PRODUCTION AND EXCRETION OF SECONDARY METABOLITES BY PLANT CELL CULTURES OF *TAGETES*

PRODUKTIE EN UITSCHEIDING VAN SECUNDAIRE METABOLIETEN DOOR PLANTECEL-CULTUREN VAN *TAGETES*

Promotor: dr. ir. J. Tramper
Hoogleraar in de bioprocestechnologie

R.M. BUITELAAR

PRODUCTION AND EXCRETION OF SECONDARY METABOLITES BY PLANT CELL CULTURES OF *TAGETES*

Proefschrift
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STELLINGEN

1. Door het toevoegen van een zorgvuldig geselecteerd organisch solvent of adsorbent aan een plantecelcultuur kan de excretie van secundaire metabolieten aanzienlijk verhoogd worden.

Dit proefschrift

2. Het begrip 'continu cultuur' wordt in de plantecelbiotechnologische literatuur vaak ten onrechte gebruikt voor systemen waarbij alleen het medium en niet de cellen continu stromen.

Rhodes, M.J.C., Robins, R.J., Hamill, J. and Parr, A.J., 1986, New Zealand Journal of Technology, 2, 59-70

3. Hoewel Stoicheva et al. schrijven dat hun resultaten met betrekking tot de relatie tussen de polariteit van solvents en de levensvatbaarheid van gistcellen in aanwezigheid van die solvents overeenkomen met de bevindingen van Brink en Tramper en van Laane et al., blijkt uit hun meetgegevens het tegenovergestelde.

Stoicheva, N.G., Davey, C.L., Markx, G.H. and Kell, D.B., 1989, Biocatalysis, 2, 245-255

Brink, L.E.S. and Tramper, J., 1985, Biotechnology & Bioengineering, 27, 1258-1269

Laane, C., Boeren, S., Vos, K. and Veeger, C., 1987, Biotechnology & Bioengineering, 30, 81-87

4. De kwaliteit van het werk van wetenschappelijk onderzoekers kan aanmerkelijk worden verbeterd door het aannemen van voldoende analisten of technici. De kwaliteit van het werk van analisten en technici kan aanmerkelijk worden verbeterd door hen in de gelegenheid te stellen aktief deel te nemen aan wetenschappelijke bijeenkomsten.

5. Het oprichten van een samenwerkingsverband leidt niet automatisch tot samenwerking.

6. De verschijning van steeds meer boekenbijlagen in kranten en weekbladen maakt het onmogelijk de daarin besproken boeken nog te lezen.

7. Het is met zwemmen als met het bedrijven van de wetenschap: zolang men vlak achter de koploper ligt, lijkt het bijzonder eenvoudig deze in te halen. Wanneer dit eenmaal is gelukt en men op kop ligt, blijkt het zeer moeilijk deze positie te handhaven.

8. Het aantal aangedreven wielen, spoilers, sportstrepen en dergelijke dat een autobezitter op zijn auto laat aanbrengen is omgekeerd evenredig met zijn mate van zelfvertrouwen.

9. Het verbod op wapenleveranties aan landen in oorlog veronderstelt ten onrechte dat wapens gebruikt kunnen worden voor vreedzame doeleinden.

10. Gezien de openingstijden van 'chemokarren' gaat men ervan uit dat in huishoudens louter bestaand uit werkende personen geen chemisch afval geproduceerd wordt.

11. Het achterblijven van de emancipatie in restaurants, tot uiting komend in het eerst bedienen van de vrouwelijke gasten, gecombineerd met de etiquette te wachten tot iedereen bediend is, leidt er nog altijd toe dat vrouwen daar hun maaltijden doorgaans enkele graden kouder moeten consumeren dan mannen.

Stellingen behorend bij het proefschrift: 'Production and excretion of secondary metabolites with plant cell cultures of *Tagetes*'.

Nettie Buitelaar, 13 december 1991

VOORWOORD

Het proefschrift dat hier voor u ligt is het resultaat van vier jaar onderzoek bij Proceskunde.

Een proefschrift heeft, net als veel andere zaken in het leven, de eigenschap dat je het niet alleen voor elkaar krijgt, en daarom wordt het voorwoord meestal voornamelijk gebruikt om alle betrokkenen te bedanken. Zo ook hier, hopelijk ben ik niemand vergeten.

Natuurlijk begin ik met Hans Tramper, mijn promotor, maar ook een hele goede vriend. Naast de vele discussies over het werk en de publikaties, waarbij vooral veel streepjes gezet en weer verwijderd werden, hebben we bijvoorbeeld ook de afgelopen jaren vele honderden kilometers achter elkaar aan gezwommen en heel wat flessen Rioja leeggedronken, al of niet onder het mom van een bridge-avond. Vooral deze combinatie van een goede zakelijke en een goede persoonlijke band heb ik altijd heel erg gewaardeerd.

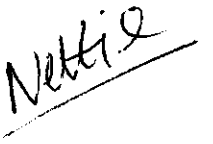
Ruime belangstelling en inzet voor mijn onderzoek is er gelukkig altijd geweest van de kant van studenten en buitenlandse medewerkers. Isabel Canales, Teresa Cesário, Peter Fredrix, Peter Geerlings, Ruud den Hartog, Renze Heidstra, Alette Langenhoff, Imke Leenen, Gijs van Rooijen, Jurriaan Schlatmann, Iñaki Susaeta, Marian Vermuë en Everhard de Vries, zonder jullie werk was dit boekje lang niet zo dik geweest.

In het dagelijkse leven is de sfeer op het werk altijd erg belangrijk; gelukkig was (is) die sfeer bij Proceskunde altijd leuk en vrolijk. Naast de gezamenlijke reizen, uitstapjes en avonden waren ook de gewone dagen altijd gezellig. Voor de samenwerking en collegialiteit wil ik alle proceskundigen, en vooral mijn kamergenoten, bedanken.

In de biotechnologie staat multidisciplinair werken altijd hoog in het vaandel. Ook bij dit projekt was dat het geval. Dit onderzoek is uitgevoerd in het kader van NOVAPLANT, en ik wil graag de leden van deze groep ook bedanken voor de samenwerking en voor de stimulerende discussies die er soms gevoerd werden op de NOVAPLANT bijeenkomsten.

Speciaal wil ik ook nog de diverse afdelingen van de Centrale Dienst bedanken voor het vakwerk dat ze altijd afleverden. Met name met de fotolokatie, de tekenkamer en de service afdeling heb ik altijd zeer goede ervaringen gehad.

Tot slot wil ik mijn beide ouders bedanken voor de mogelijkheden die ze mij geboden hebben.



CONTENTS

SHORT INTRODUCTION

The subject of this Ph.D. thesis is the stimulation of the secondary metabolite production by plant cell cultures.

This promising field does not yet know many commercial successes, due to the slow growth of the cells, the low productivity and the instability of the cell cultures. Several ways exist to alleviate these problems.

The introduction of hairy root cultures, produced by infecting plants with *Agrobacterium rhizogenes* and bringing the resulting hairy roots in culture, has been a major step forward. The hairy root cultures consist of differentiated tissue, which results in a better stability and also in higher growth rates and productivities.

The use of elicitors can also help to improve the productivity of the plant cell or hairy root cultures. The selection of the right elicitor is, however, still a matter of trial and error.

Apart from these biological approaches, the manipulation of the engineering conditions can have very valuable consequences as well.

The immobilization of plant cells can protect them from the prevailing shear forces, and it is also reported to have a stimulating effect on the productivity of the plant cell cultures in some cases.

Furthermore, the use of two-phase cultures, either liquid-liquid or liquid-solid, could also be of great importance for the in situ extraction of the product, thus facilitating more efficient downstream processing procedures.

The plant cell cultures used in the present study were derived from *Tagetes spp.* (marigolds), that produce thiophenes, compounds with a strong biocidal activity.

The organization of this thesis is as follows. The first chapter provides an overview of recent literature on the methods used to enhance the productivity of plant cell cultures, and can be seen as an introduction to the thesis. In the second chapter, the results are presented of immobilization experiments on a small and on a large scale using the resonance nozzle, with various gel types for which gelling in organic solvents is required. The reactions of four cell types (bacteria, yeast, plant cells and insect cells) to these circumstances were tested. In chapter three, the influence of the additions of various organic solvents on cell cultures of *Tagetes minuta* was tested. From there on, all the other experiments have been carried out with hairy root cultures of *Tagetes patula*. Chapter four gives a comparison of several methods available for the hairy root biomass estimation in reactors. In chapter five the influence of various organic solvents on the behaviour of the hairy roots in shake flasks and bioreactors is described. Chapter six provides similar information about the use of aqueous

two-phase systems. Chapter seven gives the results of the addition of several elicitors to the hairy root cultures and chapter eight integrates this use of elicitors with the use of a solid second phase. In the final chapter, a general discussion of the previous chapters is provided.

Chapter 1

STRATEGIES TO IMPROVE THE PRODUCTION OF SECONDARY METABOLITES WITH PLANT CELL CULTURES

A LITERATURE REVIEW

R.M. BUITELAAR AND J. TRAMPER

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Chapter 1

INTRODUCTION

The production of secondary metabolites with plant cells has been the subject of much research the last years. The expectations were high since the market introduction of the first commercial product made with this technique, shikonin made with cell cultures of *Lithospermum erythrorhizon* in Japan. In spite of the huge amount of research effort that has been put into this topic, not many products have reached the commercial stage since then. It is generally acknowledged that the main problem in this field is the lack of basic knowledge of the biosynthetic routes, and the mechanisms behind the production of secondary metabolites. There are, however, some techniques that have beneficial effects on the production and excretion in many cases, and they will be reviewed in this paper.

The subjects treated will be the application of elicitors that can stimulate production and excretion, the immobilization of plant cells, the use of two-phase systems to enhance the excretion of secondary metabolites, the use of selected fermenters for the growth of the cells and the production of secondary metabolites, models that describe the phenomena during growth and production, and the commercial processes that exist at this moment, including some economic considerations.

Although the discussed strategies have proven to work in a number of cases, there is still no general approach that guarantees a long-term, high producing process with plant cell cultures, indicating the need for an increase of the research activities in this field.

ELICITATION

Introduction

One of the methods frequently used to increase the productivity of plant cell cultures is the use of so-called elicitors. Elicitors can be all types of compounds, that provoke (the increase of) the production of phytoalexins. Phytoalexins are antibiotically active compounds, and by that important factors in

the resistance of plants to microbial attack. Many secondary metabolites belong to the group of phytoalexins. So, if the right elicitor can be found, it is possible to enhance the production of the desired secondary metabolite.

Terminology

The terminology with respect to elicitors is not well-defined. Some authors, like Eilert (1987), who cited the IAPTC VI Congress in 1986, propose to reserve the term 'elicitor' for biotic factors only. Those elicitors can be plant-derived, endogenous elicitors, or they can be micro-organism-derived. The term 'abiotic elicitor' is sometimes used for physical and chemical stress factors that can also induce product formation, factors like UV light, extreme temperatures, ethene, heavy metals and so on. According to the conclusions of the same Congress, it is better to use the term 'stress factor', or abiotic stress, in this case.

Mode of action

Although there is still much uncertainty about the mechanisms involved in elicitation, Eilert (1987) mentions four different types of action for microbe/plant interactions:

*Direct release of the elicitor by the micro-organism and recognition by the plant cell

*Microbial enzymes release plant cell wall components, which then act as elicitors

*Plant enzymes release cell wall components from the micro-organisms, which in turn induce phytoalexin formation in the plant cells

*Elicitor compounds, endogenous and constitutive in nature, are formed or released by the plant cell in response to various stimuli.

These four mechanisms are defined for whole plants and not for plant cell cultures, but it is likely that the mechanisms will be similar in both cases.

Parameters

The effectivity of an elicitor treatment is dependent on many factors. In the first place, the choice of the right elicitor is of utmost importance. Unfortunately, no general rules exist to predict whether or not a given elicitor/cell culture combination will be successful. There can even be a pronounced difference in susceptibility to microbial attack between two cultivars of the same species, as was demonstrated by Barz et al. (1988) with *Cicer arietinum* cell suspensions producing pterocarpan phytoalexins upon elicitation with *Ascochyta rabiei*.

Once a suitable elicitor is found, still many factors remain to be optimized. Important parameters are: the elicitor concentration, the cell concentration, the culture age at the time of elicitor addition, the contact period between cells and elicitor, and the culture and nutrient conditions. All of these parameters have an optimum or a most suitable range and the effect can be dramatic. Buitelaar et al. (1991c) for example, found an increase in thiophene production by hairy roots of *Tagetes patula* of over 300% between day 1 and day 2 after the elicitation with *Fusarium oxysporum* extracts (Figure 1.1). At day 4, the thiophene production was back at its initial level. So, the harvest time is a factor of importance as well.

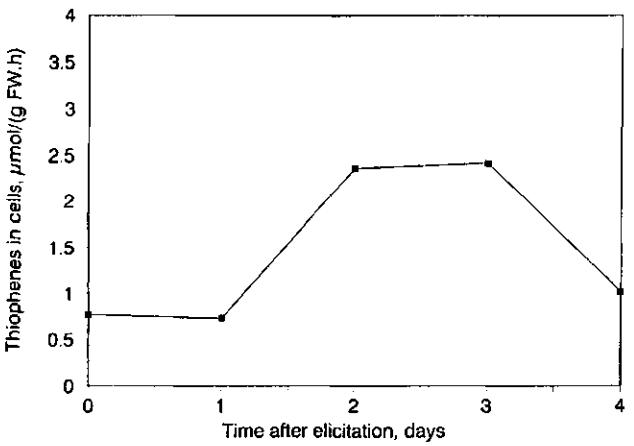

Figure 1.1
Thiophene concentration in hairy root cells of *Tagetes patula* after elicitation with 0.2% of *Fusarium oxysporum* extract. Adapted from Buitelaar et al. (1991c). FW is fresh weight.

Examples

One of the most intensively studied and commonly used biotic elicitors is derived from the fungus *Phytophthora megasperma*. Several fractions prepared from this fungus showed an increase in the activity of the enzyme phenyl ammonia lyase (PAL), generally recognized as one of the key enzymes in the process of induction of secondary metabolite synthesis. As an example, with *Glycine max* cell cultures, the production of the secondary metabolite glyceollin was increased upon addition of this elicitor (Ebel et al., 1976, Ebel et al., 1989 and Ayers et al., 1976). Although this elicitor is active in many plant cell cultures, there are some resistant species, like e.g. *Eschscholtzia californica*, at which this elicitor had no effect at all (Schumacher et al., 1987).

Another commonly used elicitor is yeast (*Saccharomyces cereviseae*) or yeast extract. This elicitor is said to be similar in both structure and absolute elicitor efficiency to an elicitor isolated from the mycelial walls of *Phytophthora megasperma* (Hahn and Albersheim, 1978). With yeast extract, the production of berberine by *Thalictrum rugosum* cells was increased up to four times and the production of alkaloids by *Eschscholtzia californica* was increased about thirty times (Brodelius et al., 1989). PAL-activity was proven to increase upon addition of yeast to *Glycine max* and *Thalictrum rugosum* cell cultures (Funk et al., 1987).

Other elicitors from microbial origin that are commonly used for elicitation are among others: *Aspergillus niger*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Penicillium expansum* (van der Heijden, 1989).

Of the abiotic stress factors, especially calcium is an interesting one. The evidence is growing that the calcium ion can act as a second messenger in the regulation of secondary metabolite production by plant cells. Kurosaki et al., (1987) were struck by the fact that many different elicitors provoked a similar phytoalexin production in cells of *Daucus carota*, indicating that this specific secondary metabolite production was mediated by so-called second messengers like Ca^{2+} and cAMP. They found that the omission of calcium from the medium resulted in a decreased 6-methoxymellein production, and concluded after numerous other experiments that calcium, together with cAMP, played a determining role in the production of secondary metabolites.

Ethene has also been suggested as an elicitor. For example, Cho et al. (1988) found that the addition of a precursor of ethene (Ethephon or 2-chloroethyl phosphonic acid) improved the production of berberine by *Thalictrum rugosum* cells by 30% and the production of caffeine by *Coffea arabica* cells with 85%. However, in experiments with hairy roots of *Tagetes patula*, Buitelaar et al. (1991c) did not find any positive effect of Etephon at all.

Conclusions

The general conclusion of the elicitor/plant cell work must be that for each specific case, the trial and error process of finding the right elicitor must be passed through. No general rules exist until now in this field. After this, all important parameters have to be optimized. Before the whole process of elicitation is fully elucidated, much research effort has to be carried out.

Chapter 1

IMMOBILIZATION

Introduction

The immobilization of biocatalysts, including plant cells, has received much attention the last decades. Reviews on immobilized plant cells have been published frequently by Brodelius (e.g. 1984, 1988b) and recently by Hulst and Tramper (1989).

There are many possible advantages of immobilization. In general, immobilization can facilitate the use of continuous-flow processes; the biocatalysts can be reused and the biocatalysts can easily be separated from the reaction medium.

Apart from these general advantages, there are some additional advantages for immobilized plant cells as well. The cell/cell contact that is induced by immobilization can be beneficial for secondary metabolite production. In this way, some differentiation can occur, which is believed to be a prerequisite for the production of secondary metabolites (Bringi and Shuler, 1990). Furthermore, immobilization protects the sensitive plant cells against shear forces, while in some cases the production and excretion can be stimulated (Wichers et al., 1983 and Asada and Shuler, 1989).

Some of the problems with immobilized cells are the introduction of gradients in the gel beads and the fact that some immobilization materials or procedures can affect cell viability in a negative way. Besides, immobilization introduces an extra cost factor.

Gel entrapment

The predominant method used for plant cell immobilization is gel entrapment. The most common gel is calcium alginate. A mixture of sodium alginate and cells is extruded dropwise into a $CaCl_2$ solution, where bead hardening starts to take place immediately. Alginate is the preferred gel because of the ease with which it is handled and because of its mildness (no high temperatures nor aggressive chemicals are needed). Moreover, calcium alginate can have beneficial effects on the production of secondary metabolites. Asada and Shuler (1989) immobilized *Catharanthus roseus* cells in calcium alginate, which resulted in a threefold increase of extracellular ajmalicine. Kim and Chang (1990b) obtained a 2.5-fold increase of shikonin production when they immobilized *Lithospermum erythrorhizon* cells in calcium alginate. Improved yields with cells immobilized in calcium alginate have also been reported by Vanek et al. (1989a) for the biotransformation of 2-(4-methoxybenzyl)-1-cyclohexanone to its glucoside by *Dioscorea deltoidea* cells, and by Subramani et al. (1989) for alkaloid production by *Solanum xanthocarpum* cells.

In many cases immobilized cells can be used for much longer periods than free cells. Naoshima and

Akakabe (1989) reused immobilized *Nicotiana tabacum* cells seven times for the biotransformation of various 3-oxobutanoates and the conversion speed increased with the number of uses. The authors attribute this to the growth of cell in the beads. These results are demonstrated in Figure 1.2. The

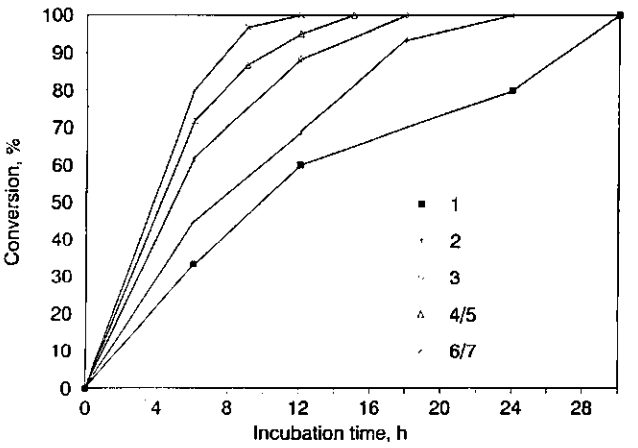

Figure 1.2
Conversion of a keto-ester to a hydroxy-ester by immobilized cells of *Nicotiana tabacum* cells as a function of incubation time. The legend indicates the number of the use. Adapted from Naoshima and Akakabe (1989).

same effect was shown by Kobayashi et al. (1988) with immobilized *Thalictrum minus* cells for the production of berberine. Although the production (in g/(dm^3.day)) by the free cells was higher, the immobilized cells kept producing for a much longer period, making the overall productivity (in g/g cells) of immobilized cells superior to that of the free cells.

The assumed diffusion limitation in the beads has been studied by several workers. Kobayashi et al. (1989) studied the effect of oxygen supply on berberine production in cell suspension cultures and immobilized cells of *Thalictrum minus*. They found that the oxygen uptake of the cells was twice as high in the production phase as in the growth phase. When they calculated the oxygen transfer rate, they concluded that the oxygen supply to the beads was high enough to support growth, but not for production. This was overcome by producing smaller beads with a lower cell load. This resulted in a longer lifetime for the immobilized cells than for the freely suspended cells.

Some other gels are used for plant cell cultures too, but the reports on them are very few. Noteworthy is the example of Vanek et al. (1989b), who immobilized *Solanum aviculare* cells in calcium pectate beads. The immobilized cells transformed cis-verbenol mainly into trans-verbenol, whereas free cells transformed cis-verbenol to verbenone as the main product. This indicates that even a mild

immobilization is not a priori an inert operation, but can have a pronounced effect on cell physiology.

Biofilms

Another application of gels for the immobilization of plant cells is their use as a support for biofilm formation. Kargi (1988) grew *Catharanthus roseus* cells on the surface of calcium alginate beads within the interspatial volume of a packed column. The cells showed some growth. In another article (Kargi and Freidel, 1988) they describe several gels for this purpose and gelatine proved to give the best adsorption results. No data were presented on growth and production.

Adsorption

Several authors (Archambault et al., 1989, 1990 and Facchini et al., 1988a/b, 1989, 1990) immobilized plant cells by adsorption to various support materials, such as fibreglass, polystyrene, sulfonated polystyrene, fluorinated ethene/propene, polyethene terephthalate, metals, plastics and ceramics. Mainly *Catharanthus roseus* cells have been used and there is ample evidence of the determining role of the surface tension of the cells, the surface and the culture liquid (Facchini et al., 1988a) in the adhesion procedure. Both groups (Facchini and Archambault) mention the importance of the culture age to obtain good adsorption. When the cells had reached the age of 8-14 days, immobilization was best (Archambault et al., 1989), probably due to the higher secretion of polysaccharides by the cells at that age. Adsorption of the cells also stimulated release of the product serpentine into the medium, in contrast to freely suspended cells. The total productivity of the adsorbed cells can surpass that of free cells (Archambault et al., 1990 suggest that the adsorbed biomass of *Catharanthus roseus* in their system contains a concentration of indole alkaloids 3-12.5 times as high as the maximum quantity found in suspended biomass in identical culture conditions).

Foam immobilization

Another material that has found widespread use in plant cell immobilization is polyurethane foam. The immobilization procedure is very simple; the cells migrate into the foam without any force, so no changes in temperature or ionic strength thus influence the viability of the cells. The foam can be used in all kinds of forms, like cubes; attached to stainless steel wire (Ishida, 1988); or wound sheets or tubes (Robertson et al., 1989). Usually, it takes about 10-15 days before all the cells are immobilized in the foam, which is a disadvantage. The effects of the immobilization in polyurethane foam are not uniform for all cell cultures. Corchete and Yeoman (1989) and Ishida (1988) mention an increase in the biotransformation activity of the cells, whereas Robertson et al. (1989) report a

decrease of the biotransformation activity and Gbolade and Lockwood (1990) find various results, depending on the substrates they use for biotransformation. So, immobilization does not necessarily have a beneficial effect on cell physiology.

Membranes

Hulst and Tramper (1989) mention five possibilities of membrane bioreactors for plant cells: hollow fibre units; flat plate systems; spiral wound units; tubular membrane reactors and multimembrane reactors. Recently, Kim et al. (1989) used a dual hollow fibre reactor to grow their *Lithospermum erythrorhizon* cells (see Figure 1.3). With this system, they reached very high biomass and secondary

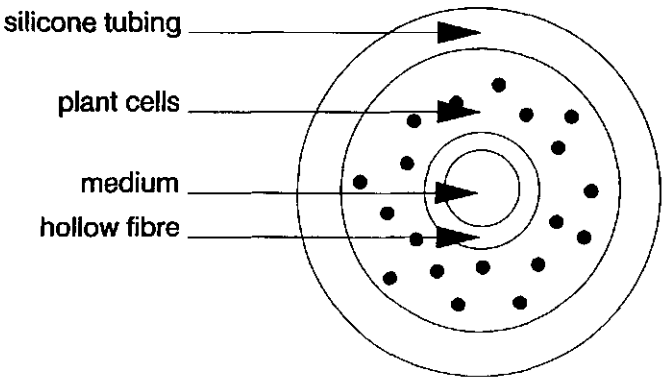

Figure 1.3
Dual hollow fibre bioreactor, as was used by Kim et al. (1989) for the immobilization of *Lithospermum erythrorhizon* cells.

metabolite concentrations; up to 325 g/dm^3 dry biomass and 221 mg/(dm^3.day) of products. This cell density is the highest ever reported for plant cell reactors, so membrane bioreactors may be considered as very important tools to reach high cell densities, which is one of the strategies to raise the overall volumetric productivity of plant cell bioreactors.

Concluding remarks

In this chapter, the predominant immobilization methods have been shortly discussed. There are more methods, like covalent linkage and natural aggregation, but there have been no substantial reports on these techniques the last years, to the author's knowledge. In general, one can say that immobilization has many theoretical advantages, but of course it introduces some extra handlings with the cells,

implying an extra risk of contamination. The advantages have to be evident, because immobilization is also an extra cost factor. The physiology of the cells is in nearly all cases more or less influenced by the immobilization, either in an advantageous or in a disadvantageous way. This indicates that for every plant cell process, the consideration has to be made whether immobilization is useful or not for that specific case.

EXCRETION OF SECONDARY METABOLITES

Introduction

The price of biotechnological products is often said to depend largely on the costs of the downstream processing part of the production process. Dwyer (1984) estimated that 50-90% of biotechnological process costs may be attributed to the recovery of purified product. This statement will be true for plant cell biotechnology as well.

The secondary metabolites produced are stored either intracellularly (in the vacuole or in other organelles), or extracellularly (bound to the cell wall or released into the medium) (Brodelius, 1990). The excretion behaviour of plant cell cultures varies from one species to another, and even within one species, from one cell line to another. As an example, protoberberine alkaloids are usually stored within the cells (Berlin, 1988). Nakagawa et al. (1984, 1986) however, selected a cell line of *Thalictrum minus* excreting their alkaloids spontaneously in the medium.

Apart from the selection of cell lines other approaches exist to trigger the efflux of secondary metabolites, mainly chemical and/or physical changes of the environment of the cells, and a number of those will be discussed in this chapter.

When the cells excrete their product, the product concentration in the fermentation broth is usually very low, which is an extra cost factor for the downstream processing. Concentration of the product stream, for instance in or on a second phase, could therefore be beneficial.

Another important consequence of the removal of the product from the aqueous phase can be the decrease of product inhibition effects, which occur even at the low product concentrations that are usually encountered in plant cell cultures (Buitelaar et al., 1991e).

Temperature

Secondary metabolites can be released with minimal loss of cell viability by the application of mild heat treatment to the cell cultures. Weathers et al. (1990) treated hairy roots of *Carthamus tinctorius*

and root disks and hairy roots of *Beta vulgaris* with temperatures ranging from 25-55°C. The release of polyacetylenes (*C. tinctorius*) and betanin (*B. vulgaris*) increased with increasing temperature. The viability of the cells however, decreased rapidly when temperatures higher than 35°C were used. The authors suggest a cyclic process, in which periods of product release are followed by a short recovery period for the cells.

Electrical permeabilization

Electroporation is a widely used technique to improve the transfer of genes into plant protoplasts for the construction of transgenic plants (e.g. Bates, 1990). The plasma membrane of the protoplasts is made permeable by electric pulses resulting in an effective uptake of foreign DNA. This technique has also been used to induce excretion of secondary products (Brodelius et al. 1986, 1988c). Products were released up to 100%, depending on the voltage applied, but viability decreased substantially. Another example of electrical permeabilization is the so-called iontophoretic release of secondary metabolites (Pu et al., 1989). Cells of *Catharanthus roseus* were subjected to a low current for 18 h. The rate of release of alkaloids almost doubled. Pretreatment with DMSO further enhanced the excretion. The authors, however, neither give any details on the viability of the cells nor on the percentage of the total production that is excreted.

Medium composition

In some cases excretion of secondary products can be enhanced by changing the composition of the medium. Berlin et al. (1988) lowered the medium concentration of inorganic phosphate, which resulted in an increased excretion of alkaloids by *Thalictrum rugosum*. The growth was retarded by lowering the phosphate concentration, but the specific production expressed per g cells increased with decreasing phosphate concentration. On the other hand, an increased ionic strength of the medium has also proved to enhance the release of intracellularly stored products (Tanaka et al., 1985). The pH of the medium can also influence the excretion of secondary metabolites. Majerus and Pareilleux (1986) observed a sharp increase of the excretion of alkaloids by *Catharanthus roseus* when the pH of the culture medium was changed from 9.0 to 4.3. However, this is not a general phenomenon in plant cell biotechnology. Other authors (e.g. Tanaka et al., 1985) found no effect on the product release when the pH was varied in the range from 4 to 10.

Chemical permeabilization

Many attempts have been made to permeabilize the plant cell membranes in a reversible way with organic solvents. Especially dimethylsulfoxide (DMSO) has been used in many cases, because it is known to extract sterols from the membranes of eukaryotic cells (Felix, 1987). Of all the cell types tested, only *Catharanthus roseus* survived the treatment with DMSO and could be used in a cyclic process, where product release and growth were alternated (Brodelius, 1988a). For the other cell types investigated, like *Cinchona ledgeriana* (Parr et al., 1986), *Chenopodium rubrum* (Knorr and Berlin, 1987) or *Thalictrum rugosum* (Brodelius, 1988a), much higher concentrations of DMSO were necessary for extraction of the products, but lead to cell death. Probably, the reason for this cell death is not the toxicity of DMSO as such, but the loss of cell compartmentation with the concomitant release of toxic compounds and degradative enzymes into the cytoplasm (Brodelius, 1988a). Apart from DMSO, other chemicals have been used, like chloroform and propanol (Berlin et al., 1989), mostly without success. The organic solvents used in successful (meaning: with preserved viability) experiments all had a lipophilic character, like hexadecane, perfluorchemicals and Miglyol (see next paragraph).

Two-phase cultures

It is of interest to selectively remove the desired products from the fermentation system. One of the ways to achieve this, is the use of two-phase cultures. One phase is the aqueous medium, the second phase can be either a water-immiscible organic solvent or a solid compound.

Criteria for the selection of suitable second phases are given by Beiderbeck and Knoop (1987): The material to be used as the second phase should be autoclavable, non-toxic, it should not influence the medium composition, it should bind the desired product, preferably in a specific manner, and finally, the product should easily be recovered from the second phase. Naturally, not all of these criteria will be met to the same extent for all cases, so optimization is required for each specific application of two-phase systems.

Another interesting aspect is the possible selectivity of the second phase. Selective extraction could be beneficial because plant cells produce a large number of biosynthetically related products, and the downstream processing of a mixture of several chemically similar solutes is difficult. Payne and Shuler (1988) used the polycarboxylic ester resin XAD-7 to selectively adsorb one alkaloid from a mixture of two, with good results, indicating the potential to use the resins for this purpose.

Two-liquid-phase systems

Basically, liquid phases other than water can be either miscible or immiscible with the aqueous phase. Because water-miscible solvents do not offer advantages for the downstream processing, this paragraph will only deal with water-immiscible organic solvents. Advantages of the use of organic solvents in biocatalysis can be (Tramper et al., 1987): the possibility of high concentrations of poorly soluble substrates or products; the reduction of substrate or product inhibition; the facilitated recovery of products and biocatalysts; the prevention of hydrolysis of substrates or products; the possibility to combine production with part of the downstream processing and the shift of reaction equilibria. Especially the first five possible advantages can be applicable for plant cell cultures. Naturally, there are also some disadvantages, like the increased complexity of the reaction system and the possible toxicity of the solvent for the cells (see e.g. Brodelius, 1988a).

Some general rules of thumb for the prediction of biocatalytic activity in the presence of a solvent are given by Laane et al. (1987). They used the log P of a solvent to predict its influence on the retention of biocatalytic activity. Log P is the logarithm of the partition coefficient of the solvent over a standard octanol/water two-phase system. From a number of experiments with enzymes and micro-

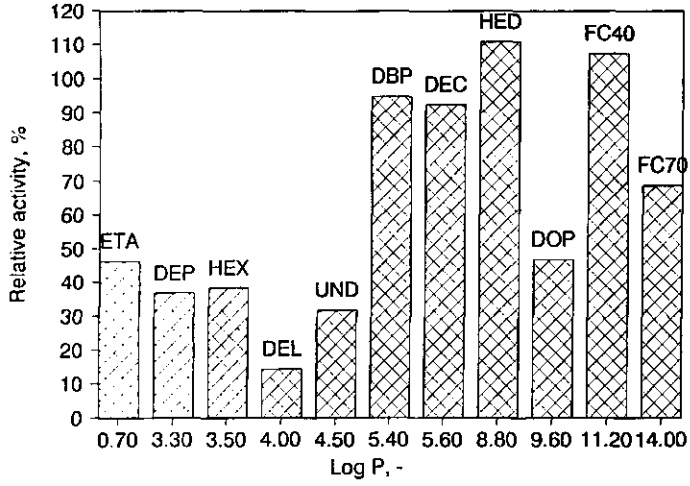

Figure 1.4
Relative respiration activity of *Tagetes minuta* cells in the presence of 1% solvent, as a function of the log P value of the solvents used. 100% is the activity in medium without solvent. ETA: ethylacetate; DEP: diethylphthalate; HEX: hexane; DEL: decanol; UND: undecanol; DBP: dibutylphthalate; DEC: decane; HED: hexadecane; DOP: dioctylphthalate; FC40 and FC70 are perfluorcompounds

organisms, they concluded that if the solvent log P > 4, indicating a rather hydrophobic solvent, then there is no negative influence of the solvent on the biocatalytic activity. If log P < 2, then hardly any activity is left. If log P is between 2 and 4 the influence of the solvent can vary. Later, this hypothesis was also tested with plant cells in our laboratory. Experiments with suspension cells of *Tagetes minuta* (Buitelaar et al., 1990) and with hairy root cultures of *Tagetes patula* (Buitelaar et al., 1991b) showed that these rules can be applied for plant cells as well, be it that the growth and production of the cells were not adversely affected when the log P value was higher than 5 instead of 4. This is demonstrated in Figure 1.4, where it is shown that all the solvents without negative effects on the activity do have a log P value higher than or equal to 5, while all the solvents with a lower log P value have adverse effects on the cell respiration activity of *Tagetes minuta* cells. Deno et al. (1987) gave a nice demonstration of this log P theory, without mentioning it, in their article on shikonin production in two-layer cultures. The cell yield and the volumetric productivity increased with increasing chain length and thus log P value of the alkanes applied. Aromatic and short chain alkanes (solvents with low log P values) gave very poor results.

A lipophilic organic phase that has frequently been used is Miglyol, a water insoluble triglyceride composed of fatty acids with 8-10 carbons. It has been reported to trap volatile lipophilic products, like monoterpenoids, that could not accumulate in medium (Berlin et al., 1984). Berlin et al. (1984) used this Miglyol with *Thuja occidentalis* cells for the production of monoterpenoids. In their case, this resulted in an increase of the total production from 0.8 mg/(g DW.day) to 3.0 mg/(g DW.day), 95% of which was excreted into the organic phase. In another case (Cormier and Ambid, 1987) it allowed a fivefold increase in the substrate load for the bioconversion of monoterpenes by *Vitis vinifera* cells.

So, although there have been many difficulties with the application of organic solvents to plant cell cultures, there now appear to be good possibilities. Many other solvents have been tested for the use in two-phase cultures like alcohols, alkanes, aromatics and many more. Kim and Chang (1990a,b) and Buitelaar et al. (1991b) selected hexadecane for further use because it had no detrimental effect on the cells at all. With *Lithospermum erythrorhizon* cells (Kim and Chang, 1990a,b) , the introduction of a second phase with hexadecane yielded a considerable increase in the shikonin production, which was even more the case when an elicitor preparation was used at the same time. When hexadecane was applied as the second phase for hairy roots of *Tagetes patula* (Buitelaar et al., 1991b) the total production did not change, but the excretion of the thiophenes increased from less than 1% to over 50% of the total production.

Perfluorchemicals can also be used as a second phase (King et al., 1990 and Buitelaar et al., 1991b). They have no negative effect on plant cells at all, but because of their inertness, hardly any product will dissolve in them to any reasonable extent, thus making them unsuitable for continuous product extraction from the fermentation broth. King et al. (1990) observed an increase in fresh and dry weight of *Solanum dulcamara* cells in two-phase systems with perfluorchemicals as the second phase, but the very heavy perfluorchemicals (the density varies around 1.9 kg/dm^3) may have influenced these results. They can, however, be used to enhance the oxygen transfer in submerged cultures of plant cells (King et al., 1990), micro-organisms (Damiano and Wang, 1985), or mammalian cell cultures (Ju et al., 1991 and references cited therein).

So, after preliminary selection of organic solvents, it is possible to use them in plant cell cultures for the extraction of products. The mechanism behind the toxicity of organic solvents is not yet understood. The solvents can permeabilize one or both of the plant cell membranes (the tonoplast around the vacuole and the plasmamembrane around the cytosol), thus removing the barriers between enzymes like proteases and their substrates, finally resulting in cell death (Brodelius, 1990 and Matile, 1990). Another, more or less related hypothesis has recently been proposed by Osborne et al. (1990) for micro-organisms. They state that there is a critical solvent concentration in the cell membrane, equal for all solvents. If the maximum solvent concentration, determined by the partition coefficients of the solvent for octanol/water and for a membrane/aqueous buffer system in the membrane is higher than this critical membrane concentration, then the progesterone 11α-hydroxylase activity of the cells stops; if this maximum concentration is less than 75% of the critical concentration, then there is no effect, with a transitional stage between 75 and 100%. They demonstrated this with cells of *Rhizopus nigricans*. However, when we used the data from organic solvent testing with plant cells (Buitelaar et al., 1990 and 1991b) we could not find any such relation at all. This indicates a different mechanism for the inactivation of plant cells as compared to microbial cells, or the need for a more elaborate theory.

For the application of two-liquid-phase cultures, Tramper et al. (1987) developed a new type of bioreactor, the so-called Liquid-impelled Loop Reactor, which is shown in Figure 1.5. This fermenter is an adaptation of the airlift loop reactor, but instead of air, a water-immiscible liquid is introduced into the fermenter, to induce mixing and circulation.

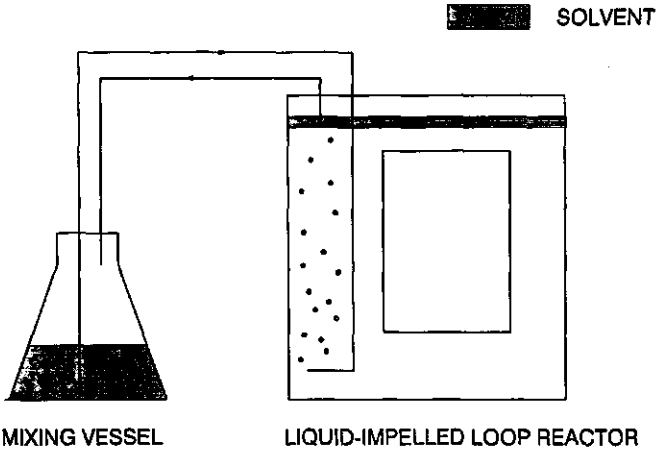

Figure 1.5
The Liquid-impelled Loop Reactor, adapted from Buitelaar et al. (1991b). In this case, a solvent lighter than water is introduced in the bottom part of the reactor. The solvent will rise and circulation and mixing takes place. Aeration can occur in an external mixing vessel or inside the reactor.

Liquid-solid two-phase systems

Next to the liquid second phases, it is also possible to use solid second phases to overcome the low product concentration in the fermentation broth. Maisch et al. (1986) tested a variety of adsorbents with cells of *Nicotiana tabacum*. From these adsorbents, (Na-Al-silicate, Mg-trisilicate, silk, polyvinylpyrrolidon, XAD-4 and XAD-7) only the latter two gave satisfactory results regarding the growth and productivity of the cells. XAD resins are used more and more with plant cell cultures with interesting results, like productivity and excretion increases (see e.g. Canales et al., 1990; Robins and Rhodes, 1986 and Asada and Shuler, 1989). Robins and Rhodes (1986) tested polyethyl and polyvinyl foams as well, but because of the better affinity and the smaller volume/mass ratio of XAD resin they preferred this resin (see Table 1.1).

Two applications of solid phases can be distinguished: one is the use of a solid phase as an adsorbent for the products, the other is stimulation of the secondary metabolite production. Amberlite resins, e.g. XAD-4 and XAD-7 not only adsorb the excreted products from the medium, they can also

enhance the total production. Robins and Rhodes (1986) used XAD-7 for the adsorption of

Table 1.1
Binding parameters for the adsorption of *Cinchona ledgeriana* anthraquinones by polymeric adsorbents (adapted from Robins and Rhodes, 1986)

Adsorbent	Capacity (g/g DW adsorbent)	Affinity (1/(mg DW))
XAD-2	0.17 ± 0.02	8.6
XAD-4	0.24 ± 0.03	35.3
XAD-7	0.18 ± 0.02	25.8
XAD-8	0.51 ± 0.11	15.6
'Foam Filtren T60'	0.44 ± 0.11	7.9
'Foam D'	0.51 ± 0.15	9.2
'Foam PR 22 60'	0.55 ± 0.18	3.6
'Nylon 66 powder'	0.31 ± 0.17	4.1

anthraquinones by *Cinchona ledgeriana* cell cultures. The addition of this resin yielded a 15-fold stimulation of the production, with over 90% excretion. The repeated addition of fresh XAD-7 can further increase the production and excretion of secondary products (Asada and Shuler, 1989). Another example of the stimulation of secondary metabolite production and excretion by a solid phase is given by Knorr et al. (1985). They used chitosan to immobilize and permeabilize cells of *Amaranthus tricolor* and *Asclepias syriaca*. From the increased excretion of low- and high-molecular weight products they concluded that chitosan can be used for this purpose. They did however, not determine the amount of secondary products released into the medium.

Aqueous two-phase systems

One two-phase system that has not been dealt with yet is that composed of two aqueous phases. Only two reports exist on the application of aqueous two-phase systems for plant cell cultures (Hooker and Lee, 1990b and Buitelaar et al., 1991a). Both articles report on good growth of plant cells in the polymer phases. In the second article, the production of secondary metabolites is also taken into account, and the level of production was equal in medium and aqueous two-phase systems. The value of the partition coefficient of the products between the two phases was not far from 1 for the

hydrophobic thiophenes produced. Probably, when aqueous two-phase systems are used for the separation of more hydrophilic plant cell products (yielding more extreme partition coefficients), they will prove to be useful, because they are used in biotechnology for the purification of proteins and cells (Albertsson, 1986).

Concluding remarks

The excretion of secondary metabolites is very important for future introduction of plant cell bioprocesses. Many attempts that have been made to force the cells to excrete their products were successful in the sense that the products came out of the cells. This release, however, can often be attributed to cell lysis caused by the treatment. Only treatments that do not affect the viability are to be considered for plant cell processes. Among them are mild heat treatment, changing the composition of the medium, the use of appropriate organic solvents and the use of suitable solid phases. For a commercial process, the ease of scaleup of the treatment is another important factor.

BIOREACTORS

Introduction

When production processes with plant cell cultures are to be commercialized in the near or far future, it is evident that shake flask cultures alone will not be sufficient any more. Therefore, bioreactors are needed, because they can be applied at large scale and because it is possible to control the conditions in bioreactors to a larger extent. The choice of the most suitable bioreactor can be determined by many factors; among them are the scale, the mixing mechanism, the method of aeration and the resistance of the cells to the shear stress generated in the bioreactor. The research into the development of bioreactors and bioreactor processes for plant cell cultures is increasing steadily. In this paragraph, the research of the most recent years will be reviewed with respect to the various aspects that can be distinguished for bioreactors.

Reactortype

Most of the bioreactors that are used to grow plant cells are more or less derived from the fermenters used for microbial cultivation. Recently, several authors compared various types of bioreactors for the growth of plant cells (Panda et al., 1989; Hong et al., 1989 and Kondo et al., 1989). One of the

most striking aspects found by the authors who executed the experiments themselves (Hong and Kondo) was that the rotating drum reactor or the roller bottle (equipped with extra baffles) proved to be among the most suitable bioreactors (see Table 1.2) for plant cells,

Table 1.2
Average specific growth rate and growth index of strawberry cell suspension cultures in different bioreactors (adapted from Hong et al., 1989; CH = carbohydrate consumed)

Fermenter	Spec. growth rate	Max DW/DW_0	Yield
	(day^{-1})	(-)	(g DW/g CH)
Shake flask	0.12	5.3	0.39
Airlift	0.14	5.5	0.44
Stirred jar	no growth	no growth	not applicable
Roller bottle with 2 baffles	0.15	6.6	0.41
Roller bottle without baffles	0.14*	2.2	not applicable
*: Growth ceased after the first 4 days			

yielding the highest growth rate and the best scaling up properties, as was also demonstrated before by Tanaka (1987). From a process engineering point of view this is somewhat surprising, because these types of bioreactors do not have very good mass transfer characteristics. On the other hand, the oxygen demand of plant cells is much lower than that of micro-organisms (so oxygen transfer needs not to be limiting), and some authors (like Payne et al., 1987) suggest that the stripping of carbon dioxide (which is more salient in airlift reactors) can reduce the growth rates of plant cells. These facts, combined with the gentle hydrodynamic environment that is created in such a rotating drum reactor, could explain the positive results with these reactor types.

Shear sensitivity

Because of the supposed shear sensitivity of plant cells, many authors choose bioreactors with a low shear environment. However, it was demonstrated by some authors (Meijer, 1989 and Scragg et al., 1988) that not all plant cells are shear sensitive. Both authors were able to grow several plant cell suspensions in stirred tank reactors at 1000 rev min^{-1}. Especially *Catharanthus roseus* suspensions, but also cell cultures of *Nicotiana tabacum*, were resistant. Other cell cultures however, like *Tabernaemontana divaricata* or *Cinchona robusta* were not able to tolerate this shear stress.

An interesting aspect is that it appeared possible to adapt cell cultures to shear stress, as was shown by Scragg et al. (1988). When they first tried to grow *Picrasma quassioides* at 440 rev min^{-1}, the viability of the cells dropped dramatically. However after a year of cultivation the culture became shear tolerant and capable of growth in stirred tank reactors at 1000 rev min^{-1}. This adaptation of cell cultures to shear was also shown by Tanaka et al. (1988). When they grew *Catharanthus roseus* cell suspensions in different environmental stress conditions, the cells in the environment with the highest shear forces appeared to contain stronger cell walls than those cultivated in a low-shear environment. Hooker et al. (1989) studied the effect of shear on cultures of *Nicotiana tabacum*. Changes in viability, cell lysis and secondary metabolite concentration were monitored. The cell culture age appeared to be an important parameter for the susceptibility of the cells to shear damage, as can be seen in Figure 1.6. Cultures in the latter part of the exponential growth phase and the early stationary

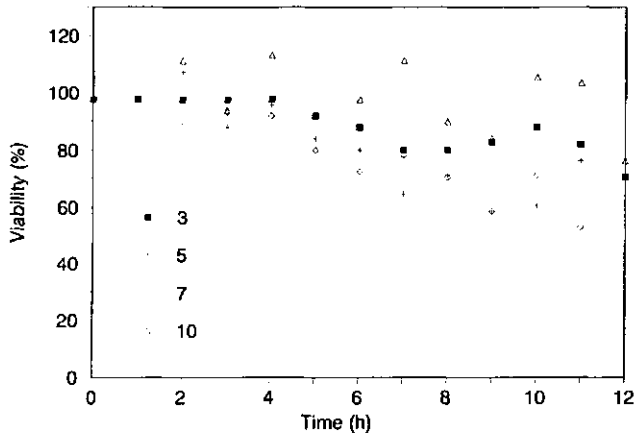

Figure 1.6
Effect of culture age on viability in a shear environment at 400 rev min^{-1}. Culture age is expressed in days in the legend. Adapted from Hooker et al., (1989).

phase (culture age 5 and 7 days) showed a higher susceptibility to shear damage than cultures in the lag phase, early exponential phase, or the later stages of the stationary phase (culture age 3 and 10 days). The excretion of their secondary metabolites (phenolics) increased with increasing shear, but this will only be useful if the viability of the cells is not affected.

Low shear bioreactors

The most salient of these low shear reactors are of course the bubble column or the airlift loop

reactors, where no moving parts are present that could damage the cells and where mixing is accomplished by the air bubbles moving upwards in the reactor. Some authors (e.g. Piehl et al., 1988, James et al. 1988 and Hegglin et al., 1990) even prefer to use bubble-free aeration systems. Piehl et al. (1988) and James et al. (1988) used a rotating membrane stirrer, while Hegglin et al. (1990) aerated their culture medium in a separate vessel. All authors report good cell growth, although no comparison is given with systems having direct aeration.

Scale

In spite of the facts stated above, the largest reactors used for plant cell cultures are still stirred tank reactors. By far the largest at present is the facility of Diversa in Hamburg, Germany, where plant cells are cultured in stirred tank fermenters with volumes up to 75 m^3. Their results with cell lines as *Echinacea purpurea* and *Rauwolfia serpentina* look very promising (Rittershaus et al., 1989 and Westphal, 1990). The first commercial product with plant cells (the production of shikonin with *Lithospermum erythrorhizon* cells) is also produced in 0.75 m^3 stirred tank reactors. The largest plant cell fermenters of other types are to the authors knowledge a 0.3 m^3 airlift fermenter, used for the biotransformation of ß-methyldigitoxin to ß-methyldigoxin by *Digitalis lanata* cells (Reinhard et al., 1989) and the 0.5 m^3 reactor to grow hairy roots, that will be discussed later in this chapter (Wilson et al., 1990).

Impeller design

The design of the impeller in stirred tank reactors for plant cell cultures is an important factor. It is obvious that there are still many plant cell cultures that are shear sensitive and especially for those cases, it is important that the culture is treated gently to preserve the viability and the production capacity. The classical, flat-bladed turbines were designed for a high mass transfer coefficient k_lA. Plant cells have a much lower respiration rate than microbial cultures; a k_lA of 20-30 h^{-1} is sufficient, whereas bacterial cultures need k_lA values of 100-1000 h^{-1} (Treat et al., 1989 and references cited therein). Several authors designed or adapted special impellers for plant cell cultures. Treat et al. (1989) used a so-called 'cell-lift' impeller, see Figure 1.7. With this impeller, suspensions with the smallest aggregates and the highest biomass concentration were obtained, when compared to standard flat-blade and marine impellers. Hooker et al. (1990) also tested various impellers, comparing the regular, flat-blade impeller with a large, flat-blade impeller and with a sail impeller, where the blades are made of nylon cloth. The fermenter culture (with *Nicotiana tabacum* cells) using the sail-cloth impeller experienced a relatively long lag phase but yielded a high maximum growth rate. The

productivity of the cells was lower in these fermenter systems than in shake flasks, but this can also

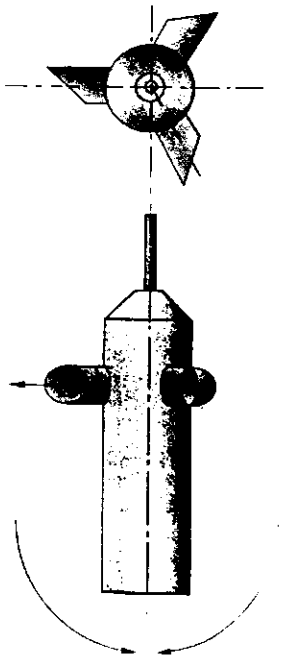

Figure 1.7
Cell-lift impeller with draft tube, adapted from Treat et al., 1989.

very well be the result of shear damage to the cells. The results obtained with the sail impeller resemble the results obtained by Tanaka (1987), where he used a large wide impeller to obtain relatively good mixing at low shear rates.

Cell density

Especially with plant cells, a high cell density is a prerequisite to obtain a high volumetric productivity. The calculated theoretical maximum cell density varies per author. Tanaka (1987) calculates 30 g DW/dm^3 to be the maximum possible, while Matsubara et al. (1989) calculate a maximum of 90 g DW/dm^3. This variation is based on different assumptions of the water content of the cells (95 and 80%, respectively), which is indeed varying between these values for plant cell cultures. Because the mixing properties of airlift fermenters are less than those of stirred tank reactors, it can be imagined that at high cell densities, the mixing in airlift reactors will not be sufficient. This was indeed reported by, among others, Tanaka (1987). Matsubara et al. (1989) achieved sufficient mixing in cultures of *Coptis japonica* with cell densities of up to 75 g/dm^3 by using a stirred tank fermenter with a stirring wing of the hollow-paddle type. To ensure good growth at these high cell densities, the medium had to contain a high concentration of nutrients, and it proved

to be compulsory to adapt the nutrient concentration to the cell concentration in the early phase of the fermenter run.

The cell density can also have its influence on the mass transfer in the reactor. Tanaka (1987) found that an increase of cell density caused a decrease in k_lA, because of insufficient mixing and insufficient gas dispersion. This phenomenon was more pronounced in airlift reactors than in stirred fermenters. With hairy roots, Kondo et al. (1989) found just the opposite effect: an increase of k_lA with increasing root concentration, caused by an increased gas-liquid interface due to the hairy roots. This effect was only present in the immobilized-cell rotating-drum reactor: for airlift fermenters the k_lA decreased again for higher root density because of poorer circulation. The mechanisms that affect the k_lA can be different for suspension cultures and hairy roots. Wilson et al. (1987) report that hairy roots excrete far less glycoproteins and polysaccharides as compared to suspension cultures. The medium thus remains a Newtonian fluid and this is certainly not the case with suspended cells. In the previously mentioned 75 m^3 stirred tank reactor (Westphal, 1990), the impeller has also been designed in a special, not specified, way to ensure low shear combined with adequate homogeneity and oxygen transfer.

Productivity

In many cases the productivity of cell cultures decreases with increasing scale for yet unexplained reasons. This can be overcome by several means. One way is the selection of special cell lines. This was done by Scragg et al. (1989), who selected a cell line of *Catharanthus roseus* that was able to produce high amounts of alkaloids when grown in bioreactors (7 and 30 dm^3) with long term retention of this ability. In contrast to the original cell line, the productivity was higher in bioreactors than in shake flasks.

Another way to keep the productivity at an acceptable level is the use of semi-continuous processes. Reinhard et al. (1989) noticed that the biotransformation rates of *Digitalis lanata* cells decreased when the concentration of the product ß-methyldigoxin in the 0.3 m^3 airlift fermenter had reached the value of 500 g/m^3. At this point, 0.18 m^3 of the culture broth was withdrawn and replenished with fresh medium. In this way, the biotransformation level remained high and six consecutive fermentation runs were performed, as can be seen in Table 1.3.

Miscellaneous reactors

Apart from the classical stirred tank reactors, bubble columns and airlift fermenters, a wide variety of bioreactors has been designed for the growth of plant cell cultures. A few examples will be given

here. The predominant 'other' reactor is the packed-bed reactor. It can be used in several ways: Kargi (1988) packed a column with sterilized alginate beads. Then, a cell suspension was added to the

Table 1.3
Semicontinuous production of β-methyldigoxin by *Digitalis lanata* cells in a 0.3 m^3 airlift bioreactor (adapted from Reinhard et al., 1989)

	Run					
	1	2	3	4	5	6
Production period (d)	14	13	14	15	16	17
Initial dry weight (g/m^3)	21.1	18.7	18.0	21.0	22.1	17.0
Suspension harvested (m^3)	0.18	0.18	0.18	0.19	0.20	0.21
Culture medium gained (m^3)	0.15	0.16	0.15	0.16	0.17	0.19
Production of β-methyldigoxin						
Level in medium (g/m^3)	505.5	558.1	583.1	580.2	669.2	559.0
Production (g/m^3)	505.5	488.0	505.0	493.2	615.5	529.0
Product yield (g)	77.8	76.6	77.3	80.2	103.3	97.9
Productivity ($g/(m^3.d)$)	30.7	32.6	31.5	28.0	32.2	27.4

column and the cells were allowed to settle and attach to the surface of the beads before the liquid circulation was started. In this way, the *Catharanthus roseus* cells formed a biofilm or aggregates in the void volume of the reactor. The cells rapidly lost their viability in this experiment, but it is not clear whether this was caused by the experimental conditions or by problems with the oxygen electrodes used. The 'classical' way to use a packed-bed reactor was reported by Schmidt et al. (1989). They immobilized cells of *Nicotiana tabacum* in calcium alginate beads and the beads were transferred to the column. In this way, the volumetric productivity in a continuously operated column was twice as high as that achieved in any batch run.

Hairy roots

The morphology of hairy roots implies that special measures must be taken to ensure an efficient growth of these cultures in bioreactors. When Wilson et al. (1987) tried to grow hairy roots of *Nicotiana tabacum* in a standard stirred tank fermenter, the roots lost their specific morphology and returned to callus-like structures, with the concomitant loss of the high productivity of the transformed roots. It is not the liquid velocity caused by the stirring itself that causes the damage but especially the direct contact between the impeller and the roots is very destructive.

Kondo et al. (1989) compared three types of bioreactors for their suitability to grow hairy roots of *Daucus carota* (see Figure 1.8): a turbine-blade reactor (a stirred tank where the stirring compartment

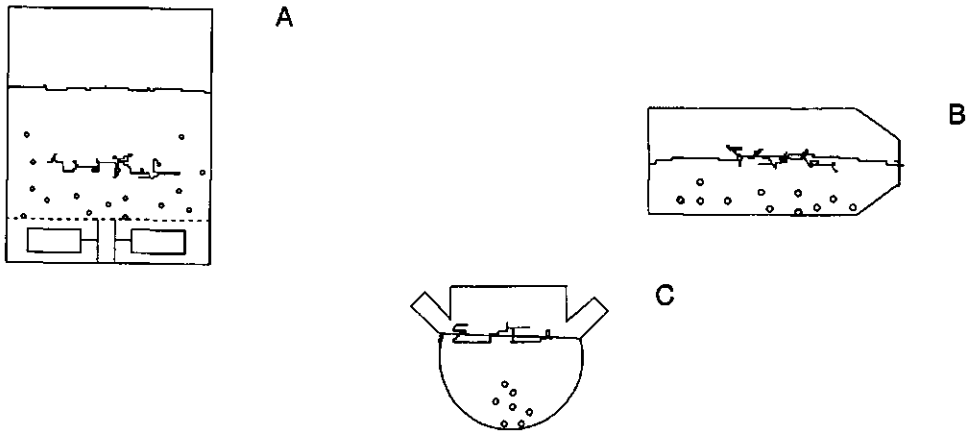

Figure 1.8
Three reactor types (simplified schemes), adapted from Kondo et al. (1989) for the cultivation of hairy roots of *Daucus carota*. A: turbine-blade reactor; B: rotating drum reactor and C: 'airlift reactor'.

was separated from the cell compartment), a rotating drum reactor with free or immobilized hairy roots and an airlift reactor, which was not a true airlift reactor but a sort of round-bottomed bubble column. The turbine-blade reactor and the 'immobilized-cell rotating drum reactor', where the cells were attached to a polyurethane foam sheet inside the reactor, gave the best growth rate. The growth rate of the cells appeared to be directly related to the mass transfer rate k_lA and the authors conclude that the k_lA value of the reactor is one of the most important factors to be considered for bioreactor design in plant cell culture, although the oxygen demand of plant cells is relatively low, as stated

before. This is demonstrated in Figure 1.9, where a graph is presented of the specific growth rate in

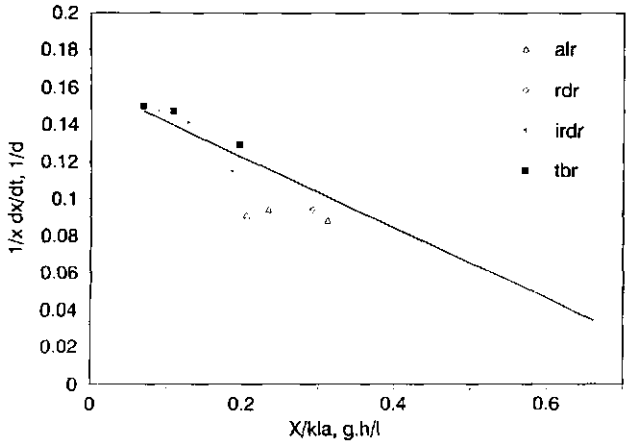

Figure 1.9
Relationship between specific growth rate (1/X dX/dt) and X/k_lA for hairy roots of *Daucus carota*, where X is the root concentration. Adapted from Kondo et al., (1989). ALR is 'airlift reactor'; RDR is rotating drum reactor; IRDR is immobilized-cell rotating drum reactor and TBR is turbine-blade reactor

relation to the root concentration divided by the mass transfer. Kondo et al. (1989) calculate from simple equations for oxygen transfer and for cell growth that this specific growth rate (1/X dX/dt) is a linear function of X/k_lA when the specific oxygen uptake rate is constant, for cell concentrations not exceeding 5 g DW/dm^3. When the rotating drum reactor was used with free hairy roots, root disruption occurred due to the fact that they adhered to the reactor wall and dropped back into the medium as the drum rotated. In other cases immobilization in polyurethane foam proved to be favourable for the growth of hairy roots in fermenters as well. Taya et al. (1989) also compared the growth of hairy roots in bioreactors and they found the airlift fermenter with cells immobilized on a polyurethane foam sheet (see Figure 1.10) to be superior to Erlenmeyer flasks, stirred tank reactors,

airlift reactors with free roots and airlift reactors where the medium was circulated along the roots, leaving the roots exposed to air for most of the culture time in the free root airlift fermenter. The roots (*Armoracia rusticana*) were blown out above the medium by the air bubbles, when they were grown as free roots in the airlift fermenter, causing severe cell disruption. When the roots were

attached to a foam sheet, these problems did not occur any more. It is evident from literature that hairy roots should be attached to a matrix when they are grown in fermenters, in order to prevent them from being damaged by the hydrodynamic forces in the reactor.

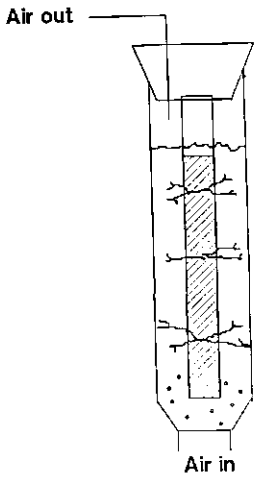

Figure 1.10
Hairy roots attached to a polyurethane foam sheet inside a bubble column, adapted from Taya et al., (1989).

Hilton and Rhodes (1990) grew their hairy root cultures of *Datura stramonium* in a stainless steel cage inside a stirred tank fermenter. Direct contact of the roots and the stirrer was prevented in this way and the cage also provided a good support matrix for the roots, allowing a more even distribution in the bioreactor.

Recently, some reports appeared about the use of trickling-bed or 'mist' reactors, where the roots are in contact with air most of the time and the medium is sprayed over the roots, but the reports are still very few (Hokama et al., 1990 and Whitney, 1990). The first results look rather promising: good growth occurs in these systems.

The largest fermenter for the culture of hairy roots is used in Norwich (UK) where Wilson et al. (1990) are using a 0.5 m^3 fermenter with internal matrix to grow hairy root cultures. Again, the first results look very promising for this approach.

Since it is not possible to take representative samples from the hairy root cultures in bioreactors to determine the biomass present, indirect ways have to be used. Buitelaar et al. (1991d) compared

several methods for the growth determination of hairy roots in bioreactors. The easiest way appeared to be the determination of the conductivity of the medium, as proposed by Taya et al. (1989) among others, but in case of cell lysis, the results were not reliable any more.

Concluding remarks

The choice of the appropriate bioreactor for secondary metabolite production with plant cells is dependent on many factors that have to be established for each individual case. The shear sensitivity of the cells, their oxygen requirements, the effects on the production of secondary metabolites of all kinds of environmental conditions are among the factors that affect the choice. For a long period, it was generally accepted that airlift reactors were the reactors of choice for plant cell processes. However, recent developments in impeller design and shear tolerance of the cells have facilitated the use of stirred tank reactors in some cases. The advantages of the latter reactor type are their presence in industry and their capability to create high mass transfer conditions. In other cases, like the growth of hairy roots or very shear sensitive cells, airlift reactors provide better conditions for cell growth and secondary metabolite production.

MODELS

Introduction

Mathematical models of biological processes are often used for hypothesis testing and process optimization. One of the most important aspects of these models is that they must enable the physical interpretation of the results, in order to yield more insight into the process behaviour. This is only possible when structured models are used, which consider several parts of the fermentation system separately.

Structured models

In general, models derived for micro-organisms consider the biomass to be a black box without examining it in more detail. For these organisms, the results of these models can be satisfactory, because their composition is relatively constant in time. For plant cells however, they appear to give unreliable results. Among the reasons for this is the fact that these unstructured (usually Monod-type) models do not describe a lag phase, which is usually encountered with plant cells; they assume steady state, which does often not occur in a plant cell culture system; and they do not acknowledge the

competition between growth and secondary product formation that often occurs in plant cell cultures (Hooker and Lee, 1990a).

So most authors reporting on plant cell culture models have used structured models. It is interesting to see that all of them use different entities to describe the cell growth and production. Bailey et al. (1985, 1989 and 1990) have developed a simple log-linear model (1985) that was merely based on curve-fitting without much physical interpretation possibilities, into a structured model (1989 and 1990), where they use a five-state mathematical model for the description of the system. They describe the limiting substrate concentration, the viable and non-viable dry weight, the fresh weight and the product concentration. Besides, they define three phases in the growth of plant cell cultures: the growth phase, the expansion phase and the lysis phase. They assume that viable dry weight reproduces with a specific growth rate that is first order in the limiting substrate concentration. With this model, they can predict among others the susceptibility of the cells to shear, which gives more information about the lysis phase. They could also design an optimal temperature control trajectory for the production of alkaloids with *Catharanthus roseus* cells, but it is not clear whether they have experimental validation for this temperature-dependence of the production.

During batch culture, nearly all parameters change with time, which makes it difficult to draw the right conclusions from an experiment. To circumvent this, Van Gulik (1990) tried to describe the growth kinetics of *Catharanthus roseus* and *Nicotiana tabacum* in a chemostat. The first model used was a black box (unstructured) model based on Monod kinetics. The results were not satisfactory and this was supposed to be due to the difference in NH_4^+- and NO_3^--uptake rate (which was prevented in later experiments by using only one nitrogen source), the changes in the elemental composition of the biomass and the byproduct formation. All these factors were not explicitly determined in the first, unstructured model. The structured model that was used later, divided the biomass in four pools: phosphate, enzymes, carbohydrates and the rest - yielding a much better correspondence between model predictions and experimental results. In this way, the changing elemental composition of the biomass and the byproduct formation were taken into account.

Hooker and Lee (1990a) use a three-compartment model to describe the batch growth of *Nicotiana tabacum*. The three pathways they describe are the structural component (biomass) production, secondary metabolite synthesis and cellular respiration. They have very good results with this model, i.e. matching experimental and theoretical values, except for the substrate concentration. The latter could be explained by the fact that cell expansion occurred, which was not included in the model.

Growth of *Echinacea purpurea* in the large (75 m^3) fermenter system at Diversa (FRG) has been described with a structured model by Posten and Munack (1989) with satisfactory results, although

they neglected the product formation rate in the mass balances.

An original idea for the modelling of plant cell suspension growth was used by Frazier (1989), who designed a 'leaky cell growth model' based on the leakage of metabolites from cells. This model gives good results, including the prediction of the lag phase with good experimental validation with cells of *Dioscorea deltoidea*. The leakage of metabolites was used as a tool to describe the interaction between growing plant cells and their environment.

Concluding remarks

Up to now, not many authors tried to model the growth of plant cells in suspension. There is general agreement now that unstructured models do not perform well enough for these cells. Good results are obtained now with structured models. However, most authors do not use cell aggregates but single cells, which is not often the form in which the cells are predominantly present.

For hairy root growth, no one has yet tried to model these cells, and most probably, the existing models will have to be adapted before an accurate description of hairy root growth can be given. One of the reasons for this is that cell division is limited to the top meristem of the roots (Wilson et al., 1987).

Another point is that the variation in and between plant cell lines is so large, that it is very unlikely that, at the present state of the art, it would be possible to develop models that have a general meaning for at least a large number of different plant cell lines.

COMMERCIAL PROCESSES

Introduction

One of the ultimate goals of the research efforts invested in plant cell biotechnology is the industrial implementation of production processes based on this technology. In recent years, quite a few authors have shed their light on this subject and, although their view in the articles range from pessimistic to optimistic, they all share the conclusion that many problems have to be solved before commercialization of plant cell biotechnology will have become a common practice.

Commercial processes

It is not easy to obtain straightforward information about the state of the art in the commercialization of products. The processes that are commercial beyond dispute, are of course the production of

shikonin by Mitsui Petrochemicals in Japan and the production of phosphodiesterase by Bethesda Research Laboratories in the USA. At the IAPTC VII Congress in Amsterdam (June 1990), Professor Yamada said that in the near future more products would go to the market in Japan. The production of berberine will be started as soon as FDA approval is obtained, while sanguinarine is now authorized for use in medicinal toothpaste. The production of scopolamine with hairy roots is almost commercial, but the productivity needs to be raised before the process will be economically feasible. Other products that have been mentioned frequently as nearly commercial, but whose status is not clear at this moment are capsaicin production in the United Kingdom and rosmarinic acid production in the FRG. Only very recently, the Californian company Escagenetics launched the sale of vanillin produced by plant cell cultures, called 'Phyto Vanilla' (Goldstein, 1990 and Shannon, 1991). Whether or not more products will reach the market, is strongly dependent on the economics of the process.

Cost price estimation

Several authors have attempted to define a reasonable cost price for plant cell biotechnology based products. Goldstein (1980, 1983, 1990) and Drapeau et al. (1987) have done the most extensive studies. From their work, it becomes clear that the final cost price is not easy to calculate and is dependent on many factors. The most important among these factors are: the fermentation time needed for the production of sufficient biomass and product, the volumetric proportion of biomass in the vessels, the appearance of the cells (single cells, aggregates), the specific productivity and the market size. By assuming minimal and maximal values for these and other variables, Goldstein (1990) calculated a manufacturing cost price of 130-90,000 US$/kg, with a production of 60-65,000 kg/year.

Table 1.4
Cost comparison for production of ajmalicine-containing biomass (adapted from Drapeau et al., 1987)

Method	Biomass cost	
	US$/kg dry biomass	US$/kg ajmalicine
Fermenter-grown cells; current technology (0.6% ajmalicine in 23 days)	16.1	3215
Roots imported from India (0.3% ajmalicine)	1.5	619
Fermenter-grown cells; improved technology (6.0% ajmalicine in 6 days)	13.8	608

For the same product produced with field grown plants, the cost price was estimated to range from

39-80,000 US$/kg, with an annual production of 45-60,000 kg. Drapeau et al. (1987) compared the costs for ajmalicine production with current technology and with improved productivity (Table 1.4). It can be seen that the cost price of the secondary metabolite is heavily dependent on this productivity. Downstream processing costs were not included, but they are assumed to be similar for both production systems. So, there can be products where it is cheaper to use plant cell culture technology than field grown plants. When we look to the practical situation, only products that cannot be made in other ways, because the original plant is nearly extinct and can not easily be grown in the field (shikonin, berberine) or because the product is not produced by the whole plant (sanguinarine), are able to survive in the market. In 1985, Sahai and Knuth calculated a minimum cost price for plant cell biotechnology products of 500 US$/kg, but they estimated that repeated use of biomass in continuous processes with immobilized plant cells could lower the costs to 20-25 US$/kg, which is a rather optimistic estimate, especially because many plant products are stored intracellularly, which not only impedes the use of immobilized cells, but also extended use of free cells.

Future research

The conclusion of all authors mentioned in the former paragraph was, that the specific productivity of secondary metabolites is in general too low to make a process commercially viable. Several ways exist to enhance this productivity, but unfortunately, no general procedure is known until now that works in all cases. Commonly used methods are (Fowler, 1988): medium engineering and optimizing the temperature, the gas composition and other physical/chemical factors. Another approach can be to collect the product in a separate phase to prevent degradation of the product in the medium, to obtain a higher net production. The product price is also influenced by the reactor configuration, the downstream processing and the process format. However, as Fowler (1988) states: 'the real limitation lies in our lack of understanding the basic physiology and biochemistry of plant cell systems'. With him, other authors, like Kurz (1989) and Rhodes (1989) plea for much more fundamental research into the physiology, genetics, technology and the excretion process of secondary metabolism with plant cells in order to gain a better understanding of the pathways and processes involved and to use this knowledge to increase the productivity of plant cell cultures.

Concluding remarks

The commercial viability of processes with plant cell cultures is largely dependent on their productivity. The production of ajmalicine could be interesting with plant cell cultures, only if the productivity can be enhanced at least 40 times (Drapeau et al. 1987). The conclusions of general

calculations, like those made by Goldstein (1980, 1983, 1990) are also largely dependent on this productivity, and in some cases the production in this way can be preferable to the production with field grown plants. However, much more research is needed. An encouraging consideration is given by Ten Hoopen (1986), who says that the amount of effort invested in plant cell biotechnology until now is only a fraction of the research that has led to spectacular improvements of the productivity of micro-organisms. So optimistic expectations are justified if research into secondary metabolites is continued.

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Chapter 2

IMMOBILIZATION OF BIOCATALYSTS IN THERMOGELS USING THE RESONANCE NOZZLE FOR RAPID DROP FORMATION AND AN ORGANIC SOLVENT FOR GELLING

R.M. BUITELAAR, A.C. HULST AND J. TRAMPER

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SUMMARY

The resonance nozzle immobilization technique was tested with thermo-hardening hydrogels (agar, gellan and κ-carrageenan) and compared with the conventional needle technique. After nozzling the warm gel solution, the formed droplets were caught in an ice-cold two-phase system consisting of an organic solvent (n-butylacetate, hexane or nonane) as top phase, in which gelling occurred, and aqueous medium as bottom phase. The cells used were yeast cells (*Saccharomyces cerevisiae*), bacterial cells (*Mycobacterium aurum* L1), plant cells (*Tagetes minuta*) and insect cells (*Spodoptera frugiperda*). The retention of the respiration activity was used as a criterion for the suitability of the conditions applied. The relatively polar solvent n-butylacetate with the low log P value gave the poorest activity retention, which is in agreement with experimentally validated theory. For all gels, the resonance nozzle technique proved to give satisfactory results, although the retention of respiration activity was generally lower than obtained with the needle technique.

INTRODUCTION

Immobilization of biocatalysts by entrapment in hydrogels has been reported frequently. A review of immobilized cells is given by Bucke (1983). In most cases, the procedure for entrapment is executed by dripping the biocatalyst-presupport mixture through a needle. Dependent on the type of gel material, the formed droplets are induced to gel in different ways. For instance, alginate will gel in a $CaCl_2$ solution and agar in a cold hydrophobic phase (Mosbach & Nilsson, 1983; Nilsson et al, 1983).

For production of immobilized biocatalysts on a small scale, the dripping method is convenient and adequate. However, if large amounts of the immobilized biocatalyst are needed, the poor production capacity of the dripping technique will be limiting. In order to eliminate this disadvantage, the upscaling of the production capacity of immobilization techniques was subject of research for several investigators. Brodelius & Mosbach (1982) designed a device with a reservoir for the alginate-cell mixture with six exit needles for accelerated droplet formation. Rehg et al (1986) used an atomizer for upscaling the immobilization technique with alginate, which consisted of six needles connected to a reservoir. The bead size was controlled by application of an air stream along the needles. Matulovic et al (1986) constructed an apparatus with a high speed rotating nozzle ring for droplet

formation of the mixture of cells with several kinds of gel types. The production capacity of the latter technique was two orders of magnitude larger than the conventional technique with one needle.
Hulst et al (1985) designed the resonance nozzle technique for production of immobilized biocatalysts in large quantities. This technique consists of breaking up a jet of the cell/presupport mixture in uniform droplets by means of a mechanical vibration. The production capacity of the resonance nozzle is also about two orders of magnitude larger than the dripping technique. Until now, the resonance nozzle technique has only been reported with alginate as gel support for the biocatalysts (Hulst et al., 1985; Schoutens et al., 1986).
In this paper the applicability of the resonance nozzle is also demonstrated for immobilization of cells in thermogelling hydrogels in combination with different hydrophobic organic solvents as the gelling medium. The retention of respiration activity after immobilization is used in this paper as the criterion for suitability. Immobilization of plant cells (*Tagetes minuta*), insect cells (*Spodoptera frugiperda*), yeast cells (*Saccharomyces cerevisiae*) and bacterial cells (*Mycobacterium aurum*) in agar, gellan and κ-carrageenan have been used as model systems. Only very recently, systematic research has been done on the effects of organic solvents on the biocatalytic activity. Brink and Tramper (1985) used the Hildebrand solubility parameter δ to relate biocatalytic activity and solvent hydrophobicity. Laane et al. (1987) used the log P value to predict the influence of the solvent on the biocatalytic capacity. Log P is defined as the logarithm of the partition coefficient of a compound, i.e. a solvent, in a standard octanol-water two-phase system. Their results show that in general biocatalysis in organic solvents is low in relatively hydrophilic solvents having a log $P < 2$, is high in hydrophobic solvents having a log $P > 4$, while the effect of solvents with a log P value between 2 and 4 seems to be rather unpredictable. A relatively polar solvent (n-butylacetate, log $P = 1.7$), a moderately hydrophobic solvent (hexane, log $P = 3.5$) and a hydrophobic solvent (nonane, log $P = 5.1$) were used in this study.

MATERIALS AND METHODS

Cells

Yeast cells (*Saccharomyces cerevisiae*) were obtained from a local bakery (trademark Gist-brocades). They were suspended in demineralized water before the immobilization to form a homogeneous paste. Bacterial cells (*Mycobacterium aurum* L1) were provided by the Industrial Microbiology Group of

the Agricultural University. The cells were grown in shake flasks in mineral medium, supplied with ethene (Habets-Crützen et al., 1984). The cells were obtained as a frozen stock culture. After thawing, the culture was suspended in mineral medium and used in the experiments.
Plant cells (*Tagetes minuta*) were provided by the research institute ITAL. They were grown in MS-medium in shake flasks (Ketel, 1987). The cell suspension was decanted and used in the experiments.
Insect cells (*Spodoptera frugiperda*) were grown in continuous culture in our laboratory (Tramper et al., 1986). After centrifugation, the pellet was resuspended in medium and used in the experiments.

Respiration media

The medium used for yeast cells consisted of: 20 g glucose, 5.2 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.55 g $MgSO_4.7H_2O$, 1 g yeast extract, 3.4 g sodium acetate/$3H_2O$ and 2.1 ml 17.5 M acetic acid per dm^3 demineralized water. The pH was 4.5.
The medium used for bacterial cells consisted of: 20 g glucose, 2 g NH_4Cl, 0.1 g $(NH_4)_2SO_4$, 0.075 g $MgCl_2.6H_2O$, 2 ml Vishniac & Santer spore solution, 2.0 g $K_2HPO_4.3H_2O$ and 0.75 g $NaH_2PO_4.H_2O$ per dm^3 demineralized water. The pH was 7.0.
The medium used for plant cells consisted of: 20 g glucose and 4.72 g MS salts (Murashige and Skoog, 1962) per dm^3 demineralized water. The pH was 5.5.
The medium used for insect cells was Hink's medium (Hink, 1982), with 10% FBS and 20 g glucose per dm^3 demineralized water.

Chemicals

The organic solvents used were n-hexane (log P = 3.5), obtained from Rathburn Chemicals, n-nonane (log P = 5.1) and n-butylacetate (log P = 1.7), both from Merck.
The hardening solutions for the formed beads were for agar: demineralized water; for gellan: a solution of 0.15 g $MgSO_4.7H_2O$ dm^{-3} demineralized water and for κ-carrageenan: a solution of 0.75 g KCl dm^{-3} demineralized water.

Gels

Thirty-five grams of agar (Oxoid no.3) were dissolved in 1 dm^3 demineralized water. Until mixing with the cells, the gel solution was kept at 55 °C.
A gellan gum (Kelco) solution was prepared by mixing 20 g of gellan with 1 dm^3 of the hardening solution. Until mixing with the cells, the gel solution was kept at 55 °C.

The κ-carrageenan solution was prepared by mixing 28 g of κ-carrageenan (Genugel) with 1 dm^3 of the hardening solution. Until mixing with the cells, the gel solution was kept at 40 °C.

Immobilisation methods

Two immobilisation techniques were used: the common, small-scale needle dripping technique and the large-scale resonance nozzle technique (Hulst et al, 1985).

Assays

Respiration of the free and immobilized cells was measured in a biological oxygen monitor, using a Yellow Springs Instruments oxygen electrode, which was calibrated in advance. After saturation of 4 ml medium with air, the free or immobilized cells were added and the oxygen consumption rate was determined. The temperature was kept at 24 °C.

Experimental procedure

For each support material, the procedure was as follows. With the needle dripping technique, 0.05 dm^3 of the gel/cell mixture was extruded into an ice-cooled 1 dm^3 flask, containing 0.2 dm^3 medium as bottom phase and 0.8 dm^3 organic solvent as top phase. With the resonance nozzle technique, 0.5 dm^3 of the gel/cell mixture was nozzled into an ice-cooled 1 dm^3 flask, containing 0.2 dm^3 medium as bottom phase and 0.8 dm^3 organic solvent as top phase. The obtained beads were sieved out of the aqueous medium, washed with demineralized water and hardened in the appropriate solution for 2 hours. As a blank, material including cells was passed through the needle or the nozzle, caught on a Petri dish and solidified at room temperature without being contacted with an organic solvent. After gelling the gel layer was cut into small cubes (about 1 mm^2). Afterwards, the respiration assays were performed with the cubes and beads.

RESULTS AND DISCUSSION

Cells

In Table 2.1, the respiration activity of the free cells is given. The activity is expressed per kg wet weight.

Table 2.2 shows the results of the immobilization experiments. The activity of the free cells is taken as 100% in all cases.

Table 2.1.
Respiration activity of free cells

Cell type	Respiration activity
	(mol $O_2.s^{-1}.kg^{-1}$) * 10^6
Saccharomyces cerevisiae	19
Mycobacterium aurum L1	12
Tagetes minuta	0.43
Spodoptera frugiperda	0.41

Apart from insect cells, bacterial cells suffer the most from the immobilization procedure. The residual respiration activity of the immobilized cells is never higher than 40%. This must be due to the temperatures used, since Habets-Crützen et al (1984) showed that immobilization of *Mycobacterium* strains at ambient temperature did not result in such a high activity reduction. With these cells, the needle technique mostly gave better results than the nozzle technique.

Yeast cells show an intermediate sensitivity to the immobilization procedure. The results show a great difference between the respiration activity after immobilization with the needle or the nozzle, probably due to higher shear forces in the nozzle.

For plant cells, in many cases the respiration activity of the immobilized cells was even higher than that of the free cells. This phenomenon has been observed earlier, with *Haplopappus gracilis* cells, when they were immobilized with the resonance nozzle technique (Hulst et al, 1985). A possible explanation can be that the aggregates of plant cells are broken up into intact individual cells by the shear forces during the immobilization procedure, which probably results in less diffusion limitation. Plant cells are apparently protected by their cell wall to short periods of relatively high temperature and shear rates.

The insect cells did not show any respiration after immobilization in κ-carrageenan. These cells are known to be resistant to a short period of 37 °C (unpublished results), but the temperature applied in this case was higher (40 °C). To investigate whether the immobilization procedure itself was responsible for the death of the cells, the insect cells were immobilized in Ca-alginate by the needle technique, where no increased temperature was used. The respiration activity of the immobilized cells was about the same as the activity of the free cells. Apparently, the insect cells are very temperature sensitive. With these results in mind, no attempts were made to immobilize the insect cells in agar or gellan, because then even higher temperatures (55° C) are needed.

Table 2.2.
Percentage activity retention of various cell types after immobilization with the needle and the nozzle technique in three different gels and after extruding them in various organic solvents. Activity of free cells is 100%, see Table 2.1.

	Gel	κ-Carrageenan		Gellan		Agar	
	Technique	needle	nozzle	needle	nozzle	needle	nozzle
Cell type							
	Solvent						
Yeast	blank	60	20	27	4	46	6
	nonane	72	22	42	3	63	9
	hexane	72	36	46	4	66	7
	butylacetate	22	7	18	2	21	1
Bacteria							
	blank	22	20	24	33	30	16
	nonane	23	15	15	28	25	4
	hexane	23	11	12	39	35	26
	butylacetate	5	16	14	24	8	4
Plant cells							
	blank	131	39	120	197	74	137
	nonane	101	55	103	191	139	55
	hexane	128	62	49	126	94	58
	butylacetate	32	14	18	135	52	40

Technique

In almost all cases, the needle technique gave a higher respiration activity than the nozzle technique. This can only be due to higher hydrodynamic forces occurring during the immobilization procedure with the resonance nozzle technique.

Solvents

The log P of a solvent appears to be a good prediction of the effect of this solvent on the respiration activity retention of cells. Butylacetate, having the lowest log P (1.7), gives the worst results in almost all cases. There is no systematic difference between hexane and nonane regarding their influence on

the respiration activity. Moreover, there is no systematic difference between the blanks and hexane and nonane, indicating their suitability for these purposes.

Gels

The gels do not show a systematic difference in their effect on the respiration activity of the cells. Apparently, the difference in temperature used between κ-carrageenan (40 °C) and gellan and agar (55 °C) does not cause an extra activity loss.

CONCLUSIONS

The resonance nozzle technique can be used with thermogelling gels, in addition to alginate.

The use of organic solvents as hydrophobic phase is convenient.

The log P value gives a rather good indication for the activity retention of the immobilized biomass.

The gels used do not show a systematic difference in their influence on the activity retention of the cells.

The cell types vary enormously in their sensitivity to the temperatures and shear forces occurring during the immobilization procedure.

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Chapter 3

THE INFLUENCE OF VARIOUS ORGANIC SOLVENTS ON THE RESPIRATION OF FREE AND IMMOBILIZED CELLS OF *TAGETES MINUTA*.

R.M. BUITELAAR, M.H. VERMUË, J.E. SCHLATMANN AND J. TRAMPER

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ABSTRACT

The influence of several organic solvents on the oxygen metabolism of *Tagetes minuta* (marigolds) was tested by incubating the cells in medium mixed with 1, 5 or 10% (v/v) of the organic solvents. The results were in good agreement with the general rules (log P) for the influence of organic solvents on biocatalytic activity. Solvents that had proved to be non-toxic at 1% (v/v), also showed no harmful effects when they were applied at a concentration of 10% (v/v). Immobilization of the cells in calcium alginate provided a slight protection of the cells against the toxic solvents.

KEYWORDS

Thiophenes; *Tagetes sp.*, plant cell cultures, secondary metabolites, two-phase bioreactors.

ABBREVIATIONS

DW: dry weight; LLR: Liquid-impelled Loop Reactor; MS: Murashige and Skoog medium.

INTRODUCTION

The use of organic solvents in biocatalysis is becoming increasingly important (Laane et al., 1987a). There are many possible advantages (Tramper et al., 1987): some of them could also apply for secondary metabolite production with plant cells. For instance, substrate and/or product inhibition can be lowered or prevented; in situ concentration of the product can be achieved, which can lower the costs of downstream processing, etc. However, organic solvents can have a negative effect on the activity of the biocatalysts. As was indicated by Freeman (1986) in an overview of solvent effects on multiphase biocatalysis, Brink and Tramper (1985a) were the first to attempt to structure the effects of organic solvents on the activity of biocatalysts. Laane et al. (1987b) proposed the use of the log P value of a solvent to predict the influence of the solvent on the activity retention of the biocatalyst.

P is the partition coefficient of a compound over a standard octanol/water two-phase system. It can be determined experimentally, but it can also be calculated using the system of hydrophobic fragmental constants developed by Rekker and de Kort (1979). From the results of many experiments with enzymes and micro-organisms, Laane et al. (1987b) proposed some general rules: if log P of a solvent is lower than 2, the effect of the solvents on the biocatalyst will be harmful; if log P is higher than about 4, there will be no negative effect and solvents with a log P value between 2 and 4 will behave rather unpredictably. The rules can be applied for various cell types, like yeast, bacterial and plant cells (Buitelaar et al., 1988; Deno et al., 1989). The influence of the solvent on the viability of the cells is not the only selection criterion; other considerations such as the polarity of the product with respect to the polarity of the solvent must also be included.

Immobilization of the cells can have a number of advantages. When slow growing cells are used in continuous systems, the immobilized cells can be retained in the bioreactor without limiting the dilution rates. also separation of the immobilized cells from the broth is much easier. Harrop et al. (1989) showed that the presence of a liquid-liquid interface can be an important factor in the loss of activity of cells. Immobilization prevents contact with the interface and thus the aggregation and clotting of the cells at the interface (Brink and Tramper, 1985b). Thus, if interfacial deactivation is the likely mechanism to occur, immobilization will provide protection against deactivation in a two-phase system. Immobilization can also have a stimulating effect on the production and/or excretion of secondary metabolites by plant cell cultures (Asada and Shuler, 1989; Wichers et al., 1983). The immobilization of cells in a gel matrix can induce differentiation or cell-to-cell contact which is often said to be compulsory for the production of secondary metabolites by plant cell cultures (Asada and Shuler, 1989; Bringi and Shuler, 1990). Both the use of organic solvents and immobilization can thus yield attractive features.

In this article, the results are given of studies with free and immobilized cells of *Tagetes minuta* in combination with organic solvents. Cells of *Tagetes sp.* can produce thiophenes, rather hydrophobic compounds (log P is about 4) with biocidal activity that can be used as biodegradable pesticides.

MATERIALS AND METHODS

Cells

Cells of *Tagetes minuta* (marigolds) were maintained in MS medium (Murashige and Skoog, 1962),

supplemented with (per dm^3): 20 g sucrose, 0.1 g myo-inositol, $5*10^{-3}$ g benzyladenine and $5*10^{-4}$ g naphtalene acetic acid. The pH was 5.8 before autoclaving (121°C, 30 min.). Usually, the pH of the medium has decreased with 0.1-0.3 units after autoclaving. The cells were grown in 0.5 and 1 dm^3 Erlenmeyers with weekly subculturing. The cultures were kept on a rotary shaker, 100 rpm, under continuous light, about 1500 lux.

Assays

Respiration of free and immobilized cells was measured in the dark in a Biological Oxygen Monitor (BOM), using a Yellow Springs Instruments oxygen electrode, which had been calibrated in advance. Six ml cell suspension or six ml MS medium plus 2 g immobilized-cell beads were aerated for 3 minutes under continuous stirring at 1000 rpm to obtain oxygen saturation, at a constant temperature of 24 °C. The optimal amount of dry weight in the BOM, corresponding to the above mentioned fresh weight values, had been determined in advance. It was also demonstrated that oxygen saturation was reached after stirring for 3 minutes. Preliminary experiments had shown that the fast stirrer speed needed to ensure initial oxygen saturation had no influence on the oxygen consumption of the cells during the short incubation period (3 min.). The stirrer speed was kept at 1000 rpm during the respiration measurement, because experiments had shown that the activity of the cells was not affected when the stirrer speed during the respiration measurement was varied from 300 to 1400 rpm. After sealing the BOM vessel to prevent the entrance of oxygen, the oxygen consumption rate was measured.

The dry weight of the cells was determined by taking a sample from the same flask as where the tested cells came from, rinsing the cells with water and drying the cells to constant weight in an oven at 115 °C.

With 'activity' we mean the oxygen consumption rate, in mole oxygen per second per kilogram dry weight of cells; 'relative activity' means activity compared to the activity of cells in standard MS medium or compared to the activity at t=0 (indicated in the headings of the figures).

Immobilization procedure

A 3.6% (w/v) sodium alginate solution (Manucol DM, Kelco) in 0.9% (w/v) NaCl was mixed with the cell suspension to a final alginate concentration of 24 g dm^{-3}. The mixture was extruded dropwise into a stirred 0.1 M $CaCl_2$ solution, where it was kept for 2 hours before transfer to fresh MS medium. Immobilization was done under sterile conditions.

Solvents

The selection of the solvents was based on the results of other researchers working with organic solvents (Brink and Tramper, 1985a and Laane et al., 1987b). Most solvents used had a log P value higher than 4 which was expected to be favourable for the cells. In order to test whether some solvents could be toxic and also to test the possible protective effect of immobilization, some solvents with low log P values were selected as well. The solvents tested are listed in Table 3.1.

Table 3.1
Organic solvents used, with their log P and density values.

Solvent	Log P	ρ	Supplier
		$kg.dm_{-3}$	
Diethylphthalate	3.3	1.12	Merck
Dibutylphthalate	5.4	1.05	Merck
Dioctylphthalate	9.6	0.99	L & I
Hexane	3.5	0.66	Rathburn
Decane	5.6	0.73	Merck
Hexadecane	8.8	0.77	Merck
Decanol	4.0	0.83	Janssen Chimica
Undecanol	4.5	0.83	Janssen Chimica
Ethylacetate	0.7	0.90	Merck
FC40	11.2	1.87	Janssen Chimica
FC70	14.0	1.94	3M

FC40 and FC70 are perfluor compounds, with the bruto formula's of $N(C_4F_9)_3$ and $N(C_5F_{11})_3$, respectively. All solvents had a purity of 99% or more. Depending on their boiling point, the solvents were either autoclaved (121°C, 30 min.) or filter sterilized.

Log P

Log P values of solvents and thiophenes were calculated from hydrophobic fragmental constants according to Rekker and de Kort (1979) and are given in Table 3.1 and Figure 3.1.

Toxicity tests

The toxicity of the organic solvents was tested by incubating cells in medium with 1, 5 or 10 % (v/v) solvent. Periodically, samples were taken and the respiration was measured as a function of time, as described in 'assays'.

Two of the solvents that had proved to be toxic (hexane, decanol) were used to test the effect of immobilization on the toxicity of the solvents for the cells. Fifteen grams of alginate beads containing *Tagetes minuta* cells were added to 50 ml medium, containing 1% (v/v) decanol or 2% (v/v) hexane. The cells were incubated on a rotary shaker (Edmund Buhler, Tubingen, FRG, type KL2) at 150 rpm. At regular intervals, samples (free cells or beads) were taken and their oxygen consumption rate was determined. The oxygen consumption rate (activity) was compared with the activity of immobilized cells that had been incubated in MS medium without solvent and with the activity of free cells that had been treated likewise.

RESULTS AND DISCUSSION

Toxicity

After determining the optimal assay conditions (see 'Materials and methods'), toxicity tests were executed with the solvents listed in Table 3.1.

In Figure 3.1, the results are shown of the experiments with 1% (v/v) solvent and free cells. The average relative activity is plotted against the log P values of the solvents tested. The activity in MS medium without solvent is taken as 100% and the results are averaged over the experimental time course of about 200 minutes. Absolute initial activities varied between 38 and 52 μmol oxygen per second per kg dry weight of the cells. As a result of the shaking, the organic phase was dispersed in the aqueous phase during the experiments except for FC40 and FC70, which formed a second phase at the bottom of the flasks. As can be seen, ethylacetate, diethylphthalate, hexane, decanol, undecanol and dioctylphthalate induce a severe decrease in the respiration activity of *Tagetes minuta* cells. Dibutylphthalate, decane, hexadecane and FC40 are not toxic in a 1% (v/v) concentration for the plant cells. The behaviour of FC70 is intermediate for these cells. Apart from one solvent (dioctylphthalate), the results agree well with the general rules of thumb on the log P value of a solvent (see Introduction). All solvents that have a log P value lower than 5 have a negative effect on the cells, whereas all non-toxic solvents do have a log P value higher than 5. Dioctylphthalate

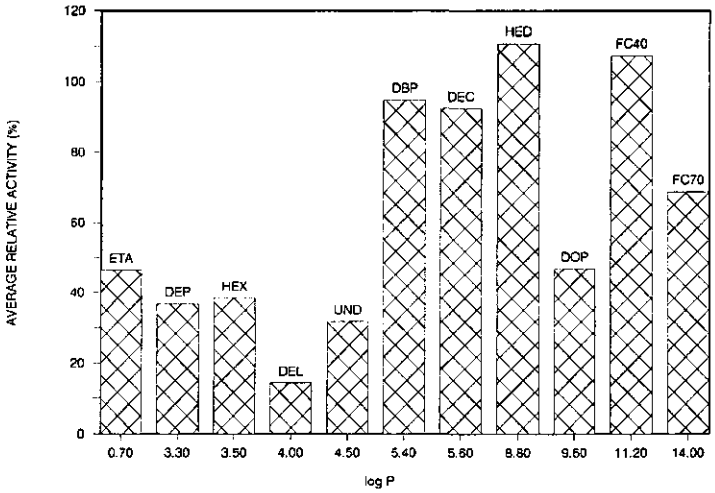

Figure 3.1.
Relative activity of *Tagetes minuta* cells as a function of the log P value of the solvent present in the system. The activity in MS medium is 100%. ETA is ethylacetate, DEP is diethylphthalate, HEX is hexane, DEL is decanol, UND is undecanol, DBP is dibutylphthalate, DEC is decane, HED is hexadecane, DOP is dioctylphthalate.

shows a somewhat exceptional behaviour. In other experiments with other cells in our lab (to be published), dioctylphthalate showed just the opposite behaviour; the cells repeatedly showed an exceptional high activity when dioctylphthalate was present. Until now, we have no reasonable explanation for these remarkable results, and experiments are being executed to elucidate this effect.

Solvent concentration

A selection of the solvents that were found to be non toxic in concentrations of 1% (v/v) (dibutylphthalate, FC40 and decane) was tested with free cells with higher concentrations, 5 and 10% (v/v), respectively. If interfacial deactivation would play any significant role in this case, raising the total amount of interface in the dispersion by increasing the amount of solvent is likely to cause an observable deactivation of the cells.

Table 3.2 shows the results of this series of experiments. The activity at t=0 was taken as 100% in all cases. The initial activity of the cells was now in the range of 29 to 37 μmol oxygen per second per kg dry weight of cells. The results show that raising the concentration and thus the interfacial area of the dispersions, does not have negative effects on the cells. This means that interfacial inactivation is not of any importance in these cases.

Table 3.2.
Concentration effects of non toxic solvents

Solvent	Concentration (% (v/v))	Relative activity time average (%)
Dibutylphthalate	1	104
	5	106
	10	100
FC40	1	88
	5	104
	10	100
Decane	1	84
	5	90
	10	84

Immobilization

The effect of immobilization on the toxicity of two toxic solvents (hexane and decanol) was tested. Again, if interfacial deactivation would be an important inactivation mechanism, immobilization could significantly protect the cells, because no clotting and aggregation of the cells can occur at the interface anymore. The initial activity of free cells was about $8*10^{-5}$ mol O_2/s.kg DW and for immobilized cells $2*10^{-5}$ mol O_2/s.kg DW.

As can be seen in Figure 3.2, the free and immobilized cells showed a similar trend of deactivation in the presence of the toxic solvents, although immobilization provided a slight protection against the toxic solvents. This indicates that interfacial deactivation is not the predominant mechanism of the toxic effect of the solvents with low log P values, because then the deactivation would have been significantly lower when immobilized cells were used.

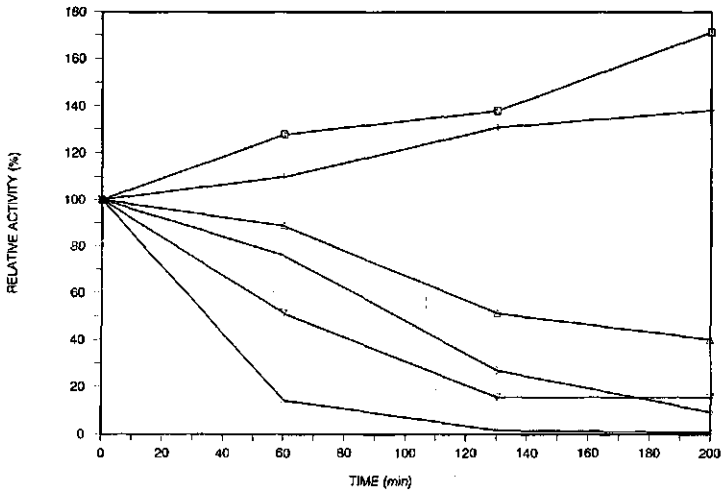

Figure 3.2.
The effect of immobilization of *Tagetes minuta* cells on the toxicity of 2% (v/v) hexane and 1% (v/v) decanol. The initial activity is taken as 100%.
Legend: o MS medium, free cells; + MS medium, immobilized cells; ♦ 2% hexane, free cells; ⌂ 2% hexane, immobilized cells, x 1% decanol, free cells; ▾ 1% decanol, immobilized cells.

CONCLUSIONS

The addition of 1% (v/v) of dibutylphthalate, decane, hexadecane and FC40 did not have any immediate toxic effect on the cells of *Tagetes minuta*.

Raising the solvent concentration of dibutylphthalate, FC40 and decane to 10% (v/v) had, likewise, no harmful effect.

Immobilization of the cells in calcium alginate provided a slight protection against the deactivating action of the toxic solvents.

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Chapter 4

GROWTH DETERMINATION OF HAIRY ROOTS IN BIOREACTORS

BUITELAAR, R.M., VAN ROOIJEN, G., DE VRIES, E.F.A.,

CESÁRIO, M.T. AND TRAMPER, J.

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Chapter 4

ABSTRACT

Three methods for the biomass determination of hairy roots growing in bioreactors were tested and compared. The methods were: the drain and weigh method, measurement of oxygen consumption by the cells and of the conductivity of the medium. All three methods had their specific benefits and shortcomings.

The drain and weigh method was easy to handle, and the results were reliable. It will be difficult to apply this method on a larger scale, however. The second method, measurement of the oxygen consumption by the cells, was easy to perform as well. Most probably, this method can be used on a larger scale without problems. The results did show some variation, but trends can definitely be distinguished this way. Additional experiments were done to test whether changes in the environmental conditions would influence the specific oxygen consumption of the cells. For the conditions tested, this was proven to be not the case, indicating a good applicability of this method. The third method, the determination of the conductivity decrease of the medium, was very easy to perform as well. The results are reliable until cell lysis occurs. Then, the cell contents influence the conductivity of the medium, yielding erroneous results.

KEYWORDS

Hairy roots, bioreactors, growth determination, *Tagetes patula*.

ABBREVIATIONS

BOM: biological oxygen monitor; DW: dry weight; FW: fresh weight

INTRODUCTION

The use of plant cell cultures for the production of fine chemicals, pharmaceuticals and agrichemicals is getting much attention the last years. Cell cultures can be initiated from leaf or stem parts. First, callus is grown on agar plates and after a number of transfers, the friable part of the callus can be used to initiate cell suspension cultures. Initially, those callus and cell suspension cultures were used

by the plant cell researchers for the production of the desired secondary metabolites. There are, however, some disadvantages connected to the work with these types of cell cultures. Basically, the tissue in these cultures is undifferentiated and the evidence that there is a relation between differentiation and secondary metabolite production is increasing (Rhodes et al., 1986). The introduction of hairy root cultures (Tepfer, 1983) was an important event for plant cell biotechnology and many researchers started to use those cultures for their secondary metabolite production.

Due to the specific morphology of the interconnected hairy roots, not all the techniques used for suspension cultures can be used for those hairy roots. For instance, when they are grown in a bioreactor, it is not possible to take representative samples during the run and the roots cannot be pumped.

In the research described in this article, hairy roots of *Tagetes patula* were grown in a bubble column. In general, since representative samples can not be taken with these cells, techniques for the determination of biomass must be used that measure macroscopic properties of the culture. Examples are exhaust gas analysis (Dalton, 1985), the mass of volume of the total cell mass, the oxygen consumption of the whole culture (Wilson, 1987), the ion consumption by the cells, which is reflected in the conductivity of the medium (Taya et al., 1987 and Yoon et al., 1988) and the peroxidase level of the medium (Shetty et al., 1990).

Three of these methods have been tested in this work. Criteria were the ease of use, the reliability and the possibilities for use on a larger scale.

The results indicate that all three methods tested have their own specific advantages and disadvantages. The drain and weigh method (measuring the total cell volume) is easy to use on a small scale, but will cause problems on a large scale. The oxygen consumption measurement is very easy to execute and is not time consuming. The results did show some fluctuation, however. The third method tested, monitoring the conductivity of the medium, was the most simple and rapid of the three. The results look very reliable until cell lysis occurs; then the contents of the cells that come into the medium cause incorrect results.

MATERIALS AND METHODS

Cells

The cells used were hairy roots, made by transforming *Tagetes patula* plants with *Agrobacterium*

rhizogenes LBA 9402 (Tp9402). The cells were maintained in Gamborg's B5 medium (Gamborg et al., 1968) (pH 5.6), supplemented with (per dm^3) 30 g sucrose and 0.1 mg biotine. The cells were grown in 250 ml Erlenmeyer flasks, filled with 50 ml of this medium. The flasks were placed on an orbital shaker (100 rpm) in the dark. The temperature was about 24°C. The cells were subcultured every two weeks by inoculating fresh medium with about 10 root tips.

Bioreactor system

In Figure 4.1, the bioreactor system is presented schematically. The reactor is a traditional bubble column, with aeration through a sintered glass sparger below. For continuous medium flow, pump 2 can be used. The use of pump 1, the weighing vessel and the oxygen electrode are explained below.

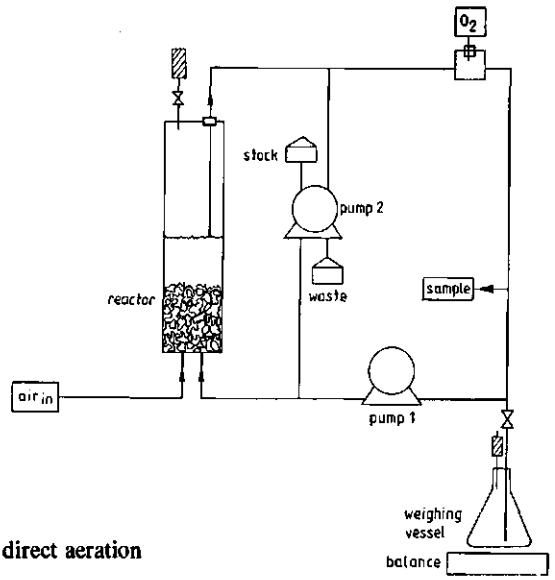

Figure 4.1
Bioreactor system with direct aeration

Biomass determination methods

Drain and weigh method

The amount of cells in the reactor can be estimated by determining the root volume. To achieve this, the aeration is switched off and all the medium in the tubes is pumped into the reactor. The total culture volume (medium plus cells) can then be read from the volumetric scale on the reactor. Subsequently, the medium is pumped out of the reactor into the weighing vessel on the balance by using pump 1 (Figure 4.1). The weight of the medium is then determined (medium density is

approximately 1 kg/dm^3) and the difference between these two measurements gives the root volume in the reactor.

Oxygen consumption rate

The amount of oxygen consumed was also used to determine the amount of biomass present in the bioreactor. In order to ensure sufficient mixing in the area of the electrode membrane, the medium was circulated from the reactor through a glass vessel containing the oxygen electrode. This galvanic probe was produced by our own workshop. First, the oxygen concentration in the medium was measured. Then, the air supply was switched off and the decrease in oxygen concentration with time was followed.

Conductivity measurement

A third indirect method to determine the biomass in the reactor is the measurement of the conductivity of the medium. This conductivity is directly proportional to the concentration of ions in the medium, with the following relation:

$$\kappa = \Sigma (c_i * \lambda_i) \qquad (1)$$

with:	κ	=	specific conductivity	(mS/cm)
	c	=	concentration	(mol/dm^3)
	λ	=	equivalent conductivity	(mS.dm^3/mol.cm)
	i	=	ion type	

To find a correlation between the increase in biomass concentration and the decrease in conductivity of the medium, a calibration curve was made (Buitelaar et al., 1991). The conductivity of the medium was determined off-line using a Schott Geräte dip type conductivity electrode (type CF 3100) combined with a Schott Geräte conductometer (type CG 854).

Fresh weight

The fresh weight of cells in shake flask experiments was determined by washing the cells three times with demineralized water to remove the medium salts, drying them for 10 minutes on filter paper and weighing them.

Dry weight

The dry weight of the cells was measured by drying a weighed amount of fresh, rinsed cells to

constant weight in an oven at 105 °C.

Biological Oxygen Monitor

The respiration activity of the cells was measured with a Biological Oxygen Monitor (BOM), consisting of an Orbisphere oxygen electrode and oxygen meter. The measuring vessel, which had a volume of 230 ml, was filled with the appropriate fresh medium. After saturating the medium with air the cells (0.5-6.5 g) were added, the vessel was closed and aeration was switched off. The decrease in oxygen concentration with time was recorded and evaluated.

Influence of medium composition

Because oxygen consumption was one of the indirect methods to determine the amount of biomass present in the bioreactor, it was important to know if there was a constant relation between oxygen consumption and amount of biomass present. Therefore, all kinds of possible bioreactor situations were simulated by varying the nutrient composition of the medium. The most important nutrients were varied: NO_3^-, PO_4^{3-}, NH_4^+ and sucrose. Depletion of these nutrients in the bioreactor was simulated by testing concentration ranges of these components varying from the original medium concentration to very low values. The respiration activity of the cells in these media was tested in the BOM and used as a measure for the influence of the medium composition on respiration of the cells.

RESULTS AND DISCUSSION

Three methods of indirect biomass determination were tested. The three methods (drain and weigh method, oxygen consumption rate and conductivity measurement) are described in detail in the Materials and methods section.

Drain and weigh method

Figure 4.2 shows the time course of the root volume for a reactor run of about 50 days. The reactor (effective volume about 1.3 dm^3) was inoculated rather densely, in order to be able to do measurements from day 0 on. It can be seen that the cells grew very well; at the end of the run the cells occupied about 50% of the total volume available in the reactor. The run was stopped after 50 days because at that time the cells had formed such a dense culture that no more medium and oxygen supply was possible in the inner part of the culture. At the end of one of the other runs, the total root

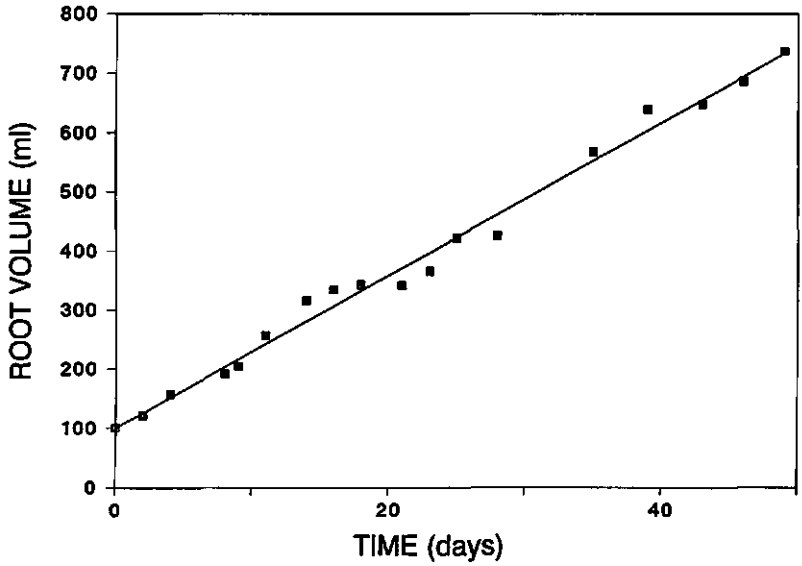

Figure 4.2
Volume of Tp9402 hairy roots in a bioreactor

fresh weight was determined and the difference between estimated root volume and fresh weight was about 3%.

For the scale used in these experiments, this drain and weigh method was easy to handle and convenient. It is clear, however, that this method will cause many practical problems when applied on a large scale. The drain and weigh method as described by Hilton and Rhodes (1990) more or less resembles the method described here.

Oxygen consumption

The second method that was tested for the determination of biomass was the oxygen consumption by the whole amount of biomass present. Figure 4.3 shows the results of these measurements for the same reactor run as is shown in Figure 4.2. The oxygen consumption measurements show more variation than the drain and weigh experiments, indicating a lower accuracy and reliability. An advantage of this method is its simplicity. If the accuracy required is not too high, this method can be used. In each of the Figures 4.2 and 4.3, one straight line is drawn as a result of regression analysis on the data presented. But, in Figure 4.3 a kind of breakpoint can be seen at day 25. The two regimes give two different regression lines, indicating two growth rates, the first one being higher than the last one. This would agree with the results obtained with the conductivity measurements, where a change of

trend is visible around the same day. The data in Figure 4.2, however, do not support such a break

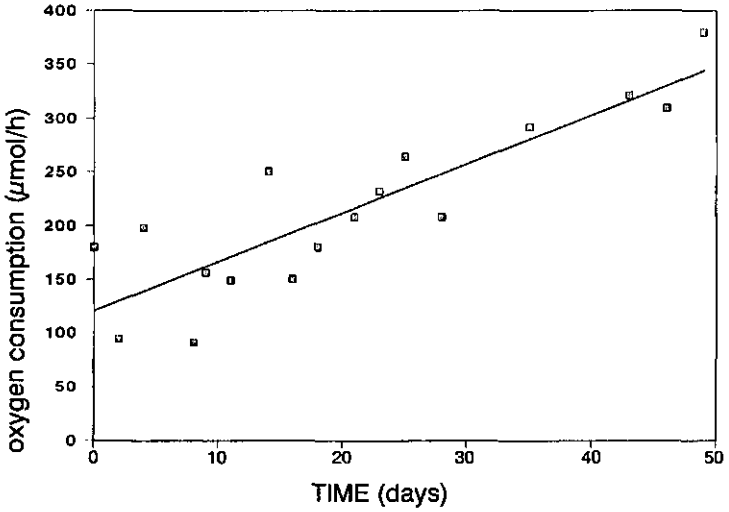

Figure 4.3
Oxygen consumption by the Tp9402 biomass in a bioreactor

of slopes. This can possibly be explained by the fact that dead cells still occupy their volume, measured with the drain and weigh method, but do not contribute anymore to the oxygen consumption or to the nutrient consumption, determined with the conductivity measurement.

In a separate experiment, the influence of the depletion of the medium components (which is likely to occur to some extent during a batch fermentation run) on the oxygen consumption was determined. Cells were incubated for five days in B5 medium where the concentration of one the components: nitrate, ammonia, phosphate or sucrose was lowered to values that could easily be found during a run. As a control, the normal B5 medium was used. In Figure 4.4, the oxygen consumption (in µmol/(g FW.h)) of the cells under these conditions, as determined in the BOM, is shown. Apparently, the oxygen consumption of the cells is not very much influenced by the concentration of the nutrients tested.

Intra- or extracellular peroxidases could influence the O_2-respiration measurement (van der Plas et al., 1987 and Shetty et al., 1990). For intact cells, intracellular peroxidases do not contribute to the oxygen uptake unless exogenous NADH is added (van der Plas et al., 1987), which has not happened in this case. The influence of extracellular peroxidases was prevented in the BOM experiments by using fresh medium for all experiments.

The oxygen consumption method was also tested in bioreactors with organic-aqueous two liquid phases with plant cells. It turned out to be not suitable for this purpose, because the oxygen capacity

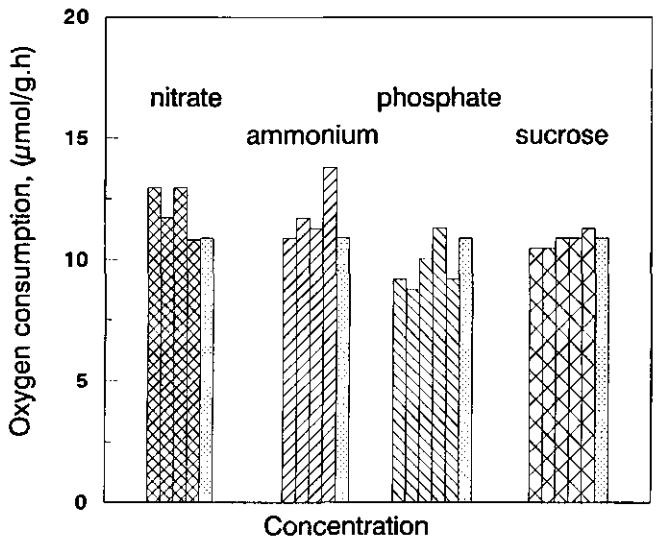

Figure 4.4
Oxygen consumption by Tp9402 hairy roots under various environmental conditions measured in the BOM. Nitrate concentration: 0.5-1.0-1.5-2.0-2.5 g/l; ammonium concentration: 25-50-75-100-134 mg/l; phosphate concentration: 25-50-75-100-150 mg/l and sucrose concentration: 5-10-15-20-25-30 g/l.

of organic solvents is usually much higher than that of water. So, the oxygen that is consumed by the cells in the aqueous phase is continuously replenished by the oxygen from the organic phase, in order to establish the partition equilibrium again. Because of this, one measurement (during which the oxygen supply has to be switched off) took about one day, which is not very practical, of course. Thus, although the oxygen consumption is a very easy parameter to measure, this method has some shortcomings which make it not always the best choice.

Conductivity measurement

For this method, a calibration curve was made to relate the biomass increase (dry weight, g/dm^3) to the decrease in conductivity (mS/cm) (Buitelaar et al., 1991). The relation between growth and conductivity can be described with the empirical equation:

$$\Delta X = A * \Delta K \qquad (2)$$

with:	ΔX	=	increase in biomass	(g/dm^3)
	A	=	constant	($g.cm/dm^3.mS$)
	ΔK	=	decrease in specific conductivity	(mS/cm)

From the experiments with Tp9402, a value for A of 3.9 $g.cm/(dm^3.mS)$ resulted, which is in the same order of magnitude as has been reported for other plant cell cultures by Taya et al. (1989), who found values of 3.6 (*Coffea arabica*), 2.8 (*Nicotiana tabacum*), 3.2 (*Withania somnifera*) and 4.1 (*Catharanthus roseus*).

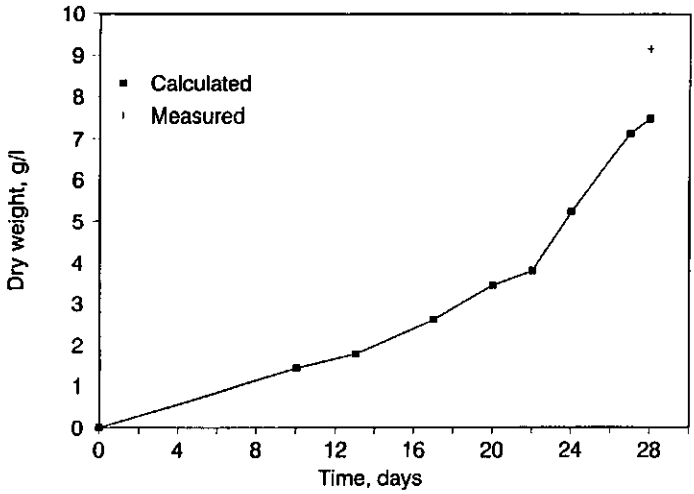

Figure 4.5
Increase in biomass dry weight as calculated from conductivity measurements for Tp9402 in a bioreactor.

Figure 4.5 shows the biomass present, as calculated with this conversion factor, during the same run as Figures 4.2 and 4.3. Up till about 25 days, the results of the conductivity measurements indicate growth. The biomass is expressed as dry weight. As is generally known, the fresh weight/dry weight ratio for plant cells is not constant throughout the whole growth cycle of the cells. This complicates the comparison of this method with the other methods. After day 25, the conductivity of the medium increased, which results in measurements that can not be interpreted. At that time, there was significant browning of the medium, indicating cell lysis. Obviously, when lysis occurs, the cell

content will come into the medium. This phenomenon is also used by Yoon et al. (1988) to indicate the beginning of cell lysis which was accompanied by a levelling off of the secondary metabolite production. In other studies, the conductivity measurement method has been used without problems for the monitoring of hairy root culture growth (Buitelaar et al., 1991)

In conclusion, all three methods tested for indirect biomass determination have their own advantages and shortcomings. The drain and weigh method is relatively laborious, and the method can not easily be scaled up. However, the results look quite well. The oxygen consumption measurement is less reproducible and gives some variation. It is, however, a very simple method that is used on-line. The conductivity measurement is also very easy to execute. In this case, an off-line electrode was used but of course, on-line electrodes can be used as well. If cell lysis occurs, the conductivity measurement can not be used anymore, which limits its applicability.

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Chapter 5

GROWTH AND THIOPHENE PRODUCTION BY HAIRY ROOT CULTURES OF *TAGETES PATULA* IN VARIOUS TWO-LIQUID-PHASE BIOREACTORS.

BUITELAAR, R.M., LANGENHOFF, A.A.M., HEIDSTRA, R. AND TRAMPER, J.

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ABSTRACT

In the work presented here, the use of aqueous-organic, two-liquid-phase systems was tested for the production of secondary metabolites with plant cell cultures. The cells used were hairy root cultures obtained by the transformation of *Tagetes patula* with *Agrobacterium rhizogenes*. *Tagetes* produces thiophenes, heterocyclic sulfurous compounds with biocidal acitivity. First, suitable organic solvents were selected with growth and secondary metabolite production as biological criteria. Physical criteria were the partition coefficient for the thiophenes and the density difference with medium. From the nine solvents tested, only those with a log P value higher than 5 proved to be suitable. FC40 and hexadecane were the solvents of choice for further experiments in two-liquid-phase bioreactors. Three fermenter types were tested, i.e. the liquid-impelled loop reactor, the stirred tank reactor and the bubble column. The liquid-impelled loop reactor was blocked by fast growing hairy root cells after one week. Immobilization of hairy roots in calcium alginate beads was not an adequate solution to this problem. In the two other reactor types, the stirred tank reactor and the bubble column, several runs of about 1 month were carried out. In the stirred tank reactor, the cells showed a higher volumetric growth rate than in the bubble column. The growth rate of the cells was higher for FC40 as the dispersed phase than for hexadecane. When thiophene production is also taken into account, the best results were obtained with the bubble column and hexadecane as the dispersed phase. The linear growth rate in this system was 0.18 g/(l.day) with a total thiophene production at the end of the run of 465 μmol in a total volume of 6 dm^3. When hexadecane was used as the organic phase, the excretion of thiophenes was 30-70% of the total thiophenes produced, in contrast to one-phase systems where the excretion is less than 1% with these cells. With FC40, the excretion was 10-20%. The distribution of the various thiophenes inside and outside the cells was not the same, indicating different excretion patterns for the various thiophenes.

KEYWORDS

Hairy root cultures; plant cell bioreactors; thiophenes; two-liquid-phase systems; *Tagetes patula*; excretion.

Chapter 5

INTRODUCTION

Plant cells offer a potential industrial use because they are able to produce compounds of commercial interest, either by the novo synthesis or by biotransformation.

Despite this potential, only a few commercial processes based on plant cell cultures exist at the moment, i.e. the production of shikonine, berberine, ginseng and sanguinarine. This is due to some drawbacks of plant cell cultures, in particular their slow growth, their low productivity and their instability.

Several approaches have been suggested to solve or reduce these problems. Among them are the selection of high yielding cell lines, genetic engineering, the use of differentiated cultures, immobilization and the use of two-phase systems. This article will deal with the latter three approaches.

Major progress has been made by the introduction of hairy root cells, obtained by the transformation of plants with *Agrobacterium rhizogenes* strains. Hairy root cells show a greater metabolic and genetic stability, they grow faster and have a higher production rate than the traditional, undifferentiated cell suspensions (Flores et al., 1988).

Immobilization of biocatalysts (enzymes, cells) is often used to easily keep them in the bioreactor, so that a more efficient repeated or continuous use is possible. Immobilization can have some advantageous physiological effects on plant cells as well. Asada and Shuler (1989) showed an increased production and excretion of ajmalicine by *Catharanthus roseus* cells immobilized in calcium alginate. Wichers et al. (1983) showed that immobilization of *Mucuna pruriens* cells in calcium alginate resulted in an increased excretion of L-DOPA. The immobilization of cells in a gel matrix can induce differentiation and/or cell-to-cell contact which is often said to be compulsory for the production of secondary metabolites by plant cell cultures (Bringi and Shuler, 1990). When working with hairy roots, it is not necessary to immobilize them for easy retention in the bioreactor, but the physiological effects can still be of importance. Furthermore, it can be expected that mixing is better in a reactor filled with beads instead of with interconnected roots.

An approach that could also help to solve some of the problems mentioned above is the use of two-liquid-phase systems, in which the cells are grown in the aqueous phase, while the second, organic phase with a more hydrophobic character can act as a product reservoir. If the product is excreted by the cells and migrates preferentially into the organic phase, several advantages may arise, e.g. a more efficient use of cells and facilitated product recovery.

Common fermenter types like the stirred tank and the bubble column can be used as two-liquid-phase bioreactor, but most salient is the liquid-impelled loop reactor. In the latter the principle of the airlift loop reactor applies, but instead of air a water-immiscible organic solvent is injected (Tramper et al., 1987).

For a successful application of two-liquid-phase bioreactors, several requirements should be met: 1) the solvent should not be toxic to the cells, 2) the product has to be excreted by the cells, 3) phase separation has to be good and fast, and 4) the product should preferentially be extracted into the organic phase.

Ad 1: To predict the toxicity of solvents to biocatalysts like enzymes and cells, the log P value of a solvent has proved to be a good indication (Laane et al., 1987). Log P is the log of the partition coefficient of a compound, e.g. a solvent, over a standard octanol/water two-phase system. Laane et al. (1987) stated that if log $P > 4$, the solvent will generally be not toxic to biocatalysts; when log $P < 2$, the solvent usually is detrimental and if log P is between 2 and 4, the retention of activity can vary. This has been demonstrated for enzymes (Laane et al., 1987), micro-organisms (Rezessy-Szabó et al., 1987 and Buitelaar et al., 1988) and plant cells (Buitelaar et al., 1988, 1990a,b).

Ad 2: If plant cell products are stored intracellularly, which is the case for many plant cell cultures (Wilson et al., 1987), several ways exist to induce excretion of the product. Two of them, production of the secondary metabolite in the presence of an organic solvent or in the presence of and adsorbent, can in principle be applied without a loss of viability of the cells (Buitelaar et al., 1990a,b, 1991, Robins et al., 1988 and Payne et al., 1988).

Ad 3: A good and fast phase separation of the two liquid phases is important because it facilitates the work-up of the product, and because it can be a prerequisite for the liquid circulation and mixing in reactor types like the liquid-impelled loop reactor. Phase separation is influenced by several factors, like the density difference between the two phases and the presence of emulsifying agents or cells.

Ad 4: A two-liquid-phase system will only be useful if the product migrates preferentially into the organic phase. Therefore, the determination of the partition coefficients of the products for the various two-liquid-phase combinations is important.

In the study presented in this paper most of the aspects discussed above have been investigated using the production of thiophenes by *Tagetes sp.* as a model. Suspensions cultures of these species have the above-mentioned disadvantages, which is less so with the hairy root cultures. Thiophenes are intracellular compounds with biocidal activity and a rather hydrophobic nature (Lam et al., 1988). It was therefore of interest to us to investigate if the introduction of an organic phase stimulates the

excretion, influences the distribution of thiophenes and facilitates the product recovery in two-phase bioreactors with free and immobilized hairy root cells. This paper reports on these aspects.

MATERIALS AND METHODS

Cells

The cells used were hairy roots, made by transforming *Tagetes patula* plants with *Agrobacterium rhizogenes* LBA 9402 (Tp9402). The cells were maintained in Gamborg's B5 medium (Gamborg et al., 1968) (pH 5.6), supplemented with (per dm^3) 30 g sucrose and 0.1 mg biotine. The cells were grown in 250 ml Erlenmeyer flasks, filled with 50 ml of this medium. The flasks were placed on an orbital shaker (100 rpm) in the dark. The temperature was about 24°C. The cells were subcultured every two weeks by inoculating fresh medium with about 10 root tips.

Cell weight determinations

The wet weight of the cells was determined by washing the cells with distilled water on a sieve, drying them for 10 minutes on filter paper and then weighing them.

For shake flask experiments, the dry weight was measured by drying the rinsed cells to constant weight in an oven at 105°C. To determine the dry weight of the cells at the end of a fermenter run, all the cells were removed from the fermenter, washed with a large amount of distilled water and then the fresh weight was determined. The dry weight of a small sample was then determined and the ratio fresh weight/dry weight of this small sample was used to calculate the dry weight of the whole amount of biomass.

The conductivity of the supernatant of cell suspensions was also used as a measure of cell weight. First a calibration curve was made. For that, many shake flasks of 100 ml filled with 25 ml medium were inoculated with a few root tips. At regular time intervals, a number of shake flasks was analysed, i.e. the dry weight (g/l) was measured together with the conductivity (mS/cm) of the medium after removal of the cells. The latter measurement was executed using a conductivity electrode (Schott, type LF 3100) combined with a conductivity meter (Schott CG 854) after equilibration of the sample in a water bath of 25°C. The thus obtained calibration curve was used to determine the amount of cells in the fermenters during the run. Samples of the medium were taken every 2-4 days to measure the conductivity.

Analysis of thiophenes

The thiophene content of the cells was determined according to the procedure of Norton et al. (1985) with modifications (Croes et al., 1989) as follows.

An accurately weighed amount of cells was homogenized in the presence of 1 ml ethanol. The homogenizer was rinsed with 1 ml ethanol:water 1:1 and 1 ml water. To these 3 ml, (x) 6 ml of a mixture of hexane:butylmethylether 1:1 was added. Extraction was done by vortexing for 1 minute. This solution was centrifuged (10 min., 3000 rpm), the top phase (hexane:butylmethylether) was removed and the procedure was repeated from point (x). The two fractions of hexane:butylmethylether were pooled and evaporated under nitrogen flushing in a water bath of 40 °C. The residue was redissolved in 1 ml ethanol which was then used for HPLC analysis. The whole extraction procedure was executed under dim light conditions to prevent the degradation of thiophenes. Medium was extracted directly with hexane:butylmethylether, according to the procedure starting at point (x).

Hexadecane is miscible with the hexane:butylmethylether mixture, but not with ethanol, so in this case direct extraction with ethanol was executed. This was not possible with FC40, because this is miscible with ethanol. The method starting at point (x) was applied to FC40. The HPLC was run under the following conditions: C18 column (Chrompack); eluens: acetonitril:water, 3:1; flow rate 0.7 ml/min.; detection at 340 nm. The HPLC system was equipped with an integrator (Spectra-Physics). Calibration curves had been made in advance using commercially available α-T (Aldrich Chemie) and BBT, BBTOAc and BBTOH that had been synthesized by the department of Organic Chemistry of the Wageningen Agricultural University. $BBT(OAc)_2$ was assumed to have an extinction coefficient similar to that of BBTOAc.

The thiophene content was calculated as μmol thiophenes per gram fresh weight or as μmol thiophenes per ml medium or solvent. Analytical data are the mean values of at least two determinations.

Solvents

The solvents used were of analytical grade. Table 5.1 shows these solvents, together with their log P values, their densities and their manufacturers. Log P values come from the literature (Laane et al., 1987) or were calculated using the hydrophobic fragmental constant method as published by Rekker and de Kort (1979).

The partition coefficients of thiophenes over medium/solvent two-phase systems were determined in shake flasks by adding a known amount of thiophenes to a two-phase system. These shake flasks were put on a rotary shaker in the dark for two days. Afterwards, the thiophene content of the aquous

phase was determined according to the above method.

Table 5.1
Solvents used, with some properties

Solvent	Log P	ρ	Supplier
		$kg.dm^{-3}$	
Diethylphthalate	3.3	1.12	Merck
Dibutylphthalate	5.4	1.05	Merck
Dioctylphthalate	9.6	0.99	L & I
Hexane	3.5	0.66	Rathburn
Decane	5.6	0.73	Merck
Hexadecane	8.8	0.77	Merck
Decanol	4.0	0.83	Janssen Chimica
Ethylacetate	0.7	0.90	Merck
FC40	11.2	1.87	3M

Since the extraction yield of the aqueous phase is known to be 100% in this way, the extraction yield of thiophenes from the organic phases (FC40 or hexadecane) could now be determined by measuring the thiophene content in the solvent with the methods described above.

Solvent testing

The influence of the various solvents on the cells was determined by growing the cells in shake flasks with medium and 5% (v/v) solvent. After about 10 days, the cells were harvested and their respiration activity and dry weight were determined. The thiophene content of cells, medium and solvent was also analysed when possible (extraction of thiophenes from the solvents only worked well with the above methods for hexadecane and FC40).

The toxicity of the solvents was also tested by adding about 10 g (fresh weight) of hairy roots to a shake flask containing 49 ml of medium and 1 ml of solvent. During several hours, at regular intervals 0.2-0.3 g hairy root cells were taken from the shake flasks and their respiration activity and exact dry weight were determined. The respiration activity was tested with a biological oxygen monitor. Five ml of medium was aerated at 30°C. After two minutes, when the medium was saturated

with air, 0.2-0.3 g hairy root cells were added. The vessel was closed with an oxygen electrode and the decrease in the oxygen concentration in the vessel was recorded.

Immobilization

Before hairy roots can be immobilized, they first have to be homogenized. This was accomplished in a blender, at 400 rpm during a few seconds. The cells were then sieved, to prevent large particles from blocking the immobilization needle. The decanted cell suspension was mixed in a 1:2 ratio with a 3.6% sodium alginate solution (Manucol DM, Kelco) and stirred to obtain a homogeneous cell/gel mixture. This mixture was extruded dropwise via a needle into a 0.1 M $CaCl_2$ solution where hardening of the beads took place. After two hours, the beads were transferred to fresh medium and were ready for use in shake flask or fermenter experiments.

Bioreactors

The liquid-impelled loop reactor used had a construction similar to that of an airlift loop reactor with external loop (Tramper et al., 1987). A schematic representation of the system can be seen in Figure

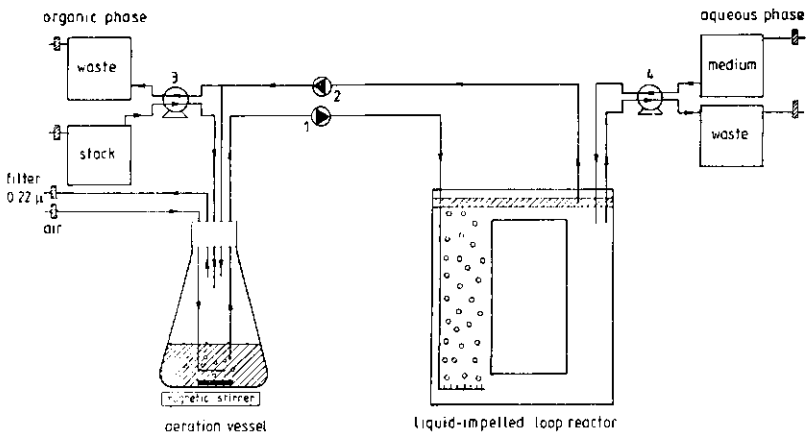


Figure 5.1
Liquid-impelled loop reactor. Detailed explanation is given in Materials and methods.

5.1. In this figure a configuration is drawn with a solvent lighter than water, with the sparger installed at the bottom of the larger tube (then functioning as the riser). When a solvent heavier than water is used, the sparger is installed at the top of the larger tube, which then functions as the downcomer.

With a solvent lighter than water, separation of the two phases takes place at the top of the fermenter. The solvent is circulated by the gear pumps 1 and 2 through an external vessel where it is aerated. If desired, both phases can be refreshed continuously using the peristaltic pumps 3 and/or 4. Engineering aspects of the LLR, like hydrodynamics, mixing and mass transfer, are presently under investigation in our department (Sonsbeek et al., 1990).

The volume of the reactor was 2 dm^3. After autoclaving (121°C, 30 min.), the fermenters were inoculated with about 1 gram fresh weight of free hairy roots or 10-15% (v/v) of immobilized hairy root cells. At regular intervals, samples of the medium and solvent were taken and analysed (medium: conductivity and thiophene content; solvent: thiophene content).

The stirred tank reactors used were standard fermenters (Applikon). The volume was 2 dm^3 and the fermenters were filled with 1 dm^3 of medium and 0.2-0.3 dm^3 of solvent. Aeration took place in the reactor. In order to prevent the cells from getting stuck behind the tubes in the fermenters, the stirrer speed was set to 450 rpm. When the stirrer speed was lower, it was not possible to keep the cells in suspension. At this stirrer speed, the two-phase system formed an emulsion, making it impossible to circulate the solvent with a pump. In contrast to a liquid-impelled loop reactor, a stirred tank reactor does not need the solvent circulation for mixing. Since preliminary experiments demonstrated that after some time browning of the medium occurred due to lysis of the oldest cells with a concomitant decrease in the growth rate of the cells, the fermenter systems were run in a semi-continuous mode. From the moment that the first browning occurred, 25% of the medium was replaced by fresh medium every day.

The bubble columns used were simple glass tubes with a sintered glass plate for air sparging in the lower part. The total volume was 6.5 dm^3. The reactors were filled with 5 dm^3 of medium and 1 dm^3 of solvent. In this case, the solvent had to be circulated through the reactor with an external pump to establish the contact between the solvent and the cells and the medium. These reactors were run in a semicontinuous mode as well; 25% of the medium was replaced by fresh medium every day after the first browning of the medium. Sampling of the solvent and the medium took place every 2-4 days.

RESULTS AND DISCUSSION

Conductivity measurements

In several recent articles (Ryu et al., 1990 and Taya et al., 1989) it is suggested that measuring the conductivity of the plant cell medium is the most suitable way to determine the amount of plant cell

biomass present in a bioreactor, which generally is a problem. In Figure 5.2, the results are plotted

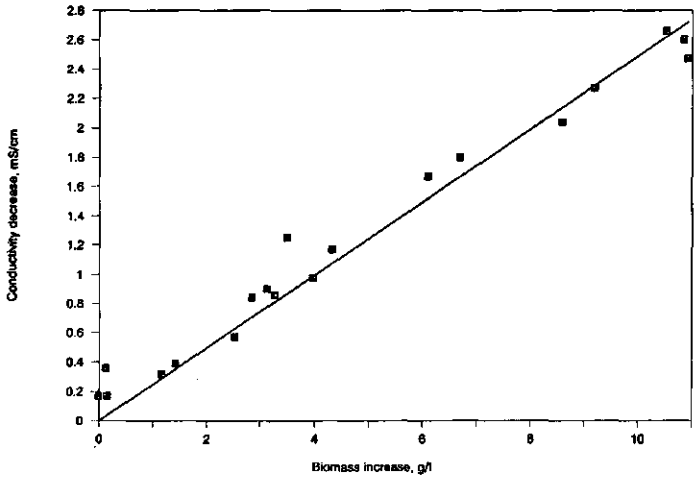


Figure 5.2
Calibration curve for conductivity vs. dry weight measurements with Tp9402 in Gamborg's B5 medium.

of a calibration experiment for our Tp9402 cells. The relation between growth and conductivity can be described with the empirical equation:

$$\Delta X = A * \Delta K$$

with:	ΔX	=	increase in biomass	(g/dm^3)
	A	=	constant	($g.cm/dm^3.mS$)
	ΔK	=	decrease in specific conductivity	(mS/cm)

From the experiments with Tp9402, a value for A of 3.9 $g.cm/(dm^3.mS)$ resulted, which is in the same order of magnitude as has been reported for other plant cell cultures (Taya et al., 1989).
The influence of sucrose, glucose and fructose on the conductivity of the medium was checked as well. Sucrose is the carbon source added in the medium, but the cells immediately split the sucrose into glucose and fructose, making their influence on the conductivity of equal importance. Figure 5.3 demonstrates this influence. At the maximum sucrose concentration (being the initial medium value of about 30 g/dm^3), the conductivity is 94% of the conductivity of medium without any sugar. The same values can be calculated from the experiments with glucose and fructose. This small deviation

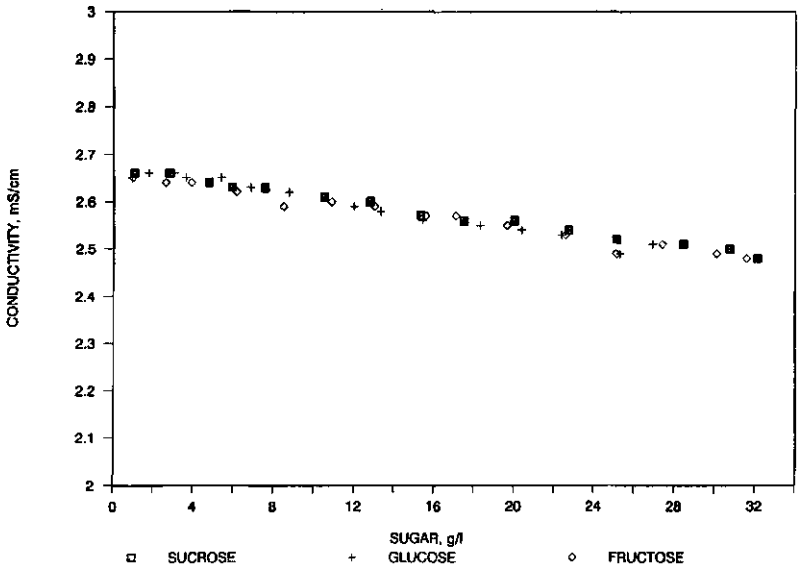


Figure 5.3
Influence of the sugar concentration on the conductivity of the medium.

is acceptable in our opinion, so no corrections were made for the sugar concentration in the medium. These results are comparable to those published by Taya et al. (1989).

Solvent selection

In order to make a proper selection of the solvents to be used, various solvents were tested for several properties. First, the influence of the solvents on the growth of the cells, the thiophene production and the respiration activity of the cells was measured. The solvents to be tested were selected based on the results of other researchers working with organic solvents (Laane et al., 1987). Most solvents used had a log P value higher than 4 which was expected to be favourable for the cells. In order to test whether some solvents could be toxic, a few solvents with lower log P values were tested as well. In Table 5.2, the results from these experiments can be seen. Growth and respiration activity give similar results in this case. The production of thiophenes per gram dry weight of cells in the presence of 5% diethylphthalate is similar to that in medium, but the growth is inhibited, so the overall production of thiophenes will be lower, which renders diethylphthalate unsuitable as a solvent for this purpose. It can be seen that the boundary value for log P is not 4, as was suggested by Laane et al. (1987), but about 5. We have observed this phenomenon before with plant cells (Buitelaar et al., 1990a,b).

Table 5.2
Growth, respiration activity and thiophene production by free Tp9402 cells in the presence of 5% solvent.

Solvent	Log P	Growth	O_2-consumption	Production
Ethylacetate	0.7	-	-	-
Diethylphthalate	3.3	-	-	+
Hexane	3.5	-	-	-
Decanol	4.0	-	-	-
Dibutylphthalate	5.4	+	+	+
Decane	5.6	+	+	+
Hexadecane	8.8	+	+	+
Dioctylphthalate	9.6	+	+	+
FC40	11.2	+	+	+

\- values much lower than controls (one-phase system without solvent)
\+ values more or less equal to the controls

Other selection criteria were physical parameters like the partition coefficient and the phase separation behaviour of a two-phase system with medium and solvents. As can be seen from Table 5.1, the density of the phthalates is very near the density of water or medium, making phase separation too slow and the driving force too small to obtain continuous circulation of the solvent in a liquid-impelled loop reactor. When the determined partition coefficients of thiophenes in two-phase systems are considered (Table 5.3), it is evident that all the thiophenes preferentially migrate into the organic

Table 5.3
Partition coefficients of thiophenes over several solvent/medium two-phase systems.

Two-phase system	Partition coefficient
Dibutylphthalate/medium	∞
Dioctylphthalate/medium	∞
Decane/medium	∞
Hexadecane/medium	∞
FC40/medium	5-10

phase, yielding a partition coefficient essentially being infinite. FC40 shows a somewhat different behaviour, the partition coefficient varies from 5 to 10 for the various thiophenes.

The log P values of the thiophenes have been calculated with the hydrophobic fragmental constant method of Rekker and de Kort (1979). Table 5.4 shows these log P values. The reason for the lower partition coefficient in an FC40/medium two-liquid-phase system can be the large difference in log P values between the thiophenes and FC40, and the well-known inertness of these FC-compounds.

At this stage, FC40, decane and hexadecane were tested for the possibility to extract and analyse the thiophenes from the solvent. Since this was easier for hexadecane than for decane (see Materials and Methods), hexadecane was selected as the solvent to be used in the experiments with a solvent lighter than medium, and FC40 for experiments with a solvent heavier than medium. For quantitative purposes, it was necessary to know the yield of extraction from the solvents to ethanol. For hexadecane and FC40, this yield was almost 100%.

Table 5.4
Calculated log P values of the thiophenes found in *Tagetes patula*

Thiophene	Log P
α-T	5.2
BBT	4.8
BBTOH	3.2
BBTOAc	3.7
$BBT(OAc)_2$	3.3

Immobilized cells

Before using immobilized cells in a bioreactor, we first had to find out if the growth and the thiophene production were influenced by immobilizing the cells. After a few days, root tips came sticking out of the beads, showing a morphology similar to that of free cells. The growth and thiophene production of the immobilized cells were identical to the results obtained with free cells (shown in Table 5.2).

Bioreactors

Liquid-impelled loop reactor

The first reactor tested was the liquid-impelled loop reactor, with hexadecane as the dispersed phase. In this system, Tp9402 cells grew very fast. Within one week the biomass had increased from a few 1 cm root tips as an inoculum to about 100 g of fresh weight. At the same time, this fast growth was a problem in this bioreactor. One week after inoculation, the clew of cells blocked the fermenter in one of the turnings, so no more circulation was possible from that time on. The scale and the dimensions of the reactor were the important factors causing this problem. The inner diameter of the riser was 6 cm and of the rest of the liquid-impelled loop reactor 4 cm. A liquid-impelled loop reactor with larger dimensions will very likely cause less problems in this respect. In order to avoid this problem, the hairy root cells were immobilized in calcium alginate beads with the aim to create a sort of fluidized bed with the beads, preventing the cell from making too many interconnections. This solution worked, but only for a short period. Now the reactor became blocked by a clew of cells growing out of the beads after two weeks instead of one.

Stirred tank reactor and bubble column

After the LLR, two other reactor types were used to study the growth and secondary metabolite production; i.e. a stirred tank reactor and a bubble column.

Both reactor types were used with hexadecane or FC40 as a lighter or heavier solvent, respectively. Table 5.5 shows the results of all the reactor runs. All the fermenters were run for about 4 weeks. The values given are the averaged values of all the similar runs (n = 2 or 3).

Figure 5.4 shows a typical run, in this case a bubble column with FC40 as the second phase. The results shown in Table 5.5 are the growth rate in g/(l.day), calculated from the conductivity measurements of the samples taken every 2 to 4 days from the reactors. With the formula given earlier, the biomass content, (dry weight, g/l) can be calculated during the whole run, making it possible to determine the growth rate in the bioreactor. In the next column, the amount of biomass present at the end of the run, in g dry weight per liter as determined by dry weight measurement is given. The end values (of calculated and measured dry weight at the end of the run) of other experiments did not always agree as well as in Figure 5. 4; the averaged difference was 1.2 g/l biomass (dry weight).

The total amount of thiophenes present in the cells at the end of the run is also given in Table 5.5, together with the total amount of thiophenes in the solvent and the total amount of thiophenes

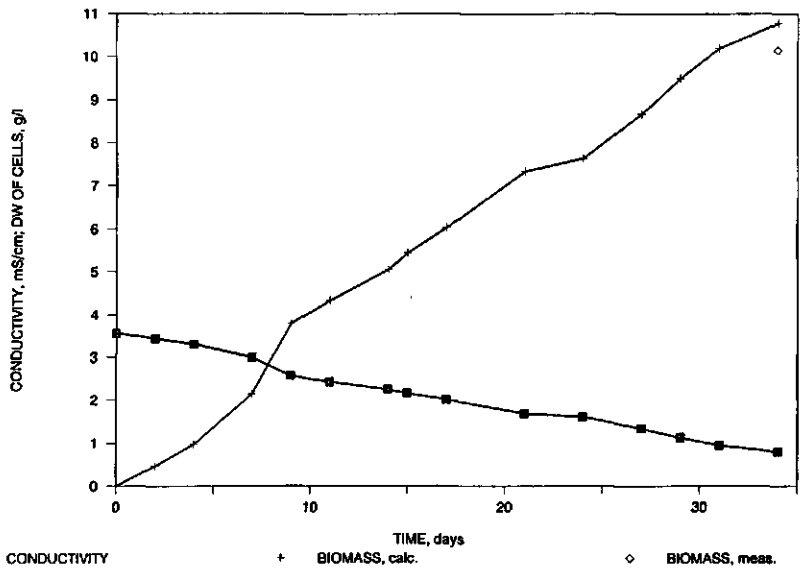


Figure 5.4
Typical bioreactor run, in this case a bubble column with FC40 as the dispersed phase.

produced per gram cells. There were never any thiophenes detectable in the medium when a solvent was present, which is in agreement with the high values of the partition coefficients for the thiophenes. The last column gives the percentage of the thiophenes that was excreted from the cells. When Tp9402 cells were grown in a one-phase system, less than 1% of the total thiophenes were excreted into the medium (Buitelaar et al., 1991). This shows the interesting and useful property of the two-phase systems, i.e. induction of excretion, especially with hexadecane as the dispersed phase. Up to about 70% of the thiophenes produced were excreted into the organic phase, facilitating continuous removal of the product stream and simultaneously offering the opportunity of a concentration step in the fermenter system itself.

Bioreactor type

For both solvents, the cells in the stirred tank reactor showed a higher growth rate in g/(l.day) and thus a higher final biomass concentration in g/l. Wilson et al. (1987) tried to grow cells of *Nicotiana rustica* in a stirred tank reactor without success; the root cells turned into callus-like structures, with a concomitant decrease in the production rate of secondary metabolites. In our case, the cells kept

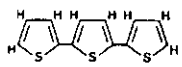
Table 5.5
Results of reactor runs

Run	n	Growth rate	Biomass, end	Total thiophenes, end			excreted
				cells	solvents	specific	%
		$g.l^{-1}.day^{-1}$	$g.l^{-1}$, DW	μmol	μmol	$\mu mol.g^{-1}$ DW	
SH	2	0.31 (± 0.16)	7.1 (± 1.7)	40 (± 25)	77 (± 42)	16.2 (± 1.8)	68 (± 3)
BH	3	0.18 (± 0.10)	5.2 (± 2.4)	334 (± 130)	131 (± 54)	21.3 (± 1.4)	28 (± 2)
SF	2	0.41 (± 0.14)	9.3 (± 3.0)	118 (± 52)	20 (± 19)	15.3 (± 1.4)	20 (± 19)
BF	2	0.26 (± 0.05)	6.8 (± 4.0)	191 (± 122)	11 (± 1)	7.2 (± 5.6)	9 (± 5)

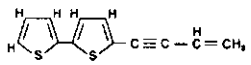
S : stirred tank reactor
B : bubble column
H : hexadecane
F : FC40
n : number of runs
DW : dry weight

their root morphology, but much more lysis occurred in the stirred tank reactor than in the bubble column, as indicated by the faster browning of the medium. Another indication of more lysis in the stirred tank reactor than in the bubble column is given by the difference in thiophene distribution in the cells and in the solvent, which is discussed below. The presented growth rates, in g/(l.day), do not really give a good indication of what occurred in the bioreactors. In the stirred tank reactor, the roots were kept relatively small by the action of the stirrer, and they were suspended through the whole reactor. In the bubble column, the cells formed one clump somewhere in the reactor. At this place, they grew out, and the fermenter had to be stopped when no more circulation of medium was possible because the cells blocked the fermenter cross section. At this time, only about 20% of the aqueous volume was filled with cells; the rest was empty. This means that the 'local growth rate' of the hairy roots in the bubble column can be considered to be 5 times as high as indicated by the values in Table 5.5.

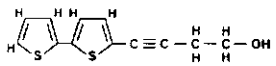
The specific production of thiophenes, in μmol/g cells, is highest in the bubble columns. So, if growth and thiophene production are considered, the bubble column is the best bioreactor of the types tested in this study.



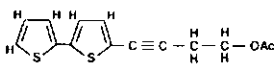
α-T
α-terthienyl



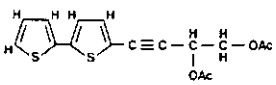
BBT
butenenylbithiophene



BBTOH
hydroxybutenylbithiophene



BBTOAc
aceloxybutenylbithiophene



$BBT(OAc)_2$
diacetoxybutenylbithiophene

Figure 5.5
Structure of five thiophenes occurring in Tp9402.

Solvent

The most striking difference between the experiments with the two solvents is the extent to which the

thiophenes are excreted by the cells. With hexadecane, the cells excrete about 30 or 70% in the BC or the STR, respectively. A similar phenomenon has been observed when *Lithospermum erythrorhyzon* cells were grown in the presence of hexadecane (Kim and Chang, 1990). With FC40, an inert liquid with a much lower solubility for thiophenes, these values are 10 and 20%, respectively. These 20% is perhaps not very realistic, because there was a considerable amount of lysis in one of the two experiments with FC40 in a STR. Probably this also explains for a part the high value of 70% of the thiophenes excreted in the STR with hexadecane. The growth of the cells appears to be better in the systems with FC40, probably because the oxygen capacity of FC40 is higher than that of hexadecane (Ju and Ho, 1989 and 3M).

Thiophene distribution

With the HPLC analysis, 5 thiophenes are detected in this system. The apolar BBT and α-T, and the more polar BBTOH, $BBT(OAc)_2$ and BBTOAC, see Figure 5.5. Under optimal conditions for the cells, meaning no lysis, as indicated by a clear, clean and colourless medium, the thiophene distribution is typical as shown in Figure 5.6. In the cells, BBT is by far the predominant thiophene. In the solvent,

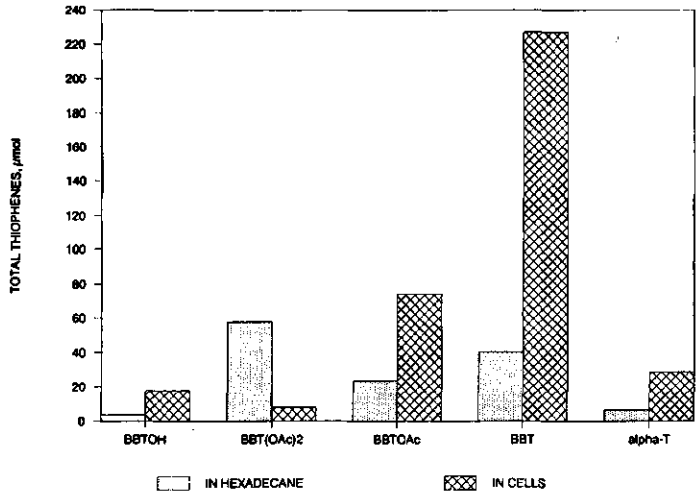


Figure 5.6
Thiophene distribution in cells and in solvent when no lysis occurs.

$BBT(OAc)_2$ is predominant, which means that some sort of active transport must be present. When no solvents are added, the thiophene distribution in the cells is similar to that in the cells of a two-

phase system, and there is hardly any excretion. When a STR is used and lysis occurs (medium is

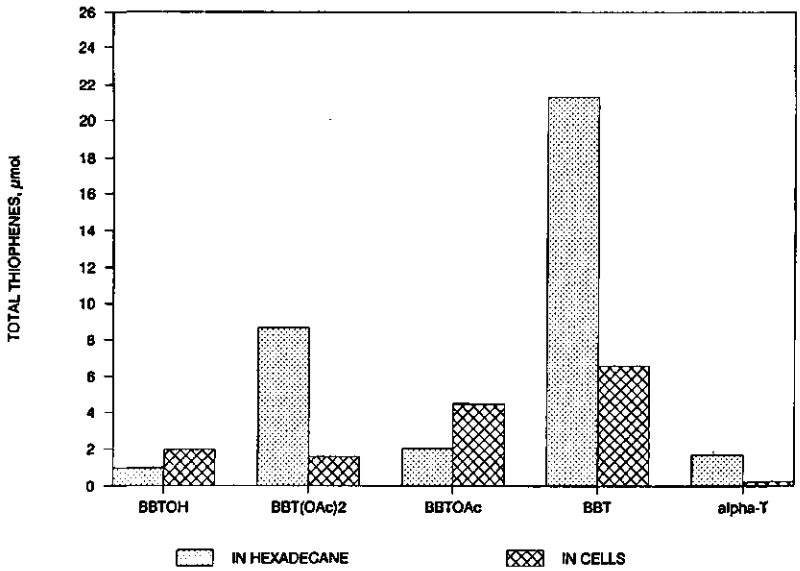


Figure 5.7
Thiophene distribution in cells and in solvent when lysis occurs.

browning), the thiophene distribution is typical as shown in Figure 5.7. The relative distribution of the five thiophenes in the cells and in the hexadecane is much more alike in Figure 5.7 than in Figure 5.6, indicating the occurrence of lysed cells.

CONCLUSIONS

It was shown in the present study that solvents with a log P value > 5 are not toxic for hairy root cultures of *Tagetes patula*. The growth of the cells, their respiration activity and their thiophene production were not influenced when these solvents were added to the amount of 5% (v/v). Immobilizing the cells in calcium alginate beads did not alter these results.

The final selection of the solvents to be used was based on the results, combined with the physical properties like density difference with water and the partition coefficient for thiophenes in a two-liquid-phase system. Hexadecane and FC40 were selected for further use. Out of the liquid-impelled loop reactor, the stirred tank reactor and the bubble column, the latter, with hexadecane as the second phase, performed best with respect to the production of thiophenes and the overall production rate. When hexadecane was used as a solvent, up to 70% of the produced thiophenes were excreted, in

contrast to less than 1% when a one-phase system is applied. With FC40, the excretion was less, 10-20%. The distribution of the thiophenes in the cells was different from that of the excreted thiophenes.

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Chapter 6

GROWTH AND SECONDARY METABOLITE PRODUCTION BY HAIRY ROOTS OF *TAGETES PATULA* IN AQUEOUS TWO-PHASE SYSTEMS

R.M. BUITELAAR, E.J.T.M. LEENEN AND J. TRAMPER

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Chapter 6

ABSTRACT

Hairy roots of *Tagetes patula* have been grown in aqueous two-phase systems. After selecting suitable polymers from single-phase experiments (in which salt phases proved to be unable to support growth in the desired concentrations), several two-phase systems were tested for their influence on cell growth and thiophene production. Cell growth occurred in all aqueous two-phase systems, but the highest growth rate was achieved in normal medium. There was no difference in thiophene production between medium and aqueous two-phase systems. The partition of thiophenes slightly favoured the more hydrophobic top phase in most cases, while the cells confined themselves to the bottom phase. One aqueous two-phase system (15% polyethyleneglycol 10.000 and 15% Reppal PES 200) was tested in a stirred tank reactor with normal medium as a control. The growth rate in medium was higher than in the aqueous two-phase system, while the thiophene production per unit cell weight was in the same range for both systems. The excretion of thiophenes in the reactor with the aqueous two-phase system was about ten times as high as in the control reactor. The amount excreted was however still not more than 3% of the total production.

KEYWORDS

Aqueous two phase systems, plant cell cultures, *Tagetes patula*, thiophenes, secondary metabolites, hairy roots.

INTRODUCTION

Plant cell cultures offer an interesting potential for the production of valuable chemicals that can, for instance, be of pharmaceutical or agricultural importance. Almost all plant cell culture production systems are operated with common aqueous media in shake flasks. In many cases, however, it could be advantageous to use a two-phase system, with an immiscible second liquid phase or with a solid second phase. The advantages can, for instance, include the prevention of substrate or product inhibition and the combination of the down stream processing with production. Aqueous-organic solvent two-phase systems have been used with plant cells (Cormier and Ambid, 1987; Buitelaar et

al., 1990a,b, 1991) with good results. Another interesting aspect of the work with two-phase systems is the possible enhancement of excretion of the desired products. This has been achieved with two-liquid-phase systems (e.g. Buitelaar et al., 1991) and with liquid-solid two-phase systems (e.g. Canales et al., 1990). One of the main objectives of the research described in this article was to see whether this effect would also occur in aqueous two-phase systems. Until now, apart from one article (Hooker and Lee, 1990), no results are published on the use of these systems with plant cells. Aqueous two-phase systems can be formed when aqueous solutions of two polymers or one polymer and one salt are mixed. They were originally developed for the separation and purification of biomolecules and cells (Albertsson, 1986), but they have gained increasing attention for use in bioconversions where enzymes (e.g. Larsson et al., 1989), bacteria (e.g. Flygare and Larsson, 1989; Fisher and Sutherland 1989) or fungi (e.g. Persson et al., 1984) are used. Aqueous two-phase systems will be particularly useful if the cells are retained in one phase, while the products partition favourably to the other phase. In the present article, results are given from experiments with hairy root cells of *Tagetes patula*. These cells are capable of producing thiophenes, compounds with biocidal activity (Lam et al., 1988). The growth of the cells and the production and partition of their secondary metabolites were tested in several aqueous two-phase systems. The use of fermenters with these systems was also studied.

MATERIALS AND METHODS

Cells

The cells used were hairy roots, made by transforming *Tagetes patula* plants with *Agrobacterium rhizogenes* LBA 9402. The roots were maintained in Gamborg's B5 medium (Gamborg, 1968), pH 5.6, supplemented with (per dm^3): 30 g sucrose and 0.1 mg biotine. The cultures (50 ml) were placed on an orbital shaker (100 rpm) in the dark in 250 ml Erlenmeyers flasks. The temperature was about 24°C. The roots were subcultured every two weeks by inoculating fresh medium with about 10 root tips.

Polymer solutions

Table 6.1 gives an overview of the polymers used, with their average molecular weight. For the preparation of aqueous two-phase systems, both phases were made separately by dissolving the

Table 6.1
Polymers and salts tested, with their molecular weight and their effect on the growth of *Tagetes patula* in a 2% solution

Compound		Molecular weight	Weight loss
		$g.mol^{-1}$	(= growth)
Control			
	Medium	-	+
Polymers			
	Dextran T-10	10000	+
	Dextran T-40	40000	+
	Dextran T-70	70000	+
	Dextran T-500	500000	+
	Dextran T-2000	2000000	+
	PEG 300	300	+
	PEG 600	600	+
	PEG 1500	1500	+
	PEG 2000	2050	+
	PEG 3000	3000	+
	PEG 6000	6750	+
	PEG 10000	10750	+
	PEG 35000	35000	+
	PVP K30	40000	-
	PVA 105	46500	+
	PVA 124	220000	+
	Reppal PES 200	200000	+
Salts			
	KH_2PO_4	136	-
	K_2HPO_4	174	-
	$MgSO_4$	120	-
	NaCl	59	-

appropriate amount of polymer into the medium and autoclaving them. Afterwards, the phases were combined to give two-phase systems.

Biomass determination

Wet weight

The wet weight of the cells was determined by washing the cells with demineralized water on a sieve, drying them for 10 minutes on filter paper and weighing them.

Dry weight

The dry weight was measured by drying a rinsed cell sample to constant weight in an oven at 105°C.

Weight loss

This method, developed by Schripsema et al. (1990), takes into account the dissimilation process, in which for every mole of oxygen consumed, a mole of carbon dioxide is formed, causing a loss of weight of the culture flask. This loss of weight is correlated with growth. This weight loss is followed by frequent weighing of the culture flasks. Because an analytical balance is essential to record the small weight loss, this method is restricted to be used with small culture flasks only. Here, 100 ml Erlenmeyer flasks with 40 ml medium were used. Blanks were included to correct for the evaporation of water. As was pointed out by Schripsema et al. (1990), this method works best when special stoppers are used that cause an enormous decrease of the evaporation loss. In that case, the variability in evaporation losses between similar flasks is negligible. Naturally, these stoppers (Silicosen plugs from Shin-Etsu Polymer Co. Ltd., obtained from Simrit Holland b.v., Naarden, The Netherlands) were used in this study as well. This method was, in this case, only used for the qualitative determination of growth in the phase of testing many polymer and salt phases.

Analysis of thiophenes

The thiophene content of the cells was determined in the way as developed by Croes et al. (1989) and Norton et al. (1985). These authors proved this method to be 100% reliable. An amount of cells was weighed and homogenized in the presence of 1 ml ethanol. The homogenizer was rinsed with 1 ml ethanol:water 1:1 and 1 ml water. To these 3 ml, (x) 6 ml of a mixture of hexane:butylmethylether 1:1 was added. Extraction was done by vortexing for 1 minute. This solution was centrifuged (10 min., 3000 rpm); the top phase (hexane:butylmethylether) was removed and the procedure was repeated from point (x). The two fractions of hexane:butylmethylether were pooled and evaporated under nitrogen flushing in a water bath of 40 °C. The residue was redissolved in 1 ml ethanol which was then used for HPLC analysis. The whole extraction procedure was executed under dim light

conditions, to prevent the degradation of thiophenes. Medium and polymer phases were extracted directly with hexane:butylmethylether, according to the procedure starting at point (x). The HPLC was run under the following conditions: C18 column (Chrompack); eluant: acetonitrile:water, 3:1; flow rate 0.7 ml/min.; detection at 340 nm.

Partitioning

Hairy roots were extracted to yield a mixture of thiophenes. This mixture was added to various two polymer systems that were shaken for two days. Then, the two phases were separated and the thiophene concentration in both phases was determined to yield the partition coefficient. The partition coefficients are presented as the concentration in the top phase divided by the concentration in the bottom phase and are the average values of all the thiophenes detected in two parallel experiments.

Reactors

The reactors used in this study were conventional stirred tank reactors (Applikon). The working volume was 0.8 dm^3, the stirrer speed was 100 rpm and the temperature was kept at 24°C. Continuous aeration took place through a metal sparger at the bottom of the reactor. The reactors were run in a semi-continuous mode; every two days 100 ml of the reactor content was withdrawn and replaced by fresh medium or aqueous two-phase systems.

RESULTS AND DISCUSSION

Single phases

In preliminary experiments single polymer and salt phases (in a 2% concentration) were tested for their ability to sustain growth of the hairy root cells. The growth was followed by the 'weight loss' method (see Materials and methods), and the qualitative results of these experiments are presented in Table 6.1. In this phase of the work, only qualitative determinations were carried out. When there was a considerable weight loss (corrected for evaporation losses), combined with a visible growth, a '+' was assigned in Table 6.1 to the polymer or salt phase under investigation. It can be seen that all polymers except polyvinylpyrrolidone (PVP) supported growth. PVP is sometimes added at the initial stage of a suspension culture to absorb polyphenols excreted by the cells (e.g. Ketel et al., 1987). The concentration is then usually about 0.1% and it is only used for as short a time as possible because useful compounds are absorbed by PVP as well. This will probably be the reason for the

growth inhibition in this case. PVA formed a very viscous solution in the concentration required (7.5%), so it was not suitable for further use. The cells did not grow in any of the salt solutions at 2%. Since salts have to be used in the much higher concentrations of 0.5-2M (Albertsson, 1986) to obtain two phases, they have not been used for further studies.

Two phases

Growth and thiophene production were tested in two-phase systems consisting of two polymers. In Figure 6.1, the thiophenes found are presented. The biosynthetic pathway of these thiophenes is not

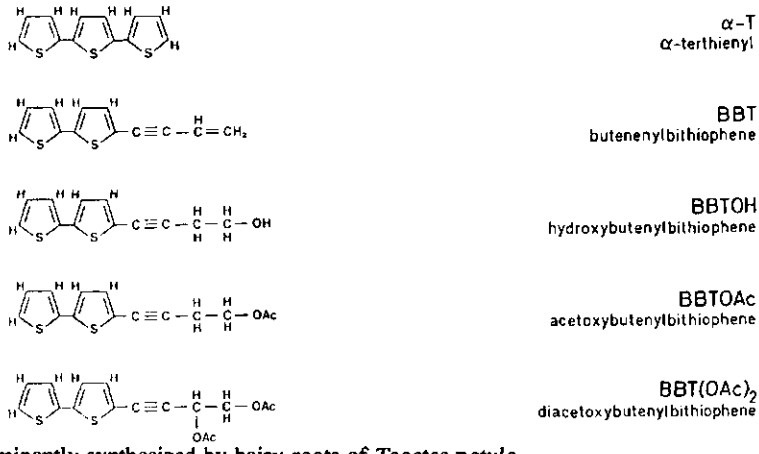

Figure 6.1
Thiophenes predominantly synthesized by hairy roots of *Tagetes patula*.

fully elucidated, and important knowledge on this point can be found in the book of Lam et al. (1988). As the top phase, several polyethyleneglycol (PEG) polymers were used, while the bottom phase contained Dextran or Reppal. In Figure 6.2, the results of experiments for growth and thiophene production in two-phase systems are presented. The cells were grown in 100 ml shake flasks with 40 ml medium. After 10 days, the cells were harvested and the dry weight was determined. The presented values are the average of three experiments. It can be seen that growth occurs in all combinations, and the highest growth rate was observed in the control medium. Thiophene production, expressed in μmol per gram fresh weight, was not different in medium than in the two-phase systems. Because of the small volume of each phase, it was not possible to detect the excreted thiophenes in a reliable way in this experiment, which is of course an indication for the very low amount of thiophenes that are excreted. The hairy root cells partitioned completely to the bottom, Dextran- or Reppal-rich, phase, in contrast to the thiophenes (see later on). In experiments

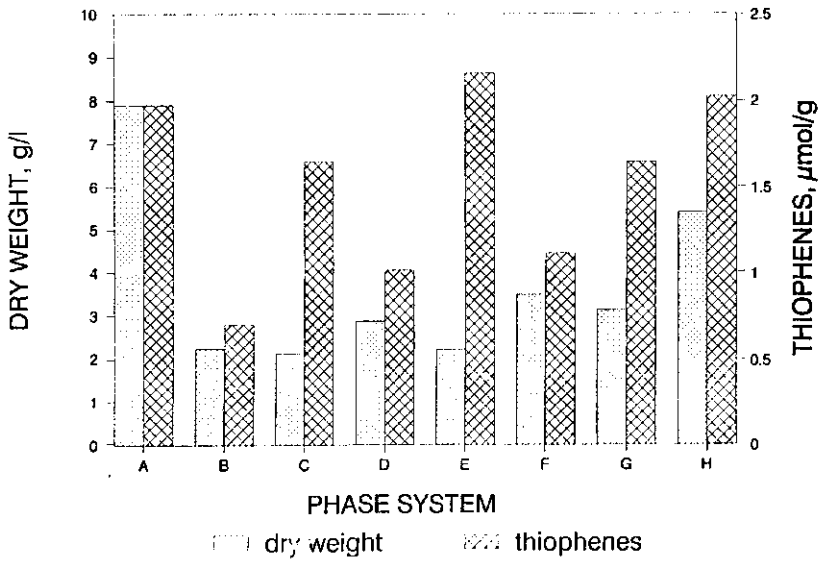

Figure 6.2
Growth (dry weight) and thiophene production by Tp9402 cells in various aqueous two phase systems. The thiophenes were predominantly found intracellularly (> 97% of the total amount of thiophenes).

A medium
B 15% PEG 6000/7.5% Dextran T-2000
C 15% PEG 10000/7.5% Dextran T-2000
D 10% PEG 35000/5% Dextran T-2000
E 10% PEG 35000/7.5% Dextran T-2000
F 15% PEG 6000/15% Reppal PES 200
G 15% PEG 10000/15% Reppal PES 200
H 15% PEG 35000/15% Reppal PES 200

with a suspension culture of *Nicotiana tabacum* cells, Hooker and Lee (1990) observed partitioning of the plant cell to the top phase and the interface, with a maximum of 25% of the total amount of cells in the bottom phase. With micro-organisms, partition of the cells occurs in the top phase, the interface or the bottom phase, depending on the species and the polymer composition of the aqueous two phases (Flygare and Larsson, 1989; Lee and Chang, 1989 and Kaul and Mattiasson, 1986).
The partition of thiophenes has been tested for several aqueous two-phase systems. In Table 6.2, these partition coefficients are presented. As can be seen, they can vary from 0.66 (slightly favouring the bottom phase) to 1.95 (favouring the top phase). Always, the partition coefficients for the more hydrophilic thiophenes (BBTOH, BBTOAc and BBT$(OAc)_2$) were slightly higher than those for the more hydrophobic thiophenes (BBT and α-T), (data not shown). A higher partition coefficient means that relatively more of the partitioned substance has moved into the upper, PEG-rich, phase. As PEG is more hydrophobic than the lower phases (Albertsson, 1986), the above results with the thiophenes

Table 6.2
Partition coefficients of a mixture of thiophenes between the two phases of some aqueous two-phase systems

Phase	Partition coefficient
	C_{top}/C_{bottom}
15% PEG 6000/7.5% Dextran T-2000	1.44
15% PEG 10000/7.5% Dextran T-2000	1.91
10% PEG 35000/5% Dextran T-2000	1.95
10% PEG 35000/7.5% Dextran T-2000	1.23
15% PEG 6000/15% Reppal PES 200	1.19
15% PEG 10000/15% Reppal PES 200	1.23
10% PEG 35000/15% Reppal PES 200	0.66

illustrate the fact that not only hydrophobicity, but also other factors like surface properties, size and the exposure of side-groups to the surrounding play a role. The PEG 10.000/Reppal PES 200 system was chosen for further work, because it was readily available and easy to use.

Reactor experiments

To test if the growth of *Tagetes patula* in aqueous two-phase systems could be scaled up, the hairy roots were grown in stirred tank reactors as well. One reactor was filled with 0.4 dm^3 of medium with 15% PEG 10.000 together with 0.4 dm^3 of medium with 15% Reppal PES 200 and the other reactor was filled with 0.8 dm^3 of medium and used as a control. The stirrer speed was set to 100 rpm, which had proved to be harmless for these cells (Buitelaar et al., 1990b). In both reactors, cell growth occurred. After about three weeks, the dry weight of the total amount of biomass was determined, yielding an average growth rate of 5.1 g/(dm^3.day) for the control reactor and 1.3 g/(dm^3.day) for the reactor with the polymer phases. So, like in the shake flask experiments, the growth in the two-phase systems was less than in medium. The thiophene content of the cells was similar in both reactors; about 2 μmol/g fresh weight. The excretion of thiophenes into the medium was 0.2 μmol/dm^3 in the control reactor and 2 μmol/dm^3 in the reactor with the polymer phases. Although this is a tenfold increase of the excretion, calculated on a volumetric scale (and a forty-fold increase

based on the excretion per gram cells), it is still only about 3% of the total amount of thiophenes produced. When these results are compared with other two-phase systems tested with *Tagetes patula* and thiophenes, it can be concluded that the excretion in the medium-organic two-liquid phase system and in the medium-solid two-phase system is higher (up to 50% of the total production), which is of course preferable (Buitelaar et al., 1990b, Canales et al., 1990).

CONCLUSIONS

Hairy roots of *Tagetes patula* can be grown in aqueous two-phase systems. Their growth rate is lower in two-phase systems than in standard medium. For this particular application (the production of thiophenes) other two-phase systems (aqueous/organic or aqueous/solid) are probably more suitable because of the hydrophobic character of thiophenes. However, this article has demonstrated that growth and secondary metabolite production with plant cells is possible in aqueous two-phase systems, and when hydrophilic substances (e.g. enzymes or other proteins) are to be produced, aqueous two-phase systems might be the systems of choice. The cells confined themselves completely to the bottom phase, while the thiophenes had a slight preference for the top phase. In the reactor system, the thiophene excretion in the aqueous two-phase system was ten times as high as in the fermenter with standard medium. However, this excretion was still only 3% of the total amount of thiophenes produced.

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ELICITATION OF THIOPHENE PRODUCTION BY HAIRY ROOTS OF *TAGETES PATULA*

BUITELAAR, R.M., CESÁRIO, M.T. AND TRAMPER, J.

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Chapter 7

ABSTRACT

Various biotic and abiotic elicitors were assayed for their effects on thiphene production by hairy roots *Tagetes patula*.

The biotic elicitors tested were extracts of the fungi: *Aspergillus niger, Fusarium oxysporum, Penicillium expansum* and *Phytophthora megasperma*, of the bacteria *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* and yeast extract. As abiotic stress factors were used: the addition of calcium ions to the medium, Ethephon (a precursor of ethene) and a high pH.

In general, the biotic elicitors were effective, although the extent to which elicitation occurred differed from elicitor to elicitor. The highest production increase was attained with the extract of *Aspergillus niger*, an increase compared to the control of 85%. Only yeast extract had no positive effect on the thiophene production.

The abiotic elicitors tested had only small effects on thiophene production: the calcium ions induced a slight increase, the Ethephon only had a negative effect on the production and the high pH yielded a temporarily high concentration but the roots were no longer viable after this treatment.

KEYWORDS

Tagetes patula, elicitation, secondary metabolites, plant cell cultures, hairy roots, thiophenes.

ABBREVIATIONS

cAMP: cyclic adenosine-monophosphate; DW: dry weight; FW: fresh weight; PAL: phenyl ammonia lyase.

INTRODUCTION

The production of secondary metabolites by plant cell cultures is potentially a very promising field. Plants are able to synthesize complex compounds, that can sometimes hardly or not be synthesized

in a chemical laboratory. A second way to obtain the desired compound would thus be to extract it from whole plants. In some cases, however, the supply of plant material may be limited, due to various factors. In that case, production by plant cell cultures could in principle be an attractive alternative. Worldwide, a great amount of effort is made to synthesize many different plant products in this way. Up till now, the commercial success of all these efforts has been very limited. One of the major reasons for this delay in commercialization is the low productivity of plant cell cultures. One of the highest productivities reported is that of rosmarinic acid by *Coleus blumei* cells (up to 20% of the dry weight of cells) (Petersen, 1989), but this value is more the exception that proves the rule. In general, productivities are too low to make a feasible economic process.

One of the methods that has been used to enhance the productivity is the use of so-called elicitors.. The term elicitors is used for compounds, of biological or non-biological origin, that cause an increase of phytoalexin production when applied to plants or plant cell cultures. Phytoalexins are in general antibiotically active compounds, which are accumulated by a plant (cell culture) in response to (microbial) attack.

Many different compounds have proven to be able to act as an elicitor. So-called biotic elicitors have a biological origin and are mostly cell wall extracts or filtrates from fungi, bacteria or yeast or purified fractions thereof. Abiotic elicitors, from non-biological origin, by some preferably referred to as 'abiotic stress factors' to reserve the term 'elicitor' for compounds of biological origin only (Eilert, 1987), can be heavy metals, UV-light, physical and chemical stress agents and so on.

One of the most intensively studied and commonly used biotic elicitors is the fungus *Phytophthora megasperma*. Several fractions prepared from this fungus induced an increase in the activity of the enzyme phenyl ammonia lyase (PAL), generally recognized as one of the key enzymes in the process of induction of secondary metabolite synthesis. Also, with *Glycine max* cell cultures, the production of the secondary metabolite glyceollin was increased upon addition of the elicitor (Ebel et al., 1976, 1989 and Ayers et al., 1976). Although this elicitor was active in many other plant cell cultures as well, there were some resistant species, like e.g. *Eschscholtzia californica*, at which this elicitor had no effect at all (Schumacher et al., 1987). Another commonly used elicitor is yeast (*Saccharomyces cereviseae*) or yeast extract. This elicitor is said to be similar in both structure and absolute elicitor efficiency to an elicitor isolated from the mycelial walls of *Phytophthora megasperma* (Hahn and Albersheim, 1978). With yeast extract, the production of berberine by *Thalictrum rugosum* cells was increased up to four times and the production of alkaloids by *Eschscholtzia californica* was increased about thirty times (Brodelius et al., 1989). PAL-activity was proven to increase upon addition of yeast to *Glycine max* and *Thalictrum rugosum* cell cultures (Funk et al., 1987). Other elicitors from

microbial origin that are commonly used for elicitation are among others: *Aspergillus niger, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Penicillium expansum* (van der Heijden, 1988). For *Tagetes sp.* and related plants, the biotic elicitors that have been tested are: *Botrytis cinerea, Cercospora lingustr* and *Pythium ultimum* (Mukundan and Hjortso, 1990a) , with an increase in production of about 100%; *Fusarium conglutinans*, which resulted in a 2-3 times higher yield of thiophenes (Mukundan and Hjortso, 1990b) and, for *Bidens sulphureus*, the fungi *Pythium aphanidermatum* and *Phytophthora dreschleri* were used with seedlings, which resulted in a production 20 times as high as without elicitor (Flores et al., 1988).

Of the abiotic stress factors, especially calcium is an interesting one. The evidence is growing that the calcium ion can act as a second messenger in the regulation of secondary metabolite production by plant cells. Kurosaki et al. (1987) were struck by the fact that many different elicitors provoked a similar phytoalexin production in cells of *Daucus carota*, indicating that this specific secondary metabolite production was mediated by so-called second messengers like Ca^{2+} and cAMP. They found that the omission of calcium from the medium resulted in a decreased 6-methoxymellein production, and concluded after numerous other experiments that calcium, together with cAMP, played a determining role in the production of secondary metabolites.

Ethene has also been suggested as an elicitor. For example, Cho et al. (1988) found that the addition of a precursor of ethene (Ethephon or 2-chloroethyl phosphonic acid) improved the production of berberine by *Thalictrum rugosum* cells by 30% and the production of caffeine by *Coffea arabica* cells with 85%.

The above shows that many examples exist of elicitors that increase the production of secondary metabolites, although no elicitor exists until now that has a general effect on different species. On the other hand, no way exists until now to predict whether or not an elicitor will be effective in a specific plant cell system.

In this article, the results of elicitation experiments with hairy roots of *Tagetes patula* are presented. Not many articles have appeared on the elicitation of hairy root cultures; most results stem from experiments with cell suspension cultures. Because of the aforementioned impossibility to predict the effectiveness of an elicitor, good results with other cell lines have been the basis for the selection of the elicitors, both from biological and non-biological origin.

The biotic elicitors tested nearly all gave a production increase, whereas the non-biotic elicitors were not very successful in this particular case.

Chapter 7

MATERIALS AND METHODS

Roots

The tissue culture used consisted of hairy roots, made by transforming *Tagetes patula* plants with *Agrobacterium rhizogenes* LBA 9402 (Tp9402). The roots were maintained in Gamborg's B5 medium (Gamborg et al., 1968) (pH 5.6), supplemented with (per dm^3) 30 g sucrose and 0.1 mg biotine. The roots were grown in 250 ml Erlenmeyer flasks, filled with 50 ml of this medium. The flasks were placed on an orbital shaker (100 rpm) in the dark. The temperature was about 24°C. The roots were subcultured every two weeks by inoculating fresh medium with about 10 root tips.

Fresh weight

The fresh weight of roots in shake flask experiments was determined by washing the roots three times with demineralized water to remove the medium salts, drying them for 10 minutes on filter paper and weighing them.

Dry weight

The dry weight of the roots was measured by drying a weighed amount of fresh, rinsed roots to constant weight in an oven at 105 °C.

Analysis of thiophenes

The thiophene content of the roots was determined according to Croes et al. (1989). An amount of roots was weighed and homogenized in the presence of 1 ml ethanol. The homogenizer was rinsed with 1 ml ethanol:water 1:1 and 1 ml water. To these 3 ml, (x) 6 ml of a mixture of hexane:butylmethylether 1:1 was added. Extraction was done by vortexing for 1 minute. This solution was centrifuged (10 min., 3000 rpm); the top phase (hexane:butylmethylether) was removed and the procedure was repeated from point (x). The two fractions of hexane:butylmethylether were pooled and evaporated under nitrogen flushing in a water bath of 40 °C. The residue was redissolved in 1 ml ethanol which was then used for HPLC analysis. The whole extraction procedure was executed under dim light conditions, to prevent the degradation of thiophenes. Medium was extracted directly with hexane:butylmethylether, according to the procedure starting at point (x). The HPLC was run under the following conditions: C18 column (Chrompack); eluens: acetonitril:water, 3:1; flow rate 0.7 ml/min.; detection at 340 nm.

Preparation of elicitors from fungal origin

The fungi used for elicitation were *Phytophtora megasperma*, six strains of *Fusarium oxysporum* (referred to, according to the suppliers, by F14, F15, F16, F17, F20 and F21), *Aspergillus niger* and *Penicillium expansum*. They were kindly supplied by the Departments of Phytopathology (*Phytophthora megasperma*), Food Microbiology (*Aspergillus niger* and *Penicillium expansum*), and Microbiology (*Fusarium oxysporum*) of the Agricultural University.

Phytophtora was grown on Erwin medium; the three other fungi were grown on Malt extract medium (Oxoid; 20 g/l). The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of the appropriate medium on a gyratory shaker at 30 °C. When the stationary phase was reached, the medium with cells was autoclaved (121 °C, 20 min) and the mycelium was filtered through Whatman no. 1 filter paper and washed several times with demineralized water. The mycelium residue was resuspended in an amount of demineralized water equal to that of the filtrate and homogenized. This homogenate was autoclaved again and used without further purification.

Preparation of elicitors from bacterial origin

The bacteria used for elicitation were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, all kindly provided by the Food Microbiology Department of the Agricultural University. All bacterial cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml Nutrient Broth medium (Oxoid; 13 g/l) on a gyratory shaker at 30 °C. The procedure for the preparation of the bacterial elicitors was equal to the one used for the fungal elicitors, with the only difference that the cells were separated from the medium by centrifugation.

Preparation of the yeast extract elicitor

A carbohydrate fraction isolated from yeast extract (Oxoid) was prepared by ethanol precipitation, as described by Hahn and Albersheim (1978). Fifty g of yeast extract was dissolved in 250 ml of demineralized water. Ethanol was added up to 80% (v/v). The precipitate was allowed to settle for 4 days at 6°C and the supernatant solution was decanted and discarded. The precipitate was redissolved in 250 ml of demineralized water, and the ethanol precipitation was repeated as described above. The second precipitate was dissolved in 200 ml of demineralized water, yielding the crude preparation that was used without further purification. This crude preparation was autoclaved for 20 min at 121°C.

Ethephon elicitation

An aqueous solution of Ethephon, a precursor of ethene, was supplied by Union Carbide, USA. The concentrations used were 0.05; 0.1; 0.2 and 0.35 g/l. Due to its volatility, the Ethephon solution was sterilized by filtration through a 0.2 μm filter.

Calcium

The influence of external calcium on the production of thiophenes was tested by varying the $CaCl_2$ concentration of the medium. The concentrations tested were 1.2 (standard B5); 50; 100; 250 and 500 mM $CaCl_2$. The calcium effect was tested as such, and also combined with 1% *Fusarium oxysporum* (F14) preparation.

pH

The influence of a pH increase on the thiophene production was tested as follows. Roots were grown in normal B5 medium for about 1 week. Then, by the addition of 1 M KOH, the pH was increased from 5.6 to 12. The thiophene production was followed for two hours by sampling roots and medium every 15 minutes.

Elicitor tests

All elicitor tests were performed in 100 ml Erlenmeyer flasks containing 25 ml of B5 medium, when appropriate supplied with biotic or abiotic elicitor in the concentrations mentioned in the separate descriptions. Initially, always approximately 0.5 g fresh weight of roots (young tips) was added to the autoclaved medium. For each data point, the contents of two or three replicate flasks were sampled and the average value reported (with $\pm$ SE values when three flasks were used).

Elicitor test optimization

With *Fusarium oxysporum* the application procedure for the elicitor tests was optimized.

The effect of elicitor concentration was tested by adding the extract of *Fusarium oxysporum* in the concentration of 0.2; 2 and 20% (v/v) and comparing the results with a control.

The influence of the incubation time was tested by adding the extract to the roots in two concentrations (0.2 and 2% (v/v)) and harvesting roots every day during a period of 5 days. The total amount of thiophenes in the roots and the medium was determined.

The root concentration might also have an influence on the response of the roots to the elicitor concentration. To test this, several concentrations of elicitor (1, 2 and 3% (v/v)) were added to several amounts of roots (from 1 to 60 g/l fresh weight (FW)) in the same growth phase, and the

production of thiophenes was determined.

At last, the effect of culture age on the response to elicitation was tested. The roots were subcultured in 250 ml Erlenmeyer flasks containing 100 ml of B5 medium and 0.5 g of roots (FW). The elicitor was added in a concentration of 0.9% (v/v) at different periods after inoculation (t = 0, 2, 8, 13, 15 and 22 days) and the roots were exposed to the elicitor during 2 days. Subsequently they were harvested and the thiophene content was determined.

RESULTS AND DISCUSSION

Selection of elicitors

Since there is still no way to predict which organism will result in elicitation (Eilert, 1987), the selection of possible elicitors has been made based on positive results from literature. *Fusarium oxysporum* had our special interest because of the results of Kourany et al. (1988), who managed to obtain a 12-fold increase in thiophene concentration in whole plants of *Tagetes erecta* by inoculating them with this fungus. The other microorganisms have been selected based on the results of van der Heijden (1988), Brodelius et al. (1989), Hahn and Albersheim (1978), Funk et al. (1987), Ayers et al. (1976) and Ebel et al. (1976, 1989). Abiotic elicitors were selected based on the literature data of Cho et al. (1988), Kurosaki et al. (1987) and Köhle et al. (1985).

Optimization of elicitation with *Fusarium oxysporum*

In a preliminary test, six *Fusarium oxysporum* strains were tested for their influence on the production of thiophenes by hairy roots of *Tagetes patula*. The controls were treated with the same amounts of medium or received nothing at all. Kourany et al. (1988) have shown that there can be a large difference between the effect of different strains of the same fungus. In our case, of the six strains tested, five resulted in an enhanced production of thiophenes (see Figure 7.1). The one with the highest production increase (F14) was used in further studies. With this strain, the elicitation procedure was optimized.

Four variables were tested: elicitor concentration, the contact time between roots and elicitor, the age of the root culture at the time of elicitor addition and the root concentration.

Elicitor concentration

Two types of dose-response curves are known for biotic elicitors. One shows a saturation pattern and the second type shows an optimum (Eilert, 1987). In both cases, it is important to find the best dose

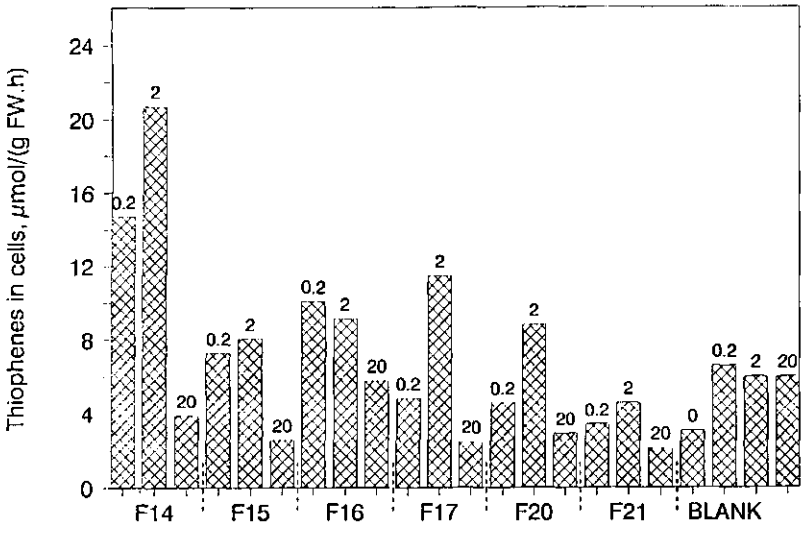

Figure 7.1
Average values (n=2) of thiophene production after elicitor treatment of Tp9402 with six different strains of *Fusarium oxysporum*, each in three different concentrations (0.2, 2 and 20% (v/v)). Blanks with 0, 0.2, 2 and 20% extra medium.

with the best response. In Figure 7.1, it can be seen that all *Fusarium oxysporum* strains except one show an optimum for a concentration of 2% (v/v). In this case, clearly the second type of response mechanism is prevalent. In Figure 7.2, again a dose-response curve with an optimum of 1% of elicitor is found. When Figures 7.1 and 7.2 are compared, two salient aspects can be seen. The

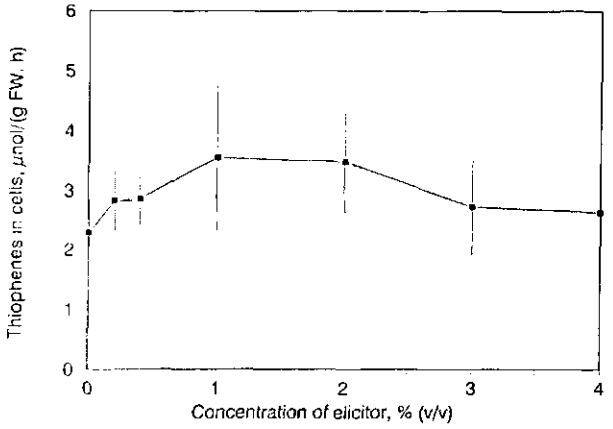

Figure 7.2
Thiophene production as a function of elicitor (*Fusarium oxysporum* F14) concentration by cells of Tp9402.

thiophene production in the blank is much higher in Figure 7.1 (about 6 μmol/g FW) than in Figure 2 (2.5 μmol/g FW). Besides, the increase (relative and absolute) of the thiophene production by *Fusarium oxysporum* F14 is much higher in Figure 7.1 than in Figure 7.2 as well. Variation in secondary metabolite production is often encountered and it is not yet clear what the mechanism behind this variation is or how it can be influenced. Apparently, the physiological state of the roots is an important factor for their susceptibility to elicitor treatment. It is clear however, that there is an optimum concentration between 1 and 2%.

Time of elicitor treatment

The incubation time of the elicitor can be an important factor. A minimal time is required to induce the process of elevated secondary metabolite production and an exposure that lasts too long could have harmful effects on growth and production. For two elicitor concentrations (0.2 and 2% (v/v)), the effect of incubation time on the thiophene production was tested. In Figure 7.3, it can be seen that

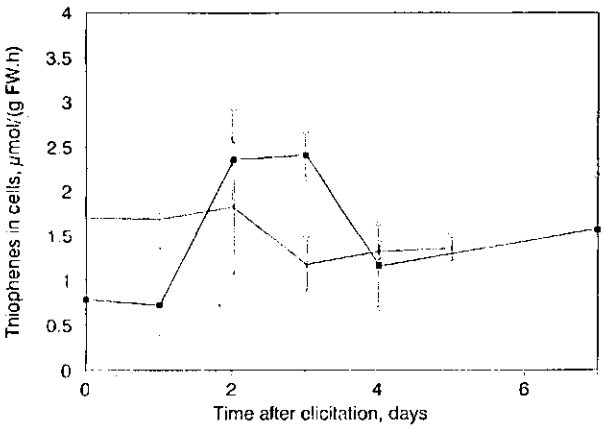

Figure 7.3
Thiophene production as a function of the incubation time. Elicitor: *Fusarium oxysporum* F14. ■: 0.2% elicitor; +: 2% elicitor.

both situations show an optimum at about 2 days of incubation. All the subsequent tests with other elicitors always had an incubation time of two days.

Root age

The response to elicitation is also dependent on the growth phase of the root culture. The growth stage of a culture may affect not only the quantitative respons to elicitation but also the product pattern (Eilert, 1987).

In Figure 7.4, the relation between growth stage and thiophene production can be seen. It is clear that

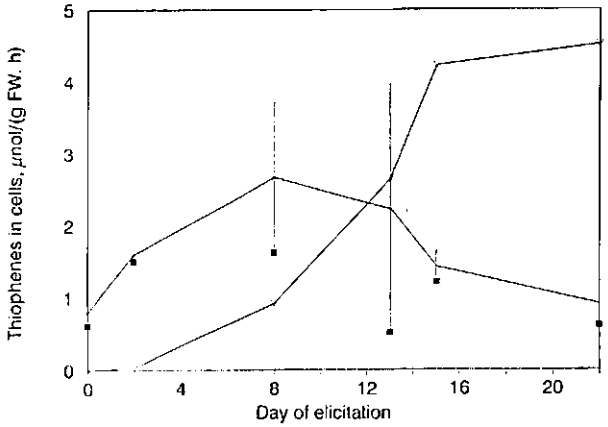

Figure 7.4
Effect of day of elicitation (*Fusarium oxysporum* F14) on the thiophene production by Tp9402 combined with a typical growth curve of Tp9402.

the optimal moment for elicitor treatment is around day 8, when the culture starts to grow more or less exponentially. There was no difference in the production pattern of thiophenes at the various days (data not shown). When comparing several of these studies with various plant species, Eilert (1987) showed that for most plant root cultures the best elicitation time was at the end of the growth phase. Most probably, this will be correlated with the fact that most secondary metabolites are produced in the stationary phase of cell growth, whereas the thiophenes are produced during the growth phase (Croes et al., 1989). Thus, it is plausible that the best time for elicitation in this case is at the onset of the exponential growth phase.

Root concentration

If the theory is true that receptor sites on the plasmamembrane recognize elicitor molecules (Yoshikawa et al., 1983), then the optimum elicitor concentration would change with the cell or root concentration. This dependence was tested for three elicitor concentrations (1, 2 and 3% (v/v)) and a blank with no elicitor. The results showed that there was no correlation between the amount of roots present and the amount of thiophenes produced for the root concentrations tested (data not shown). Whether this is due to the natural variation which is always present in secondary metabolite production or to the fact that the receptor site theory does not hold for this case, could not be decided

from this experiment.

So, the results of the optimization experiments with *Fusarium oxysporum* F14 were that optima exist for the production of thiophenes with Tp9402 regarding the elicitor concentration, the incubation time and the culture age at the moment of addition. No effect was found for the root concentration.

After these experiments, many other biotic and abiotic elicitors were tested.

Other biotic elicitors

The biotic elicitors tested are listed in Table 7.1. For all of them, dose-response curves were made to find the optimum elicitor concentration. All the biotic elicitors tested showed an increase in production, although the increase differed from case to case. All biotic elicitors tested yielded a dose-

Table 7.1
Biotic elicitors used, their optimal concentration and the production increase that was reached by their addition. The control values for all separate experiments were rather different (mean value about 6 μmol/g roots (FW)).

Micro-organism	Optimal concentration	Production of thiophenes
	% (v/v)	%
Control	-	100
Aspergillus niger	0.5	185
Fusarium oxysporum	1	155
Penicillium expansum	1.5	115
Phytophthora megasperma	1	135
Bacillus subtilis	2	150
Escherichia coli	1.5	135
Pseudomonas aeruginosa	1	135
Staphylococcus aureus	2	120
Yeast extract	0.4	105

response curve with a clear optimum. The spectacular production increase as shown in Figure 7.1 has never been reached again. From Table 7.1, the fungus *Aspergillus niger* proved to yield the highest

production increase of all the biotic elicitors tested, 85%. The results with yeast extract can hardly be seen as a production increase (only 5% more than the blank) and with all other concentrations tested (in the range of 0 to 8% (v/v)) there was only a decrease in the production of thiophenes. The results presented in Table 7.1 illustrate the variability of the effects of elicitor treatment (see Introduction).

Abiotic elicitors

The abiotic elicitors tested are listed in Table 7.2. Three types of abiotic elicitors have been tested: Ethephon (precursor of ethene), high pH and Ca^{2+} addition.

Table 7.2
Abiotic elicitors, their optimal condition and the production increase that was reached by their addition. The control value (100%) was about 3.25 μmol/g roots (FW).

Elicitor		Optimal condition	Production of thiophenes
			%
Control			100
Etephon		no increase	
pH 12		15 minutes	280
Calcium	- *Fusarium*	0.1 M	125
	+ *Fusarium*	0.1 M	120

When Ethephon was added to the roots, the production dropped dramatically, in contrast to the results of Cho et al. (1988), who found an increase in the production of alkaloids by *Coffea arabica* cells of 85%.

Changing the pH from 5.6 to 12 had a positive effect on the thiophene production. However, the roots were no longer viable after this treatment. Instead of beige, the roots turned into bright yellow-green after the addition of the KOH. After two hours of incubation, the cultures showed signals of necrosis. So, in spite of the high value reported in Table 7.2, this method is not very useful in this case.

The influence of calcium ions on the production of thiophenes was studied in the presence and the absence of a biotic elicitor (*Fusarium oxysporum* F14). It is postulated (Ebel et al., 1989), that calcium plays an important role in the elicitation process, and that the combination of Ca^{2+} with elicitors would have a synergistic effect. From Table 7.2, it is evident that in this case no such effect

occurs. The total production increase with and without elicitor is more or less equal and the production vs. calcium-concentration curve has exactly the same course for both cases.

CONCLUSIONS

In general, the conclusion of this work is that it is possible to elicit root cultures of Tp9402 by various biotic elicitors. The results with the abiotic elicitors were not very promising. For all individual cases, it is necessary to select the right conditions for optimal elicitation.

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Chapter 8

THE EFFECTS OF THE ADDITION OF XAD-7 AND OF ELICITOR TREATMENT ON GROWTH, THIOPHENE PRODUCTION AND EXCRETION BY HAIRY ROOTS OF *TAGETES PATULA*

BUITELAAR, R.M., LEENEN, E.J.T.M., GEURTSEN, G.,

GROOT, Æ. DE AND TRAMPER, J.

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Chapter 8

ABSTRACT

The effect of the addition of a solid adsorbent, XAD-7, on the growth of hairy roots of *Tagetes patula* and on their production and excretion of thiophenes is described in this article. Together with this, it was investigated whether or not the refreshment of the XAD and the medium makes any difference and finally, the combination of the above treatments with the addition of an elicitor (extract of *Aspergillus niger*) was tested for its effects.

The growth of the hairy roots was slightly increased by the addition of XAD, but slightly decreased by the addition of the elicitor. The specific production of thiophenes remained unaffected in most cases, only when XAD and elicitor where added together, an increase of the production was observed. The excretion of the thiophenes, on the other hand, was strongly influenced by the addition of XAD-7 to the cultures. Without this resin, there was no measurable excretion at all, while the presence of the XAD raised the excretion to 50% of the total thiophenes produced.

When these experiments were extended to bubble columns, the same results came out, be it that there was a surprising difference between the reactor configuration with the XAD present in the medium in the reactor and the reactor configuration with an external column containing the XAD, through which the medium was constantly circulated. In the former, about 20% of the total production was excreted, while in the latter no thiophenes could be detected outside the hairy roots.

INTRODUCTION

The production of secondary metabolites with plant cell cultures is a very promising field. The range of known products produced by plants is enormous, while until now only a small portion of the wealth of plant products has been determined. The application of plant cell cultures for the production of these compounds is still in a very early phase. Although some commercial processes exist, there are a large number of possibly commercial compounds that have not reached the market yet. There are a few, generally accepted reasons for this delay. The most important one is the low productivity of the plant cells in culture. It is not unusual that the productivity, per gram cells, drops dramatically when callus is formed from plant parts, and again when a suspension culture is started from callus culture. Another drawback is the slow growth of the plant cells in culture, combined with the fact that most plant cell products are stored intracellularly. This makes it inevitable to destroy the biomass that has been obtained so laboriously. If it would be possible to force the cells to excrete their products

combined with preservation of the cell viability and production capacity, the chances for plant cell culture based products would become much better.

There are, in the literature, some interesting examples of the combination of increased excretion and increased production when a second phase was added to the plant cell cultures. These second phases can be either liquid or solid. Organic liquid phases that are often used as a second phase in plant cell cultures with good results are hexadecane and Miglyol. Kim and Chang (1990) obtained a 7.4 times increase in the production of shikonin with cell cultures of *Lithospermum erythrorhizon* upon addition of hexadecane. Buitelaar et al. (1991a) were able to increase the excretion of thiophenes by hairy root cultures of *Tagetes patula* from 0% to 70% when a two-phase system with hexadecane was used instead of a single, aqueous phase. As a solid second phase, the Amberlite resin XAD-7 is the one that is used most. Its adsorption properties are not always the best when a number of solid second phases are tested, but it has few negative effects on the cells and it has attractive hydrophilic properties, in contrast to e.g. other XAD resins. An example of the profitable use of this resin is given by Robins and Rhodes (1986), who realized a 15-fold increase in the production of anthraquinones by cell cultures of *Cinchona ledgeriana* when XAD-7 was added to the cultures.

Another method to improve the production of plant cell cultures is the use of elicitors. Although the choice of the appropriate elicitor is still a matter of trial and error, many authors succeed to find a suitable elicitor for their specific application. For an overview of the elicitor effects, see e.g. Eilert (1987) or Buitelaar and Tramper (1991).

The combined effect of the abovementioned treatments can be synergistic, as was demonstrated by Asada and Shuler (1989). They found a synergistic effect of XAD-7 addition, elicitation with autoclaved cultures of *Phytophthora cactorum* and immobilization in calcium alginate, when applied to cell cultures of *Catharanthus roseus*. They obtained a 45-times production increase of ajmalicine when they combined these three treatments.

In the study described in this article, the influence of the addition of XAD-7, of the refreshment of XAD and medium and of the addition of an elicitor on the production and excretion of thiophenes by hairy root cultures of *Tagetes patula* was investigated in shake flasks and fermenters.

MATERIALS AND METHODS

Cells

The cells used were hairy roots, made by transforming *Tagetes patula* plants with *Agrobacterium*

rhizogenes LBA 9402 (Tp9402) (Ketel, 1987). The roots were maintained in Gamborg's B5 medium (Gamborg, 1968), pH 5.6, supplemented with (per dm^3): 30 g sucrose and 0.1 mg biotine. The cell cultures (50 ml) were placed on an orbital shaker (100 rpm) in the dark in 250 ml Erlenmeyers flasks. The temperature was about 24°C. The roots were subcultured every two weeks by inoculating fresh medium with about 10 root tips.

Second phase

The solid adsorbent used was XAD-7, a neutral polycarboxylic ester. 'Amberlite' XAD-7 was washed in methanol, rinsed in Millipore water and dried. Prior to use, the desired amount of XAD was weighed into a dialysis bag and autoclaved in medium before use, to prevent the undesired adsorption of medium components when the XAD was added to the medium with roots. The diameter of the XAD beads was 0.3-0.78 mm.

Elicitor preparation

The fungus *Aspergillus niger* was obtained from the Department of Food Microbiology of the Agricultural University Wageningen. It was maintained on plates with 12 g/l malt agar (Oxoid). Larger amounts of *Aspergillus niger* were grown in malt extract medium (Oxoid, 20 g/l) in shake flasks with 50 ml medium on a rotary shaker (100 rpm) at room temperature. After 7 days (end of the logarithmic phase) the cell suspension was autoclaved, and filtered (on Whatman no. 1 filter paper). The mycelium was washed several times with demineralized water and suspended in 50 ml water. This mixture was homogenized, autoclaved again and used without further purification.

Biomass determination

Wet weight

Before determining the wet weight of the roots, any possible remaining medium, solvent or XAD were rinsed away with demineralized water. The roots were left to dry on filter paper for 10 minutes, after which the wet weight was measured.

Dry weight

After the pretreatment used for the wet weight determination, the roots were dried to constant weight in an oven of 105 °C, and the dry weight was determined.

Conductivity

The decrease in the conductivity of the medium can be used as an indirect measure for the increase

of the biomass concentration. In earlier work (Buitelaar et al., 1991a) a calibration curve was determined to relate these two phenomena, resulting in the following equation:

$$\Delta X = A * \Delta K$$

with ΔX increase in biomass, g/dm^3

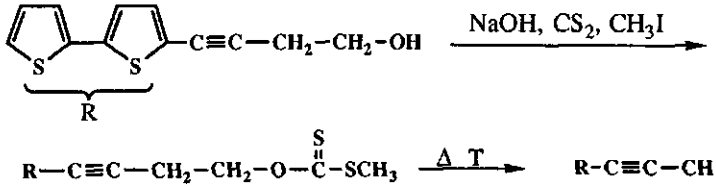
A constant, $g.cm/(dm^3.mS)$

ΔK decrease in specific conductivity, mS/cm

From the experiments with Tp9402, a value for A of 3.9 $g.cm/(dm^3.mS)$ was found and used in the measurements described in this paper.

Thiophene synthesis

The synthesis of 5-(4-hydroxybut-1-ynyl)-2,2'-bithienyl (BBTOH) and 5-(4-acetoxybut-1-ynyl)-2,2'bithienyl (BBTOAc) were carried out as described by Rossi et al. (1984). A straightforward synthesis of 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT) was planned via a Chugaev reaction of BBTOH (de Groot et al., 1968). The first step in this reaction is the formation of methyl xanthate of BBTOH, followed by a thermal decompostion of this compound at high temperature. The reaction of BBTOH with carbon disulfide and methyl iodide to the methyl xanthate was carried out at room temperature in DMSO as a solvent (de Groot et al., 1968), using powdered NaOH instead of NaH as a base.



After the regular workup of the reaction mixture, no xanthate could be detected and unexpectedly BBT was obtained directly. Apparently the decomposition of the xanthate of BBTOH is very fast and the usual high temperature is not necessary in this case. However, the so obtained BBT was contaminated with dimethyl sulfide which was not desired for our specific purpose leading to a very troublesome purification procedure. Therefore, pure BBT was finally synthesized according to method B as described by Rossi et al. (1984).

Adsorption studies with thiophenes

To determine the adsorption behaviour of thiophenes in the presence of XAD, 7.5 mg of XAD was added to 15 ml of solutions of α-T, BBTOAc, BBT and BBTOH, in concentrations ranging from 0-300 μmol/dm^3. The shake flasks containing the solutions were placed on a shaker for two days, after which the amount of thiophenes adsorbed onto the resin was determined as described below.

Feedback inhibition

The extent to which feedback inhibition of thiophene production occurred with roots of *Tagetes patula* was tested by adding thiophenes to Tp9402 hairy roots. The thiophenes added were either BBT or BBTOH, in concentrations ranging from 0 to 0.5 μmol/dm^3. After two days, the thiophene content of the roots and the medium was determined.

Analysis of thiophenes

The extraction and analysis of thiophenes from cells and medium has been described before (Buitelaar et al., 1991a). The thiophene content of the XAD was determined by adding 15 ml of ethanol to a dialysis bag (from which most of the water had been removed) with XAD and extracting the XAD for two days on a shaker. For a dialysis bag from a fermenter system, which contained much more XAD, 100 ml of ethanol was used. Five ml of the ethanol was pipetted into a tube and evaporated under nitrogen flushing in a water bath of 40°C. The residue was redissolved in 1 ml ethanol and used for HPLC analysis. The HPLC was run under the following conditions: C18 column (Chrompack); eluens: acetonitril:water, 3:1; flow rate 0.7 ml/min.; detection at 340 nm. All values presented are the average of duplicate or triplicate experiments.

XAD experiments

Shake flasks with 20 ml of medium were inoculated with Tp9402 roots. Apart from the controls, all flasks were supplied with a dialysis bag containing 0.2 g of XAD-7. After 1 week, one third of the flasks with XAD received fresh, clean XAD and one third received fresh XAD and fresh medium. Every 2 to 3 days, 2 flasks were used to determine the cell wet and dry weight and the thiophene content of the XAD, the roots and the medium.

Elicitor experiments with XAD

Shake flasks with 20 ml of medium and a dialysis bag containing 0.2 g XAD-7 were inoculated with

Tp9402 roots. Eight days after inoculation, 0.2 ml of elicitor preparation was added to the cultures. Every 2 to 3 days, 2 flasks were used to determine the cell wet and dry weight and the thiophene content of the roots, medium and XAD. The control cultures did not receive the elicitor preparation.

Fermenter experiments

The fermenters used for the larger-scale experiments were all bubble columns, with a volume of 1.8 dm^3. Aeration of the fermenters took place through a sintered glass plate at the bottom of the reactors. After autoclaving the reactors and the medium, 1.5 dm^3 of medium was added to the fermenter and aeration was started. As a sterility test, the aerated fermenter was always run for a few days without cells to be sure that sterility was achieved. In order to 'immobilize' the hairy roots, to prevent them from blocking the tubings, they were initially introduced in the reactors inside a sort of cage made of stainless steel. For inoculation, the roots were cut into very small pieces and transferred to a flask that was connected to the stainless steel cage inside the reactor through wide silicon tubing. In this way, the roots entered the reactor inside the cage. The pores of the stainless steel were wide enough to permit the roots to grow out of their cages. When XAD was used as the adsorbent, 5 g of XAD was weighed into a dialysis bag and this bag was placed in an external column, through which the medium was circulated continuously, or the bag was placed in the medium inside the reactor. Duplicate runs were performed with four systems: a control, a system with XAD present internally, a system with XAD present externally and a system with XAD present internally that received an elicitor treatment two days before the end of the run. Samples from the roots could not be taken during the fermenter run, but only at the end. Samples from the medium were taken regularly to determine the medium conductivity and the thiophene content. Samples from the XAD could only be taken when the XAD was replaced. In one system, with the XAD internally, the XAD was not replaced, so samples could only be taken at the end of the fermenter run. The thiophene contents of the roots, the medium and the second phase were determined.

RESULTS AND DISCUSSION

Feedback inhibition

Feedback inhibition is a common phenomenon in cell metabolism. This can be a problem when it is the aim to use the cell as a biocatalyst to form a product which shows feedback inhibition. To alleviate this problem a second phase, either liquid or solid, can be used to extract or adsorb,

respectively, the product either directly from the cell when stored intracellularly or from the surrounding aqueous medium when excreted. Until now, we never had the opportunity to test if thiophenes show feedback inhibition, because the only commercially available thiophene, that is also produced by the *Tagetes patula* hairy roots, is α-T. The other thiophenes (predominantly BBT, BBTOAc and BBTOH) cannot be bought and the purification of amounts large enough out of plant cells to test this feedback inhibition is rather cumbersome. To solve this problem, the Department of Organic Chemistry of the Agricultural University produced the three desired thiophenes via chemical

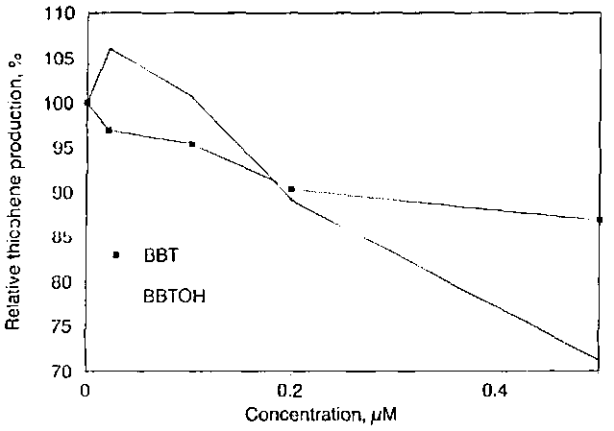


Figure 8.1
Feedback inhibition of thiophene production by hairy roots of *Tagetes patula*.

synthesis. With two of the thus obtained thiophenes (BBT and BBTOH), feedback inhibition studies were carried out with Tp9402 hairy roots. As can be seen in Figure 8.1, the presence of the thiophenes in the medium causes a decrease in the total amount of thiophenes synthesized. The spreading between the triplicate values was usually between 10 and 15% of the average value reported. The results in Figure 8.1 indicate that in situ extraction of the product is desirable. Therefore, in this study, the ion exchange resin XAD-7 was tested as an adsorbent to remove the product from the cells and the medium.

Adsorption to XAD

In order to find out whether the use of XAD was worthwhile in this case, the adsorption of the various thiophenes onto the XAD-7 was studied. This could only be done with three of the four available thiophenes (BBTOH, BBTOAc and α-T), because the fourth thiophene (BBT) was not

soluble in the concentrations as used here. In Figure 8.2, the results of these experiments are shown and it is clear that thiophenes do adsorb on XAD-7.

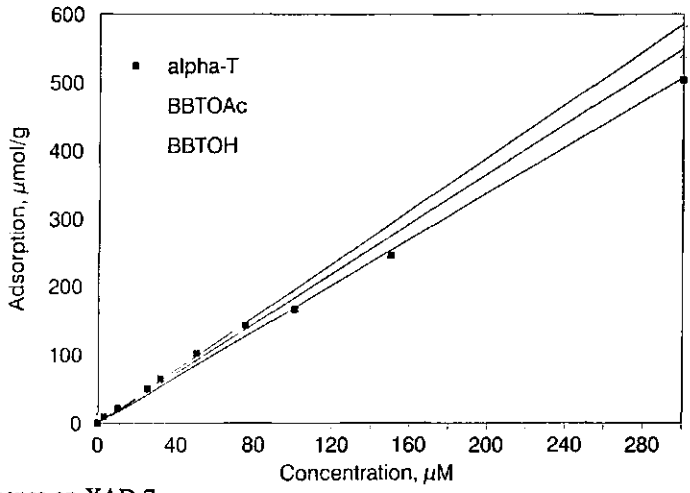


Figure 8.2
Adsorption of thiophenes on XAD-7.

Influence of XAD

The growth of the hairy roots, the production of thiophenes and their excretion were examined in the presence and absence of XAD-7. Together with this, the influence of refreshing the dialysis bags with XAD-7 or of refreshing the medium together with the dialysis bags, was also tested. Among others, Asada and Shuler (1989) obtained significant improvements in the total production of secondary metabolites when they refreshed the XAD repeatedly. We had observed regularly that the addition of fresh medium resulted in an increase of the growth rate during the first few days (data not shown). Therefore, the effects of the refreshment of XAD and medium were tested; growth might be stimulated and with that the volumetric productivity.

Now, a slight increase in growth was observed when XAD was present, as can be seen in Figure 8.3A. In the Figures 8.3A-8.3C, the spreading around the average value reported was usually between 10 and 15%. In contrast to the results presented in Figure 8.3A, Robins and Rhodes (1986) found a decrease in the growth of their *Cinchona ledgeriana* cells, when they added XAD-7 to the medium. At the concentration we used (10 g/dm^3) they already found a 35% decrease in growth rate. This is an indication that still no general rules exist that apply to plant cell cultures from different cell lines; for every new plant cell culture, the optimal circumstances have to be found.

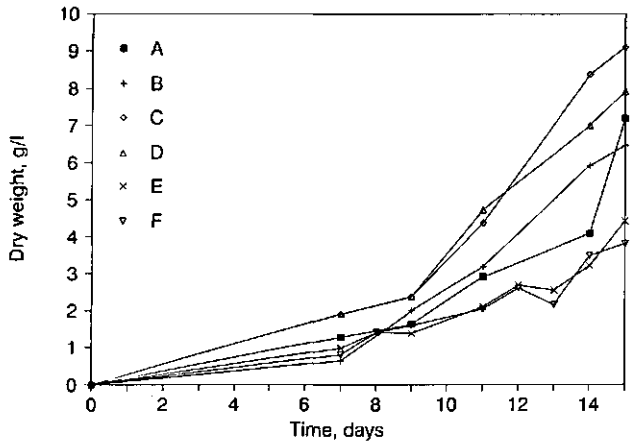


Figure 8.3A
Growth of hairy roots of *Tagetes patula* in the absence/presence of XAD-7 and an elicitor preparation of *Aspergillus niger*. A = blank, B = with XAD, C = with XAD refreshed at day 7, D = with XAD and medium refreshed at day 7, E = with elicitor added at day 8 and F = with elicitor and XAD.

The specific production of thiophenes was not significantly affected by the introduction of XAD, see Figure 8.3B. Only line B (XAD added, no refreshment) appears to be slightly higher in the beginning of the experiment. A stimulation of the production was found by other workers when they applied XAD-7 to their cultures. E.g. Payne et al. (1988) could increase the alkaloid production by *Catharanthus roseus* cells with about 40% by addition of XAD-7. The excretion of the thiophenes, however, as shown in Figure 8.3C, is stimulated substantially upon addition of XAD-7. In the control experiments, no thiophenes were found in the medium; all the thiophenes produced were stored intracellularly. With XAD-7, up to 40% of the total thiophenes produced was excreted. Naturally, this effect is of great importance for the down stream processing of the product. In none of the experiments (with or without XAD or elicitor), thiophenes could be detected in the medium.

Effect of elicitor

In addition to the effect of XAD-7, the results of the presence of an elicitor were tested. In previous experiments (Buitelaar et al., 1991a) the extract of the fungus *Aspergillus niger* had proved to give the best results for the production of thiophenes. In this case (Figure 8.3A) the growth of Tp9402 seems to be retarded when the elicitor was added. When we look at the specific production of thiophenes, however (Figure 8.3B), the combination of XAD-7 with elicitor (line F) seems to give

a somewhat higher production than the other cases. Robins and Rhodes (1986) also found a decrease in growth rate of their *Cinchona ledgeriana* cells, but the total production of anthraquinones was stimulated when XAD-7 was added to the medium.

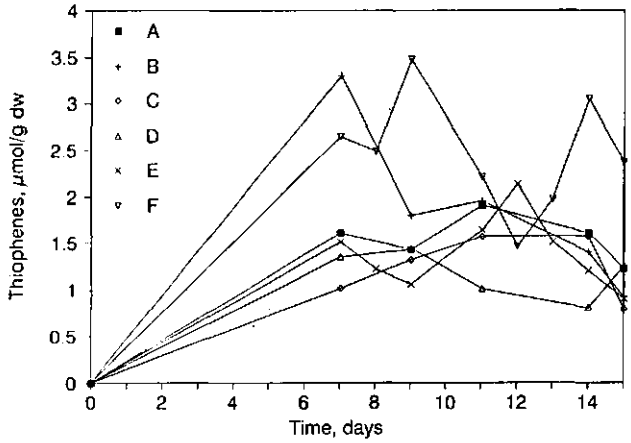


Figure 8.3B
Specific thiophene production by hairy roots of *Tagetes patula* in the absence/presence of XAD-7 and an elicitor preparation of *Aspergillus niger*. For legend, see Figure 8.3A.

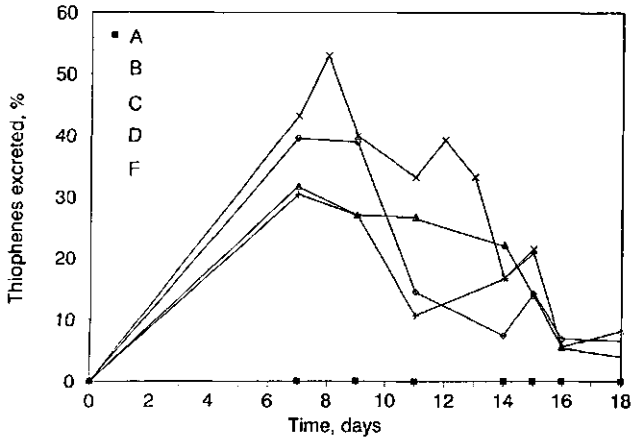


Figure 8.3C
Excretion of thiophenes by hairy roots of *Tagetes patula* in the absence/presence of XAD-7 and an elicitor preparation of *Aspergillus niger*. The results with only elicitor addition are equal to the blank. For legend, see Figure 8.3A.

The excretion of the thiophenes was again only promoted to over 50% when XAD-7 was present (Figure 8.3C). When only elicitor was added, the excretion remained zero, like in the blank. When a large number of elicitors was tested (Buitelaar et al., 1991b) for their influence on the thiophene

production and excretion, none of them stimulated the excretion of the thiophenes, although the total production was increased in most cases. The general trend that is seen in this study, is comparable to what was seen when similar experiments were carried out with hexadecane as a liquid second phase (Buitelaar et al., 1991a). In that case, no production increase was observed, but the excretion of the thiophenes produced raised from < 1% for the control to about 70% when hexadecane was added. So, both XAD-7 and hexadecane can be advantageous when the recovery procedure of the thiophenes is to be facilitated.

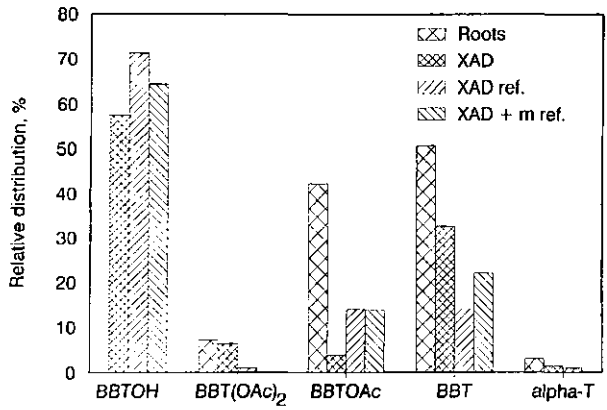


Figure 8.4
Relative thiophene distribution in the roots and on the adsorbent XAD-7 after 14 days of growth. Legend: Roots = thiophene distribution found in the roots in all cases; XAD = thiophene distribution found on the XAD in the cultures with XAD added; XAD ref. = thiophene distribution found on the XAD in the cultures with XAD refreshed at day 7 and XAD + m ref. = thiophene distribution found on the XAD in the cultures with XAD and medium refreshed at day 7.

Distribution of thiophenes

The situation that not all compounds produced by plant cell cultures are equally desirable is very likely to occur. In such a case, the selective adsorption of some of the products could be favourable. Payne and Shuler (1988) tested the selective adsorption by XAD of compounds from an ajmalicine/serpentine mixture, and they found that those two compounds showed different adsorption behaviour. When we used hexadecane as the second phase, a remarkable shift was observed from the thiophene distribution in the cells to the thiophene distribution excreted into the hexadecane. In the cells, BBT and BBTOAc were by far the predominant thiophenes, whereas in the hexadecane $BBT(OAc)_2$ was the most abundant thiophene (Buitelaar et al., 1991a).

A similar result is observed in this case with XAD. In Figure 8.4 the thiophene distribution is shown,

in the roots and on the XAD. It is obvious that in the cells, like always, BBT and BBTOAc comprise over 90% of the thiophenes present, while BBTOH could not be detected at all. On the XAD-7, on the contrary, BBTOH forms up to 70% of the thiophenes present. It must be said that the total amounts of thiophenes present (100% in the graph) when these experiments were done were about 250 nmoles in the cells and about 30 nmoles on the XAD-7, but still, it is surprising that no BBTOH at all is present in the roots, while it is present in considerable amounts on the XAD, when this resin was present. Again, this is a clear indication that two-phase cultures can be used for the selective workup of mixtures of compounds produced by plant cell cultures.

Fermenter studies

A typical growth curve of the Tp9402 roots in a 1.5 dm^3 bubble column is shown in Figure 8.5. With hairy root cultures in bioreactors, one of the main problems is the impossibility to take samples from the biomass in the fermenter. For biomass determination this problem has been solved satisfactorily by the introduction of the measurement of the medium conductivity. (Yoon et al. 1988 and Taya et al., 1989). For the determination of intracellular product, it is still necessary to sample the biomass itself, since all our attempts to find a correlation between the amounts of thiophenes in the cells and the amounts of those excreted into a second phase did not yield reliable results. Because this implies

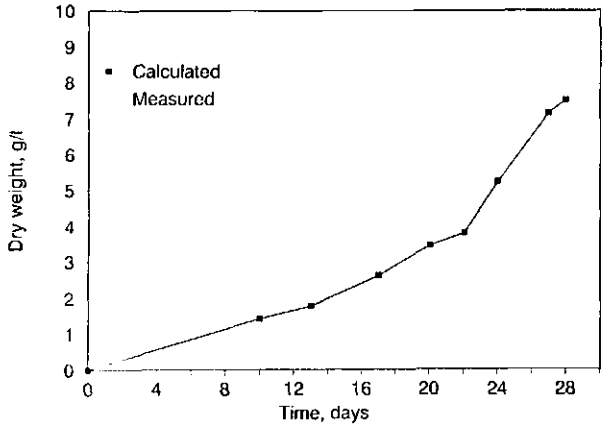


Figure 8.5
Typical growth curve of hairy roots of *Tagetes patula* in a bubble column. (+ measured at the end of the run; ■ calculated from conductivity measurements)

the rather risky action of opening the fermenter during the run, this was not done. This means that no information is available on the course of the thiophene content in the roots during their growth in

the reactor. At the end of the reactor runs, the thiophene content of the cells varied between 700 and 800 nmol/g DW, whether or not XAD-7 and/or elicitor were present. There was a consistent difference in the excretion pattern of the cells between the reactor configuration with the dialysis bag inside the medium in the fermenter and the configuration with the bag in an external column with constant circulation of the medium through it. In both cases, the cells and the bags were not in physical contact with each other. Always, in the 'internal' case, about 20% of the total amount of thiophenes was excreted and adsorbed onto the XAD, while no excretion could be detected in the 'external' case. To the authors' knowledge, this phenomenon has not been described before, and no logic explanation is available at the moment.

CONCLUSIONS

The presence of thiophenes in the aqueous medium inhibits the thiophene production by hairy roots of *Tagetes patula*.

XAD-7 can be used to adsorb thiophenes.

The addition of XAD-7 to the growth medium does not influence the growth of the hairy roots of *Tagetes patula* nor their production of thiophenes to a great extent.

The excretion of thiophenes goes from 0% to about 40% upon addition of XAD-7 to the medium.

The combined addition of XAD-7 and of the extract of the fungus *Aspergillus niger* as an elicitor seems to result in a higher production of thiophenes. Excretion in this case is always dependent on the presence of XAD-7.

The thiophene distribution in the hairy roots is different from that on the XAD-7.

When the hairy roots are grown in a bubble column, excretion of the thiophenes only takes place when the XAD is present in the medium in the column; not when it is present in an external column through which the medium is constantly circulated.

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At the time when the work described in this thesis started, the only cells available in our group were suspension cells of *Tagetes minuta* and of *Tagetes patula*. The *Tagetes minuta* cells, although easy to handle, were as such not suitable for our ultimate aim: i.e. the production of thiophenes, because they did not produce any thiophenes at all in liquid culture. The *Tagetes patula* suspension cells were very difficult to grow and produced only a small amount of thiophenes, about 10 μg per g fresh weight. In order to reach the realization of our aim, the influence of several techniques on the production of thiophenes was evaluated in this thesis. Most of the techniques used had proven their value in some other cases in plant cell biotechnology. Immobilizing the cells was one of those techniques and therefore, the *Tagetes minuta* cell suspension was used in the immobilization study described in chapter two, together with other cell types (bacteria, yeast, insect cells). This chapter can be seen as an introductory chapter for the work with organic solvents and immobilization and also as a connection to the work of A.C. Hulst, the predecessor of the author of this thesis.

The use of organic solvents to improve the total production and excretion of the secondary metabolites, for the selective removal of the desired product and for the integration of production and downstream processing, is an important aspect of this thesis. Therefore, in chapter three, the influence of organic solvents on the activity of the *Tagetes minuta* cells is described. However, good growing and good producing cells were more desirable. At that time we could obtain hairy root cultures of *Tagetes patula*, produced by other members of the NOVAPLANT group. Those hairy roots grew very well, and their production was about 100 times as high as that of the *Tagetes patula* cell suspensions. Therefore, all further experimentation was done with these hairy root cultures. Apart from the advantages mentioned above, hairy roots have the disadvantage that they grow as one clump of interconnected roots. This means that no representative samples can be taken from hairy root cultures in bioreactors. Because quantitative data on the amount of biomass present are a prerequisite to evaluate the performance, indirect ways for this biomass determination had to be found. This is described in chapter four. With the hairy root cells, we tested the influence of various environmental factors in our search for better production and excretion. The effect of product removal from the cells was tested; in chapter five by adding organic solvents to the hairy roots cultures; in chapter six by growing the hairy roots in aqueous two-phase systems and in chapter eight by adding a solid adsorbent to the cultures. All these approaches yielded a higher excretion percentage (up to 70%) of the total thiophene production, which remained unaffected itself. Another, biological, approach is described in chapter seven, where the effect of elicitor treatment on the hairy root cultures is shown. In this case, production improvements were reached by a factor 1.85.

There are more ways that may lead to improvement of cell culture growth or production and excretion of secondary metabolites. Examples are cell line selection, or genetic engineering methods. These approaches have not been used in this thesis work, because the other, more biologically-oriented members of NOVAPLANT are better equipped to do this part. The integration of biological and technological approaches is essential in the case of plant cell biotechnology. Technological solutions for the specific demands of plant cell cultures are needed, but physiological knowledge in this field must be improved too, because it is often stated that at this time, 'the real limitation lies in our lack of understanding the basic physiology and biochemistry of plant cell systems', as was written by Fowler in 1988.

In plant cell biotechnology research, still many phenomena remain unexplained. As can be seen in this thesis for example, the variation in e.g. productivity of the cell line, can be quite large, even when all environmental factors seem to be unchanged, a phenomenon quite generally encountered. The reproducibility of experiments with plant cell cultures is sometimes very low. In this thesis, a striking example of this variability can be seen when the chapter seven and eight are compared. In chapter seven, the elicitation with *Aspergillus niger* resulted in a significant improvement of the production, while the same treatment did not give a similar improvement in chapter eight. Between those experiments, the only difference was the number of subcultures that the (plant and fungal) cells had had. This, up to now unexplained, variability makes working in plant cell biotechnology not easy. On the other hand, trying to understand what happens and why and trying to get control over these processes is a major challenge, that will render great satisfaction when solved.

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SUMMARY

In this thesis, the results are presented of several approaches to improve the production and excretion of thiophenes by cell cultures or hairy roots of *Tagetes spp.*

In chapter one, most of the techniques to improve the production and/or excretion of secondary metabolites with plant cell cultures are discussed in a literature survey. From this chapter, it becomes clear that to date no general rules exist that apply for all plant species. The methods described in this chapter are the introduction of transformed cells in the form of hairy roots, the use of elicitors, the immobilization of plant cells, the use of two-phase cultures and the use of selected fermenters; all have as aim to improve the growth, production and excretion conditions for the plant cell cultures. All these methods have also been tested in the work described in this thesis, with the aim to obtain a higher production of thiophenes for less costs (meaning faster growth and better excretion).

Chapter two is the description of a side-step towards the extension of an immobilization technique for large scale operation that was introduced a few years before, the resonance nozzle technique. Until then, it had only been tested with alginate, but in this chapter, κ-carrageenan, agar and gellan gum were tested as well. The gel formation with these materials is improved when the drops are caught in an organic phase, so the reactions of four cell types (bacteria, yeast, plant cells and insect cells) to several organic solvents and the immobilization were examined. The various cell types appeared to react differently to the applied conditions, but in general hydrophobic solvents proved to be more suitable than hydrophilic solvents.

The latter phenomenon was elaborated in more detail in the third chapter. Cells of *Tagetes minuta*, in free or immobilized state, were grown in the presence of various organic solvents and their respiration activity was followed. The introduction of organic solvents can have advantageous effects, like the selective removal of the product from the cells or the aqueous phase, thereby combining production and downstream processing, while the removal of the product can relieve product inhibition effects. There appeared to be a relation between the hydrophobicity of the solvent and the residual activity of the cells - the more hydrophobic the solvent was, the more active the cells were in its presence. The immobilization of the cells provided a slight protection against harmful solvents. Although these *Tagetes minuta* cells were easy to work with (a fast growing, fine cell suspension), we changed to hairy root cultures of *Tagetes patula*, transformed with *Agrobacterium rhizogenes* LBA 9402, because those cells did produce thiophenes, in contrast to the former. These hairy root cultures are more difficult to work with, because they grow as one clump of interconnected roots, so circulation is difficult and samples cannot be taken. Therefore, in chapter four methods are compared that determine the hairy root biomass present in a fermenter in an indirect way. From the three

methods extensively tested, i.e. volume/weight balance, oxygen consumption and conductivity measurement, the latter proved to be the simplest and easiest to handle when the roots were in good condition.

In chapter five, the sensitivity of the hairy roots to organic solvents is described. The results were comparable to those obtained with the *Tagetes minuta* cell suspensions. An interesting phenomenon was observed with respect to the excretion of thiophenes. In one-phase cultures with only aqueous medium, the excretion is always practically zero. In the presence of some of the solvents tested, e.g. hexadecane, part of the thiophenes produced were excreted into the solvent. When experiments were performed with these two-phase cultures in bioreactors with volumes up to 6 litres, the excretion could rise to about 70% of the total thiophenes produced. Obviously, this is an important result, because it can facilitate the downstream processing of the thiophenes to a great extent. Of the two types of bioreactors tested, the stirred tank reactor and the bubble column, the latter gave the best results for this system.

In chapter six, similar experiments are described in aqueous two-phase systems. Again, the hairy roots were able to grow and to produce in the aqueous two-phase systems tested, i.e. in shake flasks and bioreactors. In this case, the excretion was stimulated up to 10% of the total thiophene production.

In chapter seven, the results are presented of elicitation studies with the *Tagetes patula* hairy root cultures. The biotic elicitors applied were bacteria, yeast and fungi, while abiotic elicitors were used in the form of extreme pH, Ca^{2+} addition and the addition of Ethephon, a precursor of ethene. Nearly all elicitor treatments resulted in a higher thiophene production. The highest increase was obtained when the pH was adjusted to 12 (280% of the control production), but the cells were no longer viable after this treatment. The elicitation with extracts of *Aspergillus niger* led to a production that raised up to 185% of the control, without loss of viability.

In chapter eight, the combined effects of elicitor treatment and of the use of a solid second phase, XAD-7, is described. Here, the excretion was affected by the addition of the resin, while growth and total production were not influenced, either in the presence or the absence of elicitor and/or XAD.

Finally, in the general discussion, the obtained results are shortly evaluated.

SAMENVATTING

Levende planten produceren zeer veel verschillende stoffen, waarvan een groot aantal nuttige eigenschappen heeft voor de mens. Vaak zijn dit zogenaamde 'secundaire metabolieten'; stoffen die niet direkt noodzakelijk zijn voor de groei van de plant, maar die wel een belangrijke, bijvoorbeeld ecologische, funktie vervullen. Voorbeelden van mogelijke toepassingen van deze secundaire metabolieten zijn te vinden in de farmaceutische wereld, in de levensmiddelenindustrie (natuurlijke geur-, kleur- en smaakstoffen) en de landbouw (milieuvriendelijker bestrijdingsmiddelen). Hoewel tot nu toe nog maar hooguit 10% van alle plantesoorten op de wereld is onderzocht voor wat betreft hun nuttige inhoudstoffen, wordt nu reeds 25% van alle geneesmiddelen geproduceerd uit planten. De vraag naar produkten uit planten neemt sterk toe, mede op grond van diverse maatschappelijke ontwikkelingen. Zo zijn er bijvoorbeeld veel stoffen, voorkomend in planten, die werkzaam zijn tegen diverse kankersoorten. Vaak is hun chemische structuur dermate ingewikkeld dat chemische synthese niet mogelijk of lonend is. In de levensmiddelenindustrie is er een stijgende behoefte aan natuurlijke additieven en in de landbouw gaat de regelgeving steeds meer richting afbreekbare biociden, om de milieubelasting te beperken. Vaak is het niet mogelijk om aan de vraag te voldoen door middel van het verzamelen van de planten uit de natuur of door het telen van de gewenste planten op akkers. Taxol bijvoorbeeld, een recent ontdekt middel tegen diverse vormen van kanker, wordt gewonnen uit de bast van één bepaalde *Taxus* boom. Voor de behandeling van 1 patiënt is de bast van 6 bomen nodig, terwijl deze bomen zeer zeldzaam zijn en bovendien erg langzaam groeien. Een ander voorbeeld: wanneer al het aardbeien-aroma dat in Nederland geconsumeerd wordt van natuurlijke oorsprong zou moeten zijn, dan zou daarvoor de hele wereldoogst aan aardbeien benodigd zijn. Verder kunnen seizoens- en klimaatseffecten de oogst (in kwantiteit en kwaliteit) van bepaalde gewassen zeer sterk beïnvloeden, terwijl sommige planten zich niet eens in cultuur laten brengen op akkers.

Om aan al deze beperkingen te ontkomen kan de plantecelbiotechnologie uitkomst bieden. Delen van de gewenste plant worden steriel op een voedingsbodem gebracht, die nutriënten, vitaminen en plantegroeistoffen bevat. Bij positief resultaat ontstaat na enige tijd groeiend, min of meer ongedifferentieerd weefsel vanuit het oorspronkelijke plantedeel. Wanneer relatief grote hoeveelheden benodigd zijn van de gewenste stof, moet er opschaling plaatsvinden. Dit gebeurt in stappen: de eerste stap is van Petrischaal met vaste voedingsbodem naar Erlenmeyer met vloeibaar medium. Dit kan tot een schaal van $\pm$ 1 liter goed werken, maar voor grotere hoeveelheden is het nodig de plantecellen te kweken in bioreactoren, waarin belangrijke parameters zoals de roersnelheid, de menging en de zuurstofvoorziening geregeld kunnen worden. De grootste reactor die momenteel voor plantecel-

culturen wordt gebruikt staat in Duitsland en heeft een volume van 75000 liter. Hij is met succes gebruikt voor de produktie van diverse secundaire metabolieten, met wisselend succes.
Dit proces, van hele plant tot grootschalige plantecelcultuur, verloopt meestal niet zonder problemen. Dit blijkt ook wel uit het feit dat wereldwijd zeer veel onderzoek wordt gedaan op dit gebied, door universiteiten, onderzoeksinstituten en bedrijven, terwijl er, vanaf de marktintroduktie van het eerste op deze manier vervaardigde produkt (shikonine in 1985 in Japan) nog geen tien van dergelijke produkten gecommercialiseerd zijn. De belangrijkste problemen die optreden zijn: de langzame groei van de cellen, de lage produktconcentratie (vaak veel lager dan in de oorspronkelijke plant) en de instabiliteit. Dit alles leidt ertoe dat processen op basis van plantecelculturen nog erg kostbaar zijn. In dit proefschrift staat een aantal manieren beschreven waarop deze problemen verminderd kunnen worden.
De instabiliteit van plantecelculturen, waarvan de oorzaak eigenlijk nog niet goed bekend is, kan worden verminderd door de plant te infecteren met de bacterie *Agrobacterium rhizogenes*. Hierdoor ontstaat op de infectieplaats de zogenaamde 'hairy root disease', waarbij er dus harige wortels uit bijvoorbeeld de stengel groeien. Deze hairy roots kunnen ook in cultuur gebracht worden. Nadat de overgebleven bacteriën gedood zijn, blijft een cultuur over van gedifferentieerde wortels, die veel stabieler blijkt te zijn dan de ongetransformeerde celculturen. Tevens is vaak de produktie van het gewenste secundaire metaboliet in deze hairy root culturen veel groter dan bij ongetransformeerde celculturen. Ook de groeisnelheid van deze hairy root culturen is veel hoger dan die van de gewone celculturen. Al met al is de overgang van celculturen naar hairy root culturen al een hele vooruitgang, maar voor de meeste produkten gaat die nog niet ver genoeg. Vaak zit het gemaakt produkt nog opgesloten in de cel, waardoor de hele, moeizaam verkregen, hoeveelheid biomassa gebruikt moet worden om het produkt te winnen òf het produkt wordt door de cellen of hairy roots uitgescheiden naar het omringende medium, waar de produktconcentratie zo laag is dat het winnen van het produkt uit het medium weer een hele dure stap wordt. Om dit te voorkomen, kan met zogenaamde tweefasen systemen gewerkt worden. De ene fase is het waterige medium, waarin alle voedingsstoffen en de cellen of hairy roots zich bevinden, en de tweede fase kan bijvoorbeeld een niet met water mengbare organische vloeibare fase zijn, of een vaste stof. Door het volume van de tweede fase een stuk kleiner te houden dan dat van de waterige fase, kan dan al een eerste concentratiestap uitgevoerd worden tijdens de produktie, wanneer het gevormde produkt tenminste bij voorkeur in of op deze tweede fase gaat zitten.
Nog een andere manier om eventueel de lage groeisnelheid van de plantecellen te compenseren, is het immobiliseren ervan. Dit houdt in dat de cellen in of op een dragermateriaal gebracht worden, bijvoorbeeld gelatine of zandkorrels. Een belangrijk voordeel van immobilisatie kan zijn dat medium

uit de reactor kan stromen terwijl de geïmmobiliseerde cellen erin blijven. Dit heeft uiteraard alleen zin wanneer de secundaire metabolieten uitgescheiden worden naar het medium.

Als laatste punt dient hier nog genoemd te worden het gebruik van zogenaamde elicitoren. Dit zijn stoffen die de plant in feite waarschuwen voor dreigend gevaar (bijvoorbeeld stoffen uit de celwand van een schimmel, die een aanval van die schimmel op de plant aankondigen). De secundaire metabolieten waarnaar we op zoek zijn, zijn vaak verdedigingsstoffen tegen dit soort aanvallen. Ook in plantecel- en hairy root culturen blijkt dit te werken. De produktie van het gewenste metaboliet wordt vaak verhoogd na toediening van een dergelijke elicitor.

In dit proefschrift zijn alle bovenstaande technieken uitgetest op cel- of hairy root culturen van het afrikaantje (*Tagetes patula* of *Tagetes minuta*). Deze plant produceert thiofenen, stoffen waarvan aanvankelijk gedacht werd dat ze zouden kunnen dienen als makkelijk afbreekbare biociden met een zeer breed spectrum. Intussen is men over de uiteindelijke toepassing niet meer zo zeker, maar dat is niet het onderwerp van dit proefschrift.

In Hoofdstuk 1 wordt een uitgebreid literatuuroverzicht gegeven van de mogelijkheden die er momenteel zijn om de produktiviteit van plantecelculturen te verhogen.

Hoofdstuk 2 beschrijft de resultaten van immobilisatie methodes op kleine en grote schaal, waarbij de uiteindelijke gelvorming plaats vindt in een organisch oplosmiddel. Met diverse soorten cellen (bacteriën, gisten, plantecellen en insectecellen) werd het effekt getest van diverse oplosmiddelen en van verschillende gelen. Het bleek dat de diverse celsoorten een uiteenlopende gevoeligheid aan de dag legden voor de toegepaste omstandigheden, terwijl algemeen geconcludeerd kon worden dat hydrofiele (= goed met water mengbare) oplosmiddelen schadelijker zijn dan hydrofobe (= slecht met water mengbare).

In Hoofdstuk 3 is in meer detail gekeken hoe plantecelculturen van *Tagetes minuta* reageren op verschillende oplosmiddelen, en wat de invloed was van immobilisatie van de cellen op hun gevoeligheid. Ook hier bleek er een duidelijke correlatie te bestaan tussen de mate van hydrofobiciteit en de activiteit van de cellen (hoe hydrofober het solvent, hoe beter de cellen er tegen kunnen), terwijl immobilisatie in calcium alginaat een lichte bescherming bood.

Vanaf Hoofdstuk 4 wordt er verder gewerkt met de genoemde hairy root culturen. Omdat deze wortelculturen groeien in één kluwen, is het onmogelijk om de hoeveelheid aanwezige biomassