Symbiosis, 2 (1986)
Balaban Publishers, Philadelphia/Rehovot

Large Scale Fermentation of Transformed and Non-Transformed Plant Cell Cultures for the Production of Useful Compounds

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Abstract

The results of large scale fermentations of three different culture systems (*Potato demissum*, *Nicotiana tabacum*, *Catharanthus roseus*) are described to show that different product formation characteristics (potato spindle tuber viroid, cinnamoyl putrescines and indole alkaloids) may require different fermentation strategies. All fermentation up to 800 L were carried out with fermenters equipped with flat-blade impellers.

Keywords: *Potato demissum, Nicotiana tabacum, Catharanthus roseus*, large scale fermentation, potato spindle tuber viroid, cinnamoyl putrescines, indole alkaloids

1. Introduction

Plant cell cultures are regarded as a potential source of commercially interesting natural compounds. However, despite the announcement of the first commercial process, the production of shikonins by cell suspension cultures of *Lithospermum erythrorhizon* (Fujita et al., 1982), we are still far away from a general breakthrough in this field of biotechnology. The main problem seems to be the lack of commercially interesting high yielding cell

* Scientific contractant of the Biomolecular Engineering Program of the Commission of the European Communities. Invited lecture

0334-5114/86/$03.00

culture systems. Due to the poor understanding of regulatory aspects of secondary metabolism in cultured cells, high yielding cell cultures are established mainly by chance at the moment. Consequently the list of compounds accumulating in high levels contains only structures of modest commercial interest (Zenk and Deus, 1982). As techniques such as media variation or variant screening are only partially successful, the necessity of gaining more insight into the regulation of secondary product formation and of altering expression of secondary pathways has now widely been recognized (Berlin et al., 1985a; DiCosmo and Misawa, 1985). Undoubtedly with more biochemical and molecular knowledge of the mechanism of the expression, or with the discovery of suitable elicitors for secondary metabolites, the field will make significant steps forward.

Another important question is whether highly productive cell culture systems can be scaled up to volumes required for commercial processes. Indeed the biological characteristics of higher plant cells may require special techniques. Plant cells are 200–1000 fold larger than bacterial cells and are consequently more sensitive to shear stress. Cell cultures have to be grown at rather high cell densities which may cause problems with mixing and mass transfer. Plant cells grow comparatively slowly and scale up fermentations last for weeks or months instead of days as with bacteria. This requires the maintenance of sterility over a long time period. Products of plant cells are usually stored within the cell, thus for product extraction a large portion of biomass has to be sacrificed. Despite all these complications a few cell cultures have already been grown successfully in large volumes. For example tobacco cell cultures have been grown for biomass production to 20 m^3 (Hashimoto et al., 1982). For the production of shikonins *Lithospermum* cultures are scaled up to 750 L (Fujita et al., 1982). Most other fermentations for the production of desired compounds end at volumes of 20–30 L. In the following our experiences with and observations made during large scale fermentation of three different culture systems will be summarized. These examples should demonstrate that different formation and accumulation patterns of desired products may require different fermentation strategies.

2. Production of PSTV-viroid by cell cultures of *Solanum demissum*

The most suitable systems for the production of the desired compounds seem to be those where the product is formed during the early stages of the growth cycle or where their formation parallels growth. Hence rather young

Table 1. Scale up of PSTV-producing cell cultures of *Solanum demissum* using flat-blade driven fermenters (Berlin et al., 1985b). The initial inoculum was 0.75 kg. The biomass was pumped under low pressure from one fermenter to the next.

Volume (l)	Initial biomass (kg)	Final biomass (kg)	Fermentation time (d)
30	0.75	3.7	10
80	2.8	13.4	12
300	12.6	38.5	9
800	38.2	112	7

and compact cells, less sensitive to shear stress, can be used as producers and the most suitable cell density can be chosen. A good example for this type of fermentation is the production of potato spindle tuber viroid (PSTV) by *Solanum demissum* cell cultures. Cell cultures form PSTV-infected plants have been established which produce the viroid RNA indefinitely (Mühlbach and Sänger, 1981). Thus a new reliable source for studying PSTV replication is available. The great advantage of these cells, however, is that unprecedented amounts of viroid-infected biomass can be produced which may allow the detection of the low level intermediates involved in the viroid replication process. This cell culture was scaled up to 800 L using conventional fermenters equipped with flat-blade impellers and roughly 80 mg PSTV were produced (Berlin et al., 1985b). The scale up is outlined in Table 1.

The cell line was a rather fine suspension culture with aggregates of 20–70 cells and a biomass doubling time of 3–4 days. The growth rates of the cells in the various fermenters were rather similar and were not reduced when compared with shake flask experiments (70 ml) or airlift-fermentation (20 L) (Berlin et al., 1985b). Further acceleration of this fermentation process may thus only be achieved by increasing the inoculum density and by adapting the medium concentration correspondingly. This example shows that cell cultures can be scaled up in large volume fermenters as usually used for microbial cells provided the stirring speed can be reduced to less than 100 rev/min. However, it is also evident that starting from 10 L inoculum (shake flasks or small size fermenter) it takes 6 weeks (with a minimum of 3 weeks for rapidly growing cells) to fill an 800 L-fermenter. To avoid this time consuming scale up each time, one should keep the scale up cells at the large volume. Indeed, for the development of a production process it seems necessary to establish a semi-continuous process where, by suitable dilutions, biomass production is most efficient.

3. Production of Cinnamoyl Putrescines by *Nicotiana tabacum*

A few secondary metabolites accumulate to suitable extents in cell cultures even when they are maintained on the growth medium. All compounds found in very high levels in cell cultures belong to this group. Thus, for these cultures the desired products might also be harvested at a suitable stage of the growth cycle. However, in contrast to the first example, the growth medium is not the best production medium. The specific production rates and the total yields can often be greatly increased by transferring the cells to a special production medium. With respect to large scale fermentation one may apply a two state process where the initial fermentations provide the biomass while the medium of the last fermenter stimulates the product formation. Examples for this are shikonin production on a 750 L scale (Fujita et al., 1982) or rosmarinic acid production by *Coleus* cells (30 L) (Ulbrich et al., 1985). Instead of two stage processes the technique of fedbatch fermentation may be an alternative approach to increase the productivity of such cell culture systems. The value of this rarely used technique is demonstrated by the increased production of cinnamoyl putrescines by tobacco cells (Schiel et al., 1984a,b).

Cinnamoyl putrescines accumulate spontaneously in some tobacco cell cultures in reasonable levels (0.6–1% of dry mass) on the growth medium (Berlin et al., 1982). A variant line proucing up to 10% on the growth medium has been selected (Berlin et al., 1982). Thus high levels of these compounds (e.g. caffeoyl putrescine) can be produced by normal batch growth. From detailed studies on the effects of media components on the cinnamoyl putrescine production it was known that media variation helps to increase the specific yields (Knobloch et al., 1981). From the fact that many media constituents can stimulate cinnamoyl putrescine production when they become the growth limiting component it was concluded that the medium induced increased product accumulation is a rather complex process (Schiel et al., 1984a). Under normal culture conditions phosphate becomes the first growth limiting constituent. It was also noted that medium phosphate was rapidly taken up and accumulated in the vacuoles from where it was distributed in the dividing cell population. Cells having accumulated surplus phosphate did not respond as readily with cinnamoyl putrescine biosynthesis as cells devoid of vacuolar phosphate when transferred to a production medium (Knobloch et al., 1981). Consequently it was assumed that feeding specific levels of phosphate, so that growth is not hampered and surplus phosphate is not present, would help to increase specific and total product levels. The final

Table 2. Formation of cinnamoyl putrescines by two different productive tobacco cell lines under various culture conditions after 10 days (Berlin et al., 1982; Schiel, 1985; Schiel et al., 1984a,b)

Line	Volume	Conditions	Max. specific yields mg/g dry mass	Total yields mg/L
TX1	shake flask	growth medium	12	200
		production medium	52	140
	11 L	batch	18	190
	11 L	P-fedbatch	39	400
	11 L	N, 2,4-D-fedbatch	28	190
TX4	shake flask	growth medium	100	1000
	22 L	batch	120	1250
	70 L	P-fedbatch	150	1500

outcome of various batch and fedbatch fermentations are given in Table 2.

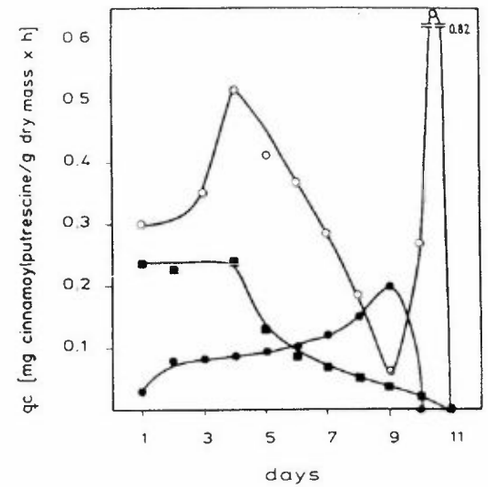

Figure 1. Specific cinnamoyl putrescine production rates q of shake cultures (•), batch (○) and P-bedbatch (■) grown tobacco cells. The absolute values may vary depending upon the preculture but production pattern was found to be reproducible (Schiel, 1985; Schiel et al., 1984a). The dramatic increase of the specific production rate of fedbatch cells at day 10 was due to the fact that the phosphate feeding rate for 2 days was added within 5 hr at day 9.

In summarizing our experiences of many fermentations of tobacco cell cultures for cinnamoyl putrescine production one can draw several conclusions

of general importance. In contrast to many predictions the product yields in batch fermentations (up to 70 L) were not reduced but even increased by 20–30% in comparison to shake flask experiments. The production of other compounds accumulating spontaneously in high levels in cell cultures, such as rosmarinic acid (Ulbrich et al., 1985) or protoberberines (Breuling et al., 1985) were also increased in fermenters. When grown on the same medium similar dry masses (here 15 g/L) were achieved in slowly stirred batch fermenters or shake flasks. Thus it is evident that the mean specific production rate was increased in the fermenter grown cells. Indeed the specific production rates are very different in shake flask cultures and fermenter grown cells. At present there is no explanation of the fact that the highest production rates in the fermenters are at the beginning of growth and in shake flasks at the end (Fig. 1). For process design it is important to prevent the decline of the specific production rate in the fermenter. Fedbatch fermentation is one way of increasing and of shifting the production rates (Table 2/Fig. 1) and thus might also be helpful for maintaining the initially high specific production rates for a longer period. With respect to total yields, however, only the P-fedbatch fermentation can be regarded as successful. In the N, 2,4-D-fedbatch cultures only increased specific yields were observed, but the total yields remained unchanged due to poor growth. Although the cells had received the same amount of nutrients at the end of feeding as the cells in batch culture, a minimum of 20–30% growth reduction was always noted in fedbatch cultures. Clearly, optimization of feeding rates has to be performed to fully realize the potential of fedbatch fermentations. As fine tuning is required, we conclude from our observations, that the fedbatch technique for increased product formation should only be applied for systems which respond rather rapidly so that feeding rates can also be adapted rapidly. Thus, all products accumulating on growth media during early phases of the growth cycle and whose levels can be altered by media variation should be suitable for the fedbatch technique. Whether a two stage process or fedbatch is used for highest product yields has to be checked experimentally. In the case of cinnamoyl putrescines highest specific yields were achieved in the production medium and highest total yields by fedbatch fermentation (Table 2). The value of selecting highly productive and stable cell lines before large scale culture is readily seen when the product levels of wild type TX1 cells and derived TX4 cells are compared.

4. Production of indole alkaloids by *Catharanthus* cells

Many secondary metabolites are only produced in culture by resting or late stationary phase cells. Usually those products will accumulate in rather low levels under these conditions. In such cases cells may be transferred from growth media to production media so that a higher and earlier production by better prepared cells is achieved. The formation of indole alkaloids by *Catharanthus roseus* cells represents a typical example of this. As some indole alkaloids of this plant are of medicinal interest, several groups have tried to establish highly productive cell lines. Today product levels of 30–70 mg/L have been reported for the main alkaloids ajamalicine and serpentine when grown in vessels over 5 L in capacity (Smart et al., 1982). Although such product levels are far too low for commercial processes, we have studied the behaviour of this culture when grown up to large volumes. The scale up of our line CP-3 to 750L is shown in Fig. 2 and detailed information is presented elsewhere (Schiel and Berlin, 1986).

In the context here such observations are stressed which may also apply to related culture systems. A technological complication is that the effect of the production medium with respect to alkaloid formation is only seen after 5–8 days after transfer of the cells to the production medium. The productivity (time course of product accumulation and levels) depend greatly upon the physiological state of the cells when transferred to the production medium (Berlin et al., 1985a). Thus the cells of the last growth fermenter should be brought to a state for the most rapid and highest induction. In shake flasks the best state is easy to find by transferring differently aged cells into the production medium. However, the same conditions may not be realized in the fermenter. We were able to grow the cells to the same dry masses but the fresh masses were distinctly lower due to the fact that enlarging cells were readily lysed in the flat-blade stirred bioreactors. Thus not only the phase of cell enlargement was not realized but the nutritional situation was quite different (Schiel, 1985; Schiel and Berlin, 1986). For establishing a physiological state which yields in the production fermenter the same or better alkaloid levels as in shake flasks one may have to alter the medium composition of the last growth fermenter. Thus only a detailed characterization of the cultural requirements for bringing the cells into the optimal state may give the results expected from shake flask experiments in large fermenters. Such characterization would also be required if biotic or abiotic elicitors, instead of production media, would be used for induction of secondary product formation (Kombrinck and Hahlbrock, 1985). In the case of *Catharanthus* cells it

was also noted that the fermentation itself had a negative effect on secondary product formation. Suspensions removed from the production fermenter and kept for the same time on the shaker usually yielded 50–100% more indole alkaloids as growth was much higher (Schiel and Berlin, 1986). The growth of the cells in the production medium dropped with every stage of the scale up when the same dilutions of 1:8 were chosen. Thus the stress imposed by a production medium developed for shake cultures might be too high for the fermenter grown cells. Again a special fermenter production medium seems to be necessary for optimal results. The good growth achieved in 750 L-production fermenter (Fig. 2) may be explained by the fact that a dilution of 1:3 instead of 1:8, as with shake flasks, was chosen. When the fermentation had to be stopped for technical reasons the maximum yield possible had evidently not been achieved (Fig. 2).

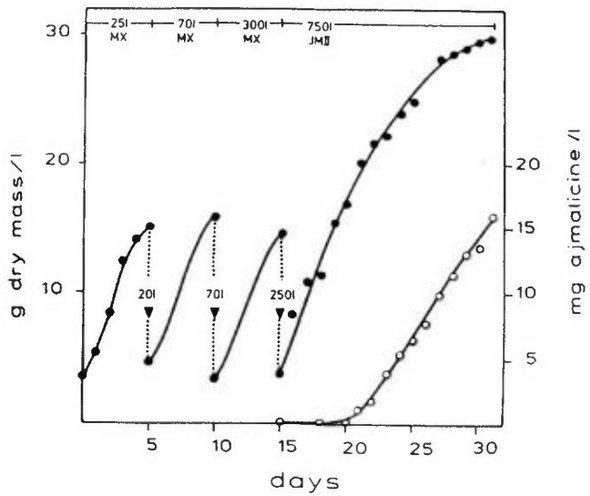

Figure 2. Scale up of *Catharanthus* cells for ajamalicine production from 5 L shake flask inoculum to 750 L. Note: the large increase of biomass in the production medium is due to the incorporation of the increased level of sucrose (8%)! Suspensions removed at the beginning from the production fermenter yielded 35 mg ajamalicine as shake cultures after the same time period.

5. Conclusions and Outlook

It has now repeatedly been shown that rather fine suspension cultures can successfully be scaled up, even in mechanically stirred bioreactors as usually used for microorganisms (Berlin et al., 1985b; Fujita et al., 1982, Hashimoto et al., 1982; Ulbrich et al., 1985). The performance of the cells can normally be further improved by the use of low shear-stress impellers which allow low stirring rates (Ulbrich et al., 1985). The possibilities of better control of factors influencing the productivity of cultures (e.g. aeration) even suggest that at the end of process development higher yields will be produced by fermenter grown cells than in shake cultures. On the other hand the results of shake flask experiments will often not be directly transferable to large scale fermentations and may therefore have to be modified. Independent of the fermenter type the growth rates of cultured plant cells are difficult to improve in large volume reactors. Thus for technical processes continuous or semi-continuous fermentation, with all its problems, seems to be necessary. However, the various technical problems will be solved as soon as commercially interesting products or biotransformation reactions are found in high levels in rapidly growing shake cultures which are worth scaling up. The most important task, therefore, in this field is the establishment of new ways for obtaining more meaningful high yielding cell culture systems (Berlin et al., 1985a). As well as manipulating plant cells by genetic transformation one may also search for *specific* elicitors of commercially important compounds (DiCosmo and Misawa, 1985) which may then be added to a culture system after biomass production. Thus the overall progress of this field will depend mainly upon further progress in expressing desired products or enzymes.

Acknowledgement

The financial support, from the EEC within the Biomolecular Engineering Programme, of our efforts to improve secondary product formation in cultured plant cells by genetic engineering is gratefully acknowledged. I would also like to thank Dr. V. Wray for his linguistic advice.

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