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Large scale fermentation and alkaloid production of cell suspension cultures of *Catharanthus roseus*

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Abstract. Cell cultures of *Catharanthus roseus* were scaled up to volumes of 5000 l using conventional reactors equipped with flat-blade impellers. The behavior of the fermenter grown cells was compared with corresponding shake flask experiments with respect to growth and indole alkaloid inducibility and production. The limits and problems of transferring shake flask experiments of culture systems such as *Catharanthus*, in which alkaloid production depends greatly upon the physiological state of the cells, to large scale multistage processes is discussed.

Introduction

The biotechnological impact of plant cell cultures, as a new source of natural compounds, depends upon further progress in the establishment of cell lines expressing a desired, commercially interesting compound [2]. An additional requirement for these is that they maintain or even improve their productivity when grown in larger volumes. That the latter is indeed possible has been demonstrated by the improved production of shikonins [4] and rosmarinic acid [24] by two stage processes, and of cinnamoyl putrescines by the fedbatch-technique [16, 17]. Common to all three systems was that the rapidly growing, finely dispersed cells were able to synthesize and accumulate the desired compounds in reasonable levels on their maintenance medium during early stages of their growth cycle. An improvement in productivity was rather easily achieved by optimization of the media constituent concentrations and the inoculum size.

However, many secondary products are not spontaneously expressed in dividing cell populations. For example, diosgenin production occurs only in late stationary phase cells of *Dioscorea* [21], while rapidly growing *Catharanthus roseus* cells have to be transferred to growth limiting production media [26] for initiation of indole alkaloid formation. The response of *Catharanthus roseus* cells to the production medium depends greatly upon the physiological state of the cells [3, 20] and the effect upon product

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levels is only seen after 5 to 7 days. An important problem is the recognition of the 'best' physiological state for highest induction of alkaloid biosynthesis. As the expression of alkaloid biosynthesis is a rather complex process influenced by parameters such as the levels of 2,4-D, phosphate, sucrose, nitrogen, cell age or inoculum size [1, 5, 6, 10, 11, 15, 20] the measurement of only one parameter may not be sufficient. Thus, the amount of free phosphate stored in the vacuole as determined by in-vivo ^{31}P-nmr spectroscopy was a good indicator for predicting low or delayed inducibility [3] but its absence did not guarantee rapid or good inducibility. Nevertheless in shake flask experiments or in smaller fermenters fairly "reproducible" product yields can be achieved by strict control of culture conditions. For example, our unselected 9 year old cell line CP-3 regularly accumulated over the last 4 years 0.8 to 2.0 mg gram dry ajmalicine corresponding to 15–35 mg/l when transferred to the production medium IM 2. Therefore, we wanted to see how the cells would behave when gram in to large volumes. For this purpose conventional fermenters were used.

Materials and methods

Cell cultures and media

Initiation and maintenance of cell line CP-3 (*Catharanthus roseus* L. Don) on MX-medium (MS-medium plus 2 μM 2,4-D) have been described recently [5]. Fermenter inoculum was provided from 12–15 400 ml shake flask suspensions.

Fermentation of Catharanthus roseus *cells*

All fermenters were equipped with three six flat-bladed turbine impellers. The conditions were the following: b20 (Braun, Melsungen, FRG) working volume 25 l, revolutions 90 rpm, rotation speed 42 cm/sec, aeration 0.3–0.5 Nm^3/h; b50 (Giovanola freres, Monthey, CH) 70 l, 80 rpm, 57 cm/sec, 0.5–1.5 Nm^3/h; b200 (G) 300 l, 55 rpm, 58 cm/sec, 0.5–1.5 Nm^3/h; b500 (G) 750 l, 55 rpm, 84 cm/sec, 2.0–3.0 Nm^3/h; b 3150 (G) 5000 l, 30 rpm, 79 cm/sec, 3.0–5.0 Nm^3/h. All fermenters were equipped with pO_2-poloragraphic electrodes. The cells were pumped under low pressure into the next size fermenter. Harvesting of the large volume fermentations was performed by filtration (process filter PTFE 250 μ, flow rate 2000 l/h).

