Prace przeglądowe

Secondary metabolites from plant cell and tissue cultures

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1. Introduction

Plant material produces primary and secondary metabolites. The primary or general metabolites, such as compounds involved in the energy metabolism, are essential for the life of the cell. However, the cell can produce large numbers of compounds, which are not essential for the cell's life. The function of these secondary metabolites is less obvious. Bennet and Bentley (1) reviewed the literature on this subject and proposed the following definition: "A metabolic intermediate or product, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metab-

olites by a wider variety of pathways than is available in general metabolism". This definition still does not reveal the function of secondary metabolites. The function is sometimes evident, such as attraction of pollinators by flowers through colour and/or fragrance, in other cases the function is very presumable as for several anti-herbivoric compounds. A common phenomenon is the formation of secondary metabolites upon stimulation of the cell by an elicitor. These elicitors are mainly cell wall fragments from either organisms attacking the plant, or the attacked plant itself. The signal of the elicitor induces significant changes in the secondary metabolism of the plant cell. The formed defense products are known as phytoalexins. The various *in vivo* functions established for secondary metabolites are shown in table 1. Several secondary metabolites are applied by mankind; some of these applications are known for thousands of years. In a number of cases, secondary metabolites are applied in their natural function, but mostly secondary metabolites have an application different from their original function. Application fields of secondary metabolites are summarized in table 2. The total number of known secondary metabolites is huge. In table 3 a division is given in seven groups, each containing several sub-groups: for each group a representative sub-group and a member of this sub-group is presented. Only the group of the alkaloids consists already out of over 15.000 unique structures (3). Several alkaloids are used as pharmaceuticals (table 4). Obviously, it is impossible to discuss all secondary metabolites and their various pathways within the scope of this paper. A general picture of the origin and destination of the most important pathways is given in figure 1, in figure 2 the pathway of the alkaloid ajmalicine is shown (this example is chosen, because the production of this compound will be dealt with somewhat more in detail in this paper). There are probably many products in this plethora of chemically complex compounds of which the valuable application is unknown. High through-put screening programmes are a possibility to detect such compounds for applications such as drugs.

Until now, most research effort on secondary metabolites in plant cell biotechnology has been focused on products with an already known function. The advantage of this approach is that the elaborate screening procedures are avoided, but it has the disadvantage that the development of commercial plant cell biotechnology has to compete with the fully optimized traditional production techniques of these compounds. This traditional production of secondary metabolites is in most cases still comparable with the medieval approach: extraction or distillation of plant material. Although this technology has been developed to the 20th century level, some disadvantages occur. The supply of raw material may be unstable in quality and quantity. The raw material is in several cases a very slow growing plant or tree, with a very narrow ecological niche. Production of a secondary metabolite from such a source ends up in extermination of the species. In these cases production from cell or tissue culture could be an attractive alternative.

TABLE 1
SOME NATURAL FUNCTIONS OF PLANT SECONDARY METABOLITES

allelophatic substances, inhibiting germination growth or occurrence of other plants
predators control
infectious diseases control
pollinators attraction

TABLE 2
APPLICATION FIELDS OF PLANT SECONDARY METABOLITES

* allelopathic substances	* flavours
* fragrances	* herbicides
* insecticides	* lubricants
* pharmaceuticals	* pigments
* poisons	* sweeteners
* waxes	* cosmetics

TABLE 3
MAJOR GROUPS OF NATURAL PRODUCTS

Section	Number of classes	Typical class	Typical compound
Quinones	2	Anthraquinones	alizarin
Phenyl propanoids	9	Dopa and betalains	l-DOPA
Isoprenoids	5	Triterpenes	digitoxin
Alkaloids	8	Indole alkaloids	vincristine
Other Compounds	6	Volatiles	alliin (garlic flavour)

From B. E. Ellis, 1988,(2)

TABLE 4
PHARMACOLOGICALLY IMPORTANT ALKALOIDS

Alkaloid	Pharmacological effect
1	2
Morphine, codeine	Analgesic
Emetine	Antiamoebic
Quinidine, ajmaline	Antiarrhythmic
Ajmalicine	Cerebral circulation improvement
Berberine	Antibacterial
Vinblastine, vincristine, harringtonine, camptothecin, taxol	Anticancer
Colchicine	Anti-inflammatory

1	2
Quinine	Antimalarial
Glaucine, noscapine	Antitussive
Physostigmine, pilocarpine, atropine, ephedrine, nicotine, scopolamine	Act on autonomic nervous system
Cocaine	Local anaesthetic
Tubocurarine, papaverine, theophylline	Muscle relaxant
Vincamine	Vasodilator

From C. A. Hay et. al., 1988, (4)

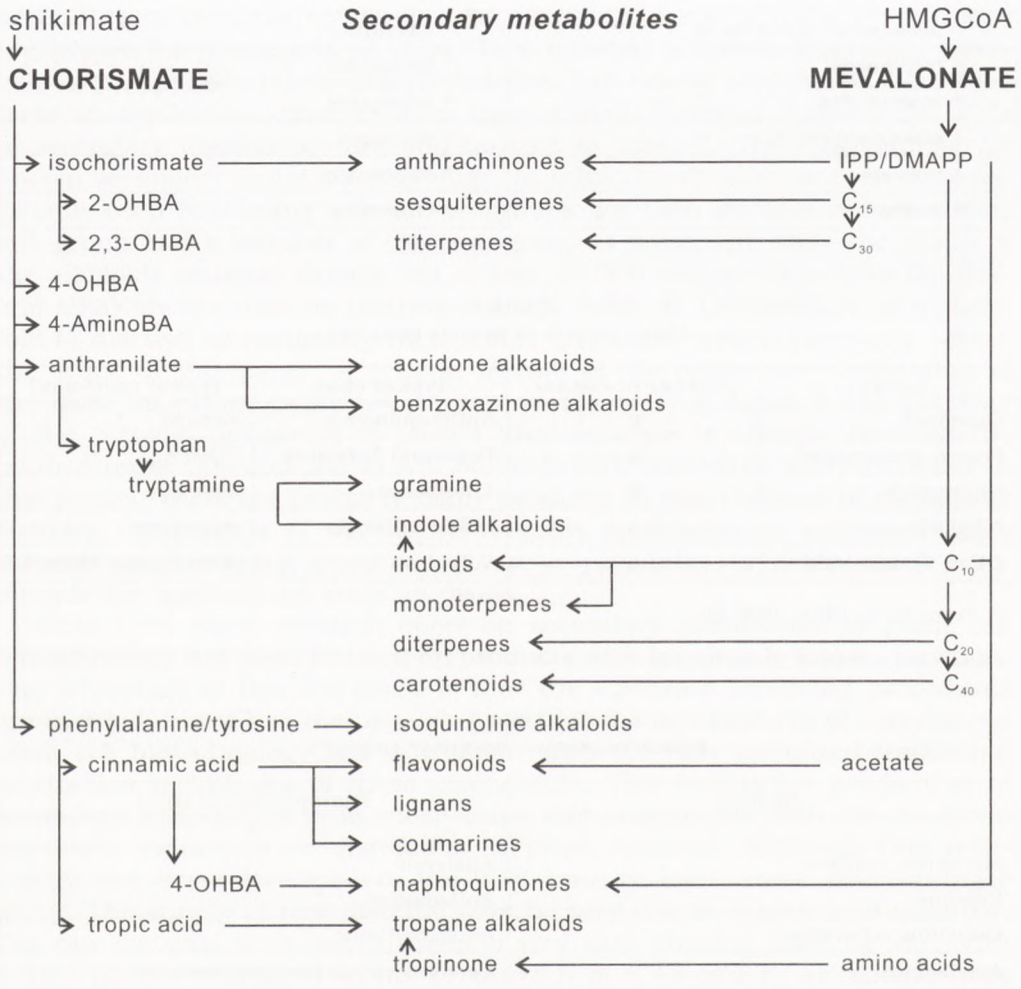

Fig. 1. Schematic relationship of major secondary metabolite pathways (derived from chorismate and mevalonate) in plants. HMGCoA = 3-hydroxy-3-methylglutaryl-coenzyme A, IPP = isopentenyl diphosphate, DMAPP = 3,3-dimethylallyl diphosphate, OHBA = hydroxybenzoic acid, 4-AminoBA = 4-aminobenzoic acid.

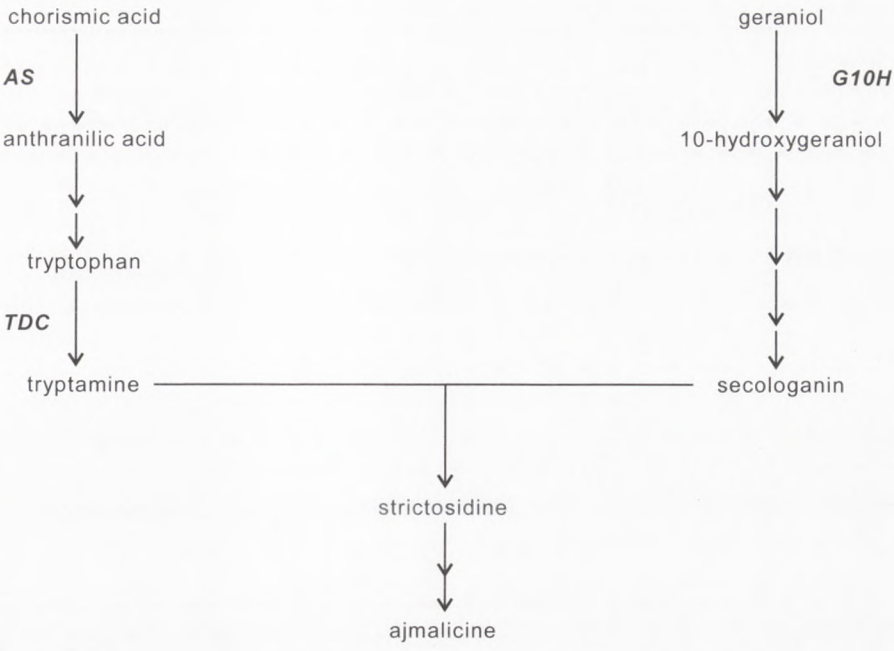

Fig. 2. Some intermediates and key enzymes in the biosynthesis of ajmalicine. AS: Anthranilate Synthase, TDC: Tryptophan Decarboxylase, G10H: Geraniol-10-Hydroxylase.

From the beginning of the 20th century studies have been carried out on the *in vitro* culture of plant cells. In the fifties the state of the art was that culture of suspended cells on artificial media was possible and that the production of secondary metabolites by *in vitro* cell cultures was established. These were the essential building blocks for a new technology for the production of plant secondary metabolites by cell cultures in bioreactors, circumventing the disadvantages of the 'classic' production as mentioned above. Scaling-up the process appeared no problem according to a paper of Noguchi et al. in 1970 reporting the culture of tobacco cells in a 20,000-l bioreactor (5). A fast development of processes employing this new technology was expected. However, only one industrial process has been realised until 1995: the production of shikonin by cell cultures of *Lithospermum erythrorhizon* in 750-l fermenters in Japan. Currently, the production of taxol by cell cultures on a large scale is close to commercialisation. Experimental runs at a 60,000-l scale are carried out in Germany.

2. Process economy

An economic analysis may answer the question: what is hampering the development of this technology? An approach for such an analysis is to design

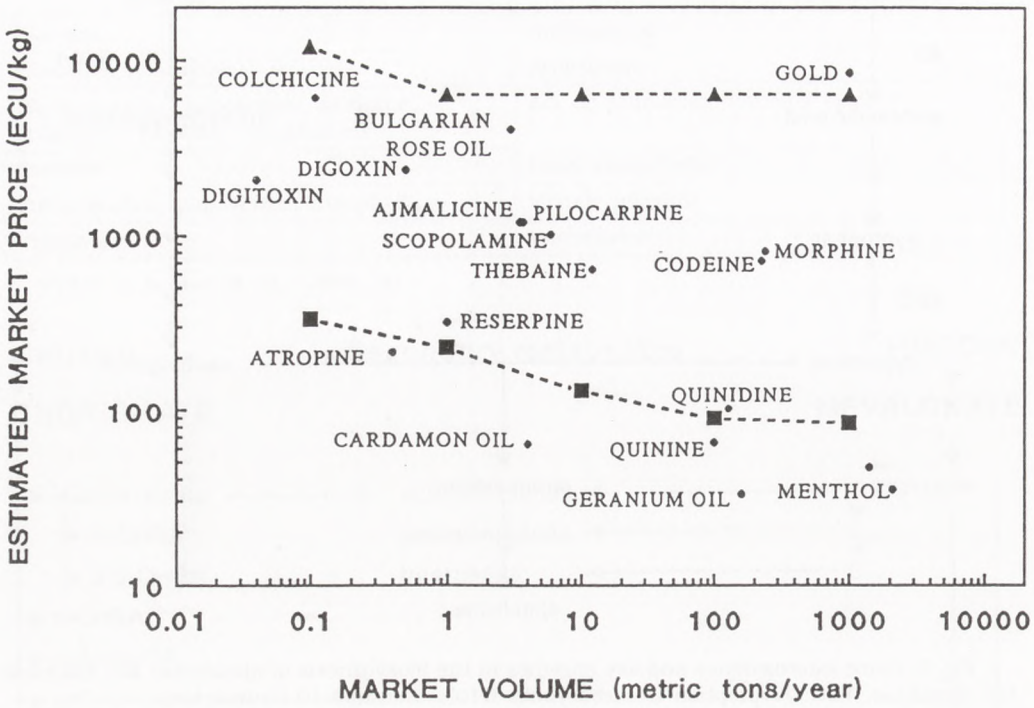

Fig. 3. Relation between market price and volume for plant secondary metabolites in comparison with theoretical cost prices (squares: current productivity data, triangles: optimised productivity data).

a theoretical process for the production of the compound of interest with well-defined parameters and calculate the total production costs. The sizes of the process equipment are of course dependent on the quantity of product demanded, the growth rate of the cell material, the production rate of the cells, the type of process (e.g. one-stage, two-stage, batch, continuous), and the type of equipment (e.g. stirred tank or air-lift reactors). The equipment costs, energy costs, medium costs etc. can be derived from the size of the equipment and the run time of the process. A cost price per kg product results from an addition of these costs and can be compared with the current market price of the product. Elaborated examples of this approach were presented by Goldstein et al. (6). During the last 15 years there has been a considerable progress in plant biotechnology. Therefore it was worthwhile to conduct a comparable calculation on the basis of the current knowledge. The following assumptions were made: a secondary metabolite is produced in a two-stage batch process; in the first stage biomass is grown under optimal growth conditions, in the second stage the biomass is transferred to an induction/production medium, where growth is suppressed and production starts under

optimal conditions. The bioreactors are standard stirred tank reactors, this choice is motivated by the opinion that standard fermentation equipment will support acceptation of this new technology in fermentation industry. The often mentioned disadvantage of the stirred tank reactor for plant cells is the possible shear damage to the cells, but this problem is now considered to be less important (7). Two series of growth and production parameters were used in these calculations: 1) the average current parameters of cell cultures, 2) a combination of optimal values, characterising an imaginary plant cell system with the highest possible productivity. The results of such calculations for four different production volumes are combined with price figures and production volumes of well-known plant products in figure 3. The line presenting the cost prices with current productivity data is situated above the price-production points of most known products, which means that plant biotechnology is too expensive to compete with the traditional technology. However, the line calculated from the maximum productivity data reveals that competitive production by plant biotechnology could be possible. The conclusion from this exercise is undoubtedly that plant biotechnology is an option only if the productivity of the cell cultures is increased considerably. Some very interesting compounds for plant biotechnology are not plotted in the figure 3: vinblastine, vincristine, taxol. They fall outside the scope of the figure, because their product volumes are very low and the prices of these products are very high. This makes plant biotechnology an option for these compounds. But, three other constraints for plant biotechnology can be illustrated with these products. Firstly, the cell suspension must produce indeed the required product (for vincristine and vinblastine this is not the case until now). Secondly, the price of the product should be stable if more product comes on the market by another production technology. Thirdly, although the price of the product is significant, the total product value is limited, because of the low production volume. This is a rather general observation for the field of secondary metabolites. In table 5 this point is illustrated for three products with a large variation in prices and production volumes. The limited total market value sets of course also a limitation on the acceptable investments in a production process.

TABLE 5
ECONOMIC IMPACT OF SOME ALKALOIDS

Compound	Yearly production kg/year	Market value ECU/year
Vincristine	2	10 000 000
Ajmalicine	3 500	5 000 000
Quinine	100 000	15 000 000

1 ECU = 1.3 US$ = 1.8 DM

An economic analysis can reveal the most important cost factors of the production process. In figure 4 results are presented for a cost price esti-

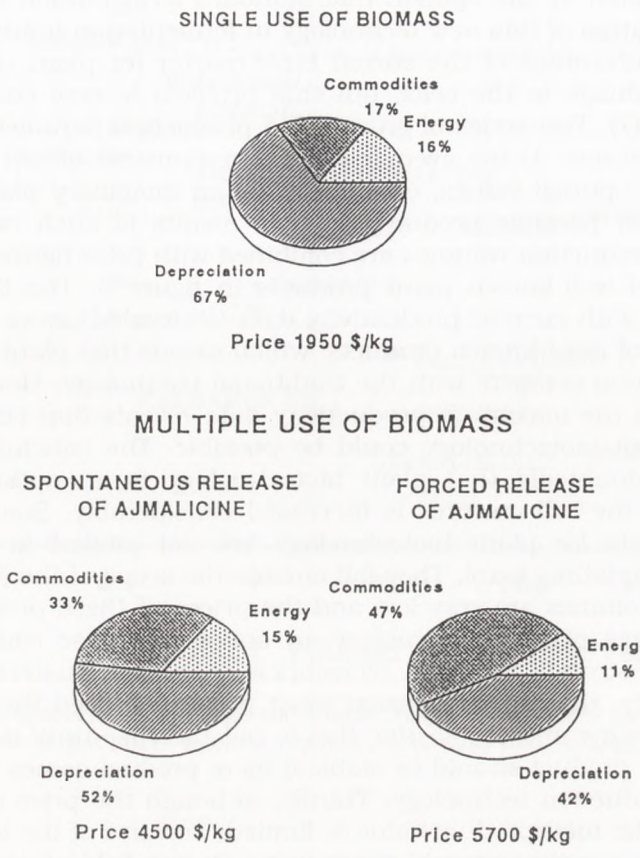

Fig. 4. Cost breakdown of ajmalicine production by plant cell culture technology. Comparison of three different production systems.

mation of ajmalicine through different production processes: 1) single use of biomass in a two-stage process, 2) recirculation of the biomass in processes were the product ajmalicine spontaneously or forced (by a permeabilising agent) is released in the medium and the biomass is used again. Surprisingly, the product price by the latter technology is higher than by the single-use technology. This is caused by some cost-increasing actions necessary in the recirculation processes (addition and separation of the permeabilising agent or low biomass concentration to facilitate release of the product). From this figure it is also evident that the investment in expensive fermentation equipment is cost factor number one, followed by the medium costs. Therefore, production costs can be reduced by increasing the productivity of the plant cell system, which generates savings on equipment and medium.

3. Productivity increase

There are several methods to increase productivity in the complete process from plant to product. It starts by the choice of the plant species and the specific cell suspension (screening and selection), and eventually the possibility of increasing the yield by genetic modification of the involved pathway. The choice of the process type is a next step; examples mentioned before are single use of biomass vs. multiple use of the biomass. In a fermentation process there is a limited number of control factors, that can be manipulated: medium and gas composition, temperature, pH, mixing, etc. Downstream processing is another process operation were probably some yield increase can be realised. Several examples of integrated fermentation and product recovery on a laboratorium scale have been reported. In table 6 the available techniques for productivity increase are summarised. In this paper a few examples will be discussed illustrating the possibilities and importance of the optimisation of process conditions in the bioreactor. The process of interest is the production of the alkaloid ajmalicine by a cell suspension culture of *Catharanthus roseus* in a two-stage batch process. In the first stage biomass is grown in a growth medium. In the second stage the production of ajmalicine is induced by transferring the cells to a induction/production medium.

TABLE 6
STRATEGIES TO IMPROVE PRODUCTIVITY

Biological approaches
* Screening and Selection
* Genetic modification
Process options
* Bioconversion
* Differentiation (e.g. hairy roots)
* Immobilization
* Permeabilization and Extracellular storage
Process conditions
* medium composition
* gas composition
* inoculum condition (two-stage process
* cell density
* temperature
* light

In figure 5 the effect is shown of the transfer of a producing system from a shake flask into a bioreactor. The ajmalicine production is almost com-

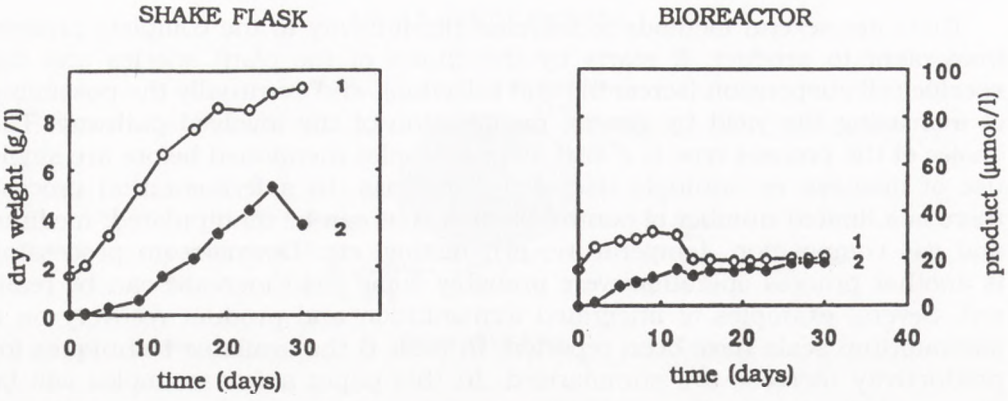

Fig. 5. Time courses of total ajmalicine (1) and dry weight (2) in shake flask and bioreactor experiments.

pletely lost. This observation has been described for several plant cell systems. The main cause is that the gas exchange between gas and liquid phase and thus the coupled dissolved gas concentrations in a aerated and stirred bioreactor are quite different from those in a shake flask. One conclusion from this observation is that production experiments should be carried out preferably in a bioreactor: the behaviour at a large scale is easier to predict from these bioreactor experiments, and the conditions in the bioreactor can be manipulated much better than in the shake flask. The production of ajmalicine could be restored to a great deal in the bioreactor by recirculating partly the gas phase: obviously there were important gaseous compounds that had to be kept in the bioreactor (figure 6). On the other hand, some refreshment of the gas phase in necessary to keep the oxygen concentration at the necessary level.

Experiments were run to determine the effect of the dissolved oxygen concentration on the ajmalicine production. Therefore, a control system was designed to keep the oxygen concentration at a set point value. The results in figure 7 show that the ajmalicine production needs an oxygen concentration above 40% saturation. This is much higher than the oxygen need for growth (above 10%). From these results the assumption can be made that oxygen acts as a substrate for one or more enzymes in the ajmalicine pathway, and that these enzymes have a lower affinity for oxygen than the enzymes of the primary metabolism.

Induction/production media for the production of secondary metabolites by plant cell suspensions have an high initial concentration of the carbon source in common. These media are applied in batch experiments. This implies that this initial sugar concentration decreases during the production

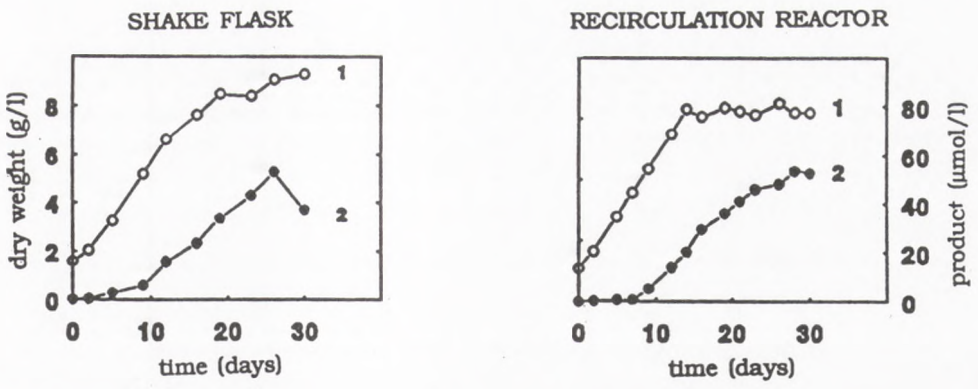

Fig. 6. Time courses of total ajmalicine (1) and dry weight (2) in shake flask and recirculation bioreactor experiments.

period through consumption by the cells. In fact, the optimal sugar concentration is unknown. In a fed-batch experiment where the sugar concentration was kept at constant levels the optimum concentration for the ajmalicine production by *Catharantus roseus* cells was established. Figure 8 shows that the optimum concentration is around 30 g/l, this is considerably lower than the concentrations between 80 and 100 g/l suggested by several authors. In batch experiments the optimal concentration is reached apparently after some time and maintained only for a short period.

The oxygen addition to the culture demands a specific fresh air flow. However, the recirculation experiments mentioned before, revealed that a large flow decreases the concentration of other important gaseous metabolites in the suspension. In experiments where the gas flow is controlled at various values and all other variables are kept constant it could indeed be proven that there is an optimum in the gas flow.

In a two-stage production process the cells have to be transferred from the growth medium to the production medium. The optimal moment for this transfer is hardly studied. In fact most researchers assume that late exponential cells will be optimal because they are in a very active state. In the case described in this paper however this assumption was tested and for the ajmalicine it was shown that surprisingly late stationary cells were much better equipped for the production phase. Enzyme levels from the ajmalicine pathway did not clearly correlate with this result (but not all enzymes of this pathway were determined). Another hypothesis is, that the older cells are better equipped to withstand the osmotic shock coupled with the transfer from growth medium to production medium.

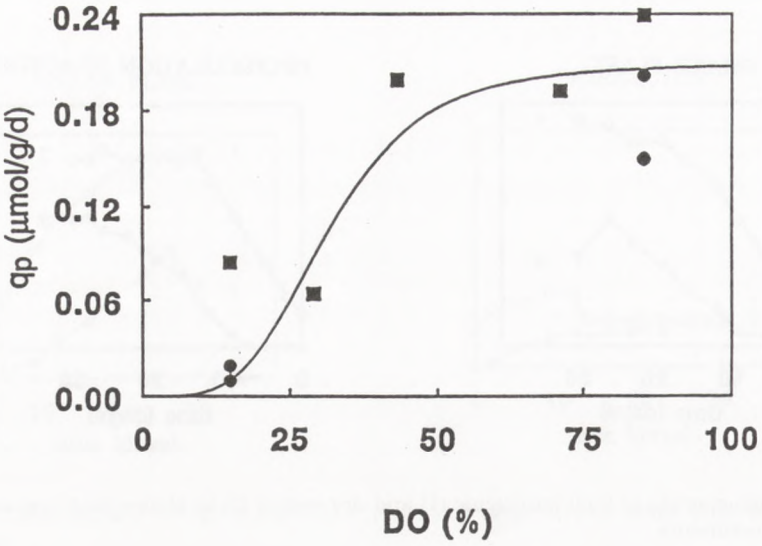

Fig. 7. The experimental relation between the DO concentration and the specific ajmalicine production rate q_p of *Catharanthus roseus* cells. The line represents a model equation. The different markers represent data from different experiments.

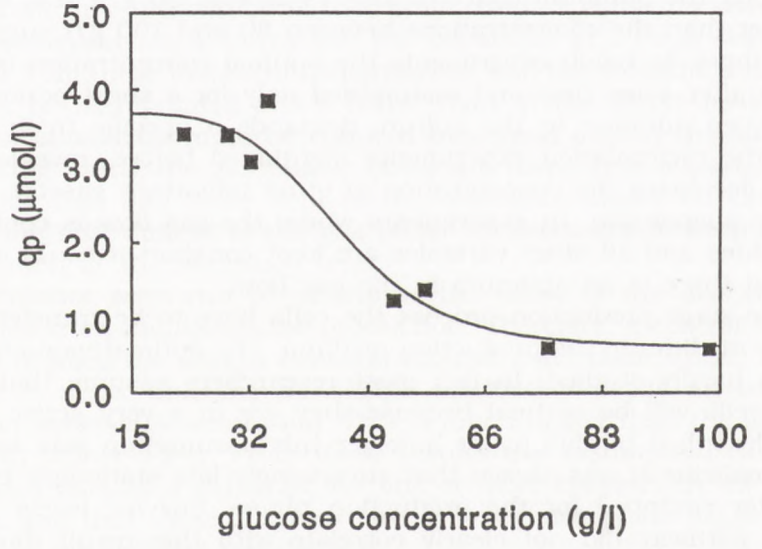

Fig. 8. The experimental relation between the glucose concentration and the specific ajmalicine production rate q_p of *Catharanthus roseus* cells. The line represents a model equation.

4. Conclusions

Plants produce a plethora of secondary metabolites. Many secondary metabolites are applied as pharmaceuticals, food additives, etc. Elaborate screening programmes will reveal more interesting plant products. Plant cell biotechnology is a promising alternative for the production of these products. Although the fundamentals of this technology are available for more than 40 years, there are only a few commercial processes. An economic analysis reveals that the productivity of plant cell and tissue culture systems is too low in most cases. The productivity may be increased by biological, genetical, and technological approaches. The results of investigations on the ajmalicine production by *Catharantus roseus* in laboratory scale bioreactors showed that optimisation of the bioreactor controls, like aeration, can already result in an increase in productivity.

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Summary

A vast number of secondary metabolites can be found in the vegetable kingdom. Only the group of the alkaloids includes already over 15.000 unique structures. The function of secondary

metabolites for the plant is in many instances obscure. A small number of this plethora of unique compounds has been applied as pharmaceuticals, food additives, herbicides, etc. Intensive screening programmes would probably reveal a lot more valuable compounds. Traditionally, these products have been purified from plants. Since the development of plant cell and tissue culture, these techniques provide an alternative. However, an economic analysis shows that in many cases the productivity of cell and tissue cultures is too low to compete with the traditional production. There are various techniques to increase the productivity. In this paper, the possibilities of productivity enhancement by an optimized control of the process conditions in the bioreactor is illustrated for the production of ajmalicine by *Catharanthus roseus* in a two-stage batch process. During the second (production) phase, the productivity could be increased considerably by optimization of the glucose concentration in the medium and the aeration rate in the bioreactor. Both the dissolved oxygen concentration, and the concentration of other dissolved gaseous metabolites were controlling the productivity. Furthermore, the age of the inoculum appeared to be an essential factor.

Key words:
secondary metabolites, plant and tissue culture, process economy, productivity.

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