that with suitable culture conditions, the activities of the biosynthetic precursor pathways were better coordinated, and thus the undesirable accumulation of tryptamine was prevented or diminished. We were not able to increase the total yields of ajmalicine by the altered preculture conditions. However, we succeeded in reducing the period required for alkaloid biosynthesis. The slight modification of the preculture system made the induction process more reliable and generally resulted in higher specific yields.

In efforts to increase the total yields, several dilutions of the suspension with IM 2 medium were tested. We found that at a low dilution of 1:3, resulting in final dry masses of ca. 30 g/l, the productivity was nearly as good as at 1:6 or 1:8. By further fine-tuning of the culture conditions the total product levels can probably be improved above the 45 mg/l we detected, but will probably not surpass 60–80 mg/l within 10 days. Whether or not further optimization of shake-flask cultures for biotechnological purposes is useful, is at least questionable. We observed during scaling up of CP-3 cells in several stages to 5 m^3 that such fermenter grown cells may require different, or adapted production media for optimal responses [9].

It was originally intended to select cell lines producing ajmalicine on their growth medium. Indeed, this was achieved with the selection of two CP-3NK lines which possess similar capacities for ajmalicine production. However, as indole alkaloid formation is observed in *C. roseus* cells only in stationary phase cells, or when one or more constituents of the growth medium have been depleted, a relatively long lag phase before initiation of alkaloid biosynthesis may be expected. By improving the ratio of cell inoculum : medium constituents [32] or by applying fed-batch techniques [33] the production period may be shortened and the yields may be increased. To our knowledge Morris [6] was the first to describe

C. roseus cell lines growing permanently on NAA/kinetin. Although it is not clear if the described lines were the result of selections from existing lines or if the cell lines were originally established on NAA/kinetin, his observations of high indole alkaloid production on this medium are fully supported by the characteristics of the CP-3NK lines we describe. In our case it must be assumed that the new cell lines are an event of selection. It is probably the aim of all those working with *C. roseus* and indole alkaloid production to have that biosynthetic ability expressed in the growth medium because product levels of such cultures might be easier to optimize. We suspect that some researchers will take Morris' lead [6] and will establish 2,4-D independent *C. roseus* cell cultures; such cell lines will probably be described with good growth and production characteristics. Optimization of alkaloid levels of such better determined cell lines by elicitation or development of adapted production media is expected to be not only more rewarding, but may also help to shift alkaloid production distinctly towards the goal of biotechnological application.

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