Analytical procedures

25 mg freeze dried cells were extracted twice with 1.5 ml MeOH at 70 °C for 30 min. The combined extracts were dried under a stream of nitrogen and dissolved in 0.4 ml MeOH and 0.6 ml 10% CH_3COOH. The solution was purified on an Extrelut 1 column [8]. After elution with hexane the

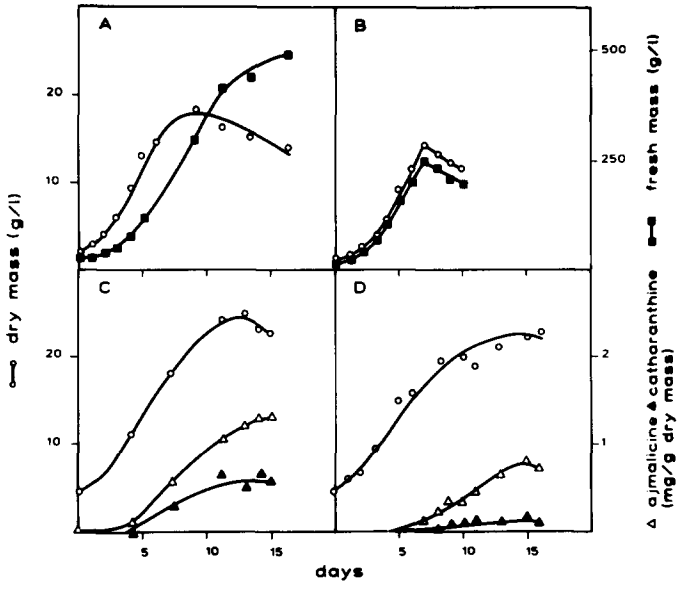

Figure 1. Growth and production characteristics of cell line CP-3. (A) shake flasks, 70 ml growth medium MX; (B) mechanically stirred fermenter, 25 l MX; (C) shake flasks, 70 ml production medium IM 2; (D) fermenter, 25 l IM 2.

column was flushed with a stream of ammonia. Afterwards the indole alkaloids were eluted with 6 ml $CHCl_3$. The internal standard was ibogain. After removal of $CHCl_3$ the samples were dissolved in 0.5 ml MeOH and used for HPLC or TLC. HPLC-conditions: Pre-column Lichrosorb RP 2–30 (30 × 4.0), separation column Lichrosorb RP 18–10 (250 × 4.0), solvent Ch_3CN/0.01 M triethylamine formate 1:1 (v, v), pH 8.5, flow rate 2 ml/min, detection 280 nm. Under these conditions ajmalicine was eluted after 5.5 and catharanthine after 6.6 min. Other smaller peaks were neglected. Alkaloids were also measured by co-chromatography on silica gel plates KG-60 F_{254} with xylol-ethylmethyl ketone (2:1 v, v) + 2% diethylamine, or toluene-MeOH (6:1, v, v) and absorption was measured at 280 nm with a Shimadzu-TLC scanner.

Results and discussion

Before describing the scale up some characteristics of this cell line which was initiated in 1977, should be given. Indole alkaloids do not accumulate on the MX maintenance medium. Alkaloid formation is readily induced by transfer of the cells to various division limiting production media [5, 26]. More recently only the production medium IM 2 has been used for inducing alkaloid biosynthesis [3, 12]. This production medium is based on three frequently made observations: (1) 2,4-D prevents indole alkaloids

biosynthesis and its deletion alone promotes alkaloid formation (5, 14, 15); (2) stored phosphate impairs induction of alkaloid biosynthesis [3, 5, 6] and (3) increased levels of sucrose have beneficial effects on product levels [5, 11, 26]. Typical growth and production kinetics are given in Figure 1A and C. In dark grown suspension cultures ajmalicine is the main $CHCl_3$-extractable indole alkaloid. Under light regimes a part of the ajmalicine seems to be converted to serpentine [7]. The second most abundant alkaloid of CP-3 has been identified as catharanthine [8]. However, this alkaloid accumulated only in shake flask experiments and in small size fermenters but was never found in scaled up fermentations.

As 2,4-D has a negative effect on indole alkaloid production, it was determined whether growth of CP-3 cells at lower 2,4-D levels was possible and whether this would improve production rates. Therefore, cells were maintained for three passages on MS-media containing 2,4-D concentrations of between 0.2 and 2 μM before being transferred to IM 2. Indeed the lower levels of 2,4-D yielded more cells. On the other hand, the cells aggregated and growth in the production medium decreased. Thus a prolonged preculture on low 2,4-D-media caused no significant increase in total yields of alkaloids. To prevent the reduction of biomass yield, only the last growth fermenter was run at reduced 2,4-D levels as accumulated 2,4-D might impair inducibility of alkaloid biosynthesis. The inducibility was also greatly influenced by the amount of inoculum. Addition of 2–4 g cells to 70 ml production medium resulted in specific yields of 2–2.5 mg indole alkaloids/g dry mass while only 0.2 mg accumulated at 0.5 g inoculum/flask. The importance of the age of the inoculum for inducibility of CP-3 cells has recently been shown [3].

Up to now airlift-fermenters have been mainly used for growth of cell cultures of *Catharanthus roseus* at larger volumes and alkaloid levels of 30–50 mg/l have been reported [19, 25, 26]. For the scale up, however, only impeller driven reactors were available. In order to use only one type of impeller during scale up, conventional fermenters equipped with flat-bladed impellers had to be used. However the decision to use this type of impeller should not be regarded as an indication that it is the most suitable for plant cells. Other types, as suggested by Ulbrich et al. [24] or Tanaka [22], might have given as good or even better results. The growth and production curves (Figure 1B/D) shown for the initial fermenter with a working volume of 25 l are typical results. The specific yields oscillated between 0.5 and 1.2 mg ajmalicine/g dry mass (shake flasks 0.8–2.0 mg/g). This variation, however, must not be confused with strain instability. While initial growth rates of fermenter or shake flask grown cells were quite similar with respect to dry mass, great differences were seen at the fresh mass level (Figure 1A/B). This fermenter grown cells were evidently not able to enlarge as only one half of the fresh mass of shake flasks was achieved. Such enlarged cells may be more sensitive to the shear stress

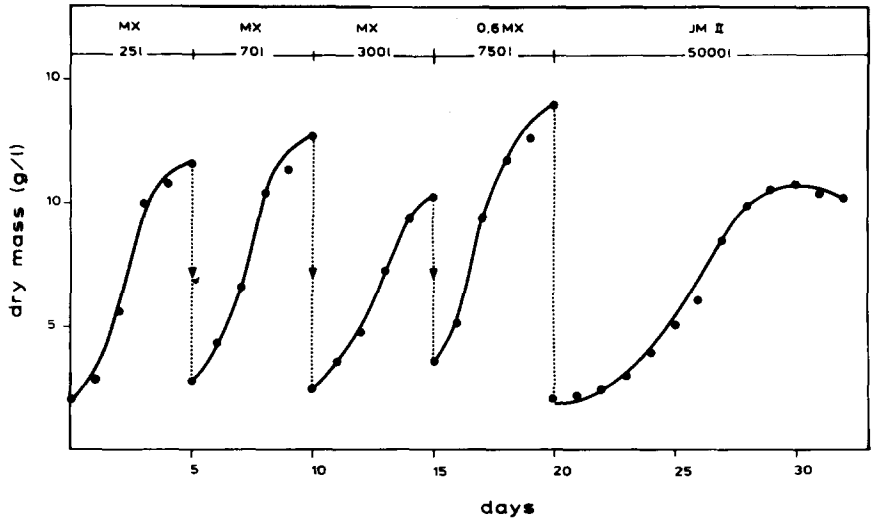

Figure 2. Growth of CP-3 cells during scale up. 1. stage 20 l growth medium MX + 5 l shake flask inoculum, 2. stage 50 l MX + 25 l of stage 1, 3. stage 230 l MX + 70 l of stage 2, 4. stage 500 l MX + ca. 280 l of stage 3, 5. stage 4250 l production medium IM 2 + ca. 750 l of stage 4.

imposed by the impellers and continuous cell lysis may occur. Comparison of vacuolor phosphate levels of shake flask cultures and fermenter grown cells by in vivo 31P-nmr spectroscopy (data not shown see 3) revealed that only the latter showed a large signal for vacuolar phosphate while in shake flask cultures the signal for stored phosphate was very low between 5–10 day and appeared again later in deteriorating suspensions. This indicates that the nutrional situation of the fermenter grown cells could be quite different to shake flask cells and that accumulation of constituents negatively effecting alkaloid formation (eg. phosphate and 2,4-D) may impair production. Whether the reduced productivity of fermenter grown cells as shown in Figure 1C/D is indeed due to this or to other factors cannot determined at the moment.

Scale up of suspension cultures of Catharanthus roseus

According to the small scale experiments it was concluded that cells should be transferred rather early from one growth fermenter to the other to prevent cell enlargement during later phases. Thus the cells were pumped every 5 days to the next size fermenter. Figure 2 shows that growth was not reduced in larger culture volumes. As the dry mass of fermentations was generally 10–25% lower than that of shake flasks the last growth fermenter contained MX-medium with a reduced medium concentration. Hence it was assumed that nutrient overloading, with negative consequences on inducibility, would be avoided. These cells were then pumped to the

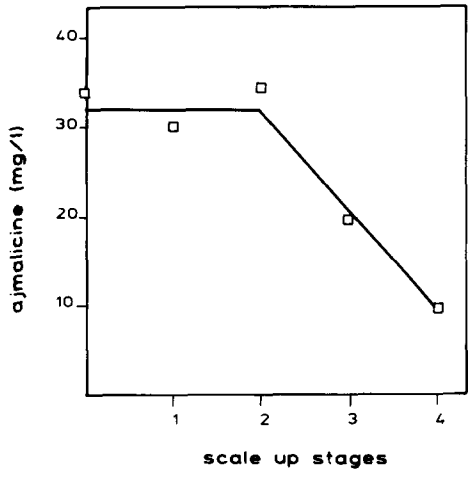

Figure 3. Inducibility of CP-3 cells for indole alkaloid formation during scale up. At the end of every stage 10 ml samples of initial inoculum or fermenter suspension were added to 70 ml IM 2 and were maintained for 8–16 days on the shaker. The highest yield of indole alkaloid (mg/l) reached at each stage is given.

5000 l production fermenter. Only traces of alkaloids had accumulated when a fungal infection stopped further growth and production. The experiment was repeated and the growth behaviour of the cells was quite similar. During the scale up the inducibility of the cells at every stage was tested at the shake flask level (Figure 3). While the cells of the 25 and 70 l fermenters showed a similar productivity as the initial shake flask inoculum (Figure 3), the cells of the 300 and 750 l fermenters showed distinctly decreased yields. This was mainly due to the decreased growth of the cells in the production medium (from 25 to 10 g dry mass/l) while the specific yields varied only between 1.0 to 1.4 mg/g dry mass. As a nutritional limitation should not account for this it is assumed that the fermentation cells suffered 'too much' stress during the scale up such that they could not fully tolerate the additional stress caused by the production medium. The 5000 l fermenter was again infected after 12 days and neglectable traces of alkaloids were detected. It was not apparent whether the lack of alkaloids was due to the slow appearance of the infection or due to the high stress (initial sucrose concentration 6.8%, great increase of hydrodynamic pressure). Even if the induction in the fermenter is lower than that of shake flasks containing the identical suspension one would have expected to find more than traces of indole alkaloids in the 5000 l fermenter. To demonstrate that alkaloid production can be achieved in large scale fermenters a final scale up to 750 l was performed. For this the cells were scaled up to 300 l as before and 250 l were pumped into 500 l IM 2-medium. At this dilution the osmotic stress in the production fermenter

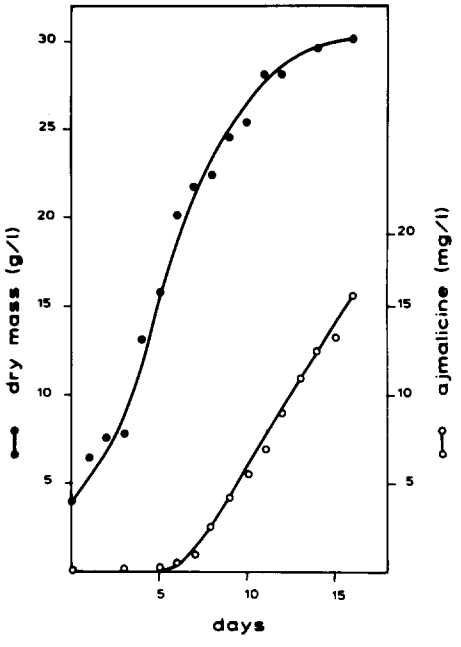

Figure 4. Production of ajmalicine during a 750 l fermentation. Cells were grown as in Figure 2. 250 l of the third stage was added to 500 l IM 2 medium.

was reduced as the inititial sucrose concentration was only 5.3%. This production fermenter showed good growth (Figure 4) and after 5–6 days alkaloid accumulation resumed. After 16 days a product level of 16 mg/l (specific yield 0.5 mg/g) was achieved when for technical reasons the fermentation had to be halted. Again it was noted that the productivity in the fermenter was lower than in the corresponding shake flasks. An aliquot of the suspension from the production fermenter was taken 1 h after inoculation. These shake flasks yielded 35–40 mg ajmalicine (1.3–1.6 mg/g) within 12–15 days. This clearly indicated that additional yet unknown factors occurring during fermentation prevented maximal inducibility of the cells. A further reduction of the stirring speed or a different impeller may helf to reduce the shear stress. When productivities of *Catharanthus* cells in various mechanically agitated reactors were compared with results of airlift fermenters [25], the latter had distinctly higher alkaloid levels which indicates that the stirring may have negative effects. Aeration and CO_2-levels may also influence the overall productivity [9, 13, 18, 23]. During all fermentations the pO_2 was below 15% after a few hours. By increasing the aeration rate a level of 5–10% was maintained to prevent oxygen limitation. Without further precautions higher aeration was not possible as large bubbles caused severe beating of the suspensions in the

reactor. This problem may be solved by the installation of suitable aeration equipment. Additionally one may need to develop production media especially adapted to the physiological state of the cells which leave the growth fermenter. As growth (according to dry mass increase) of the cells during the scale up was very reproducible, the state of the cells entering the production fermenter should be characterized. Under such conditions the productivity of the cells in production fermenters may reach those of the shake flasks. Starting from 50 g dry mass more than 100 kg dry mass of alkaloid containing *Catharanthus* cells can be produced within 30 days (Figure 4). Provided the cell culture has stable production characteristics at the shake flask level, a predictable product level should be feasible. For the development of a commercial process, however, the main problem remains the lack of high yielding and rapidly growing suspension cultures of *Catharanthus roseus* with stable production characteristics. This might be more difficult to achieve than an optimization of the scale up.

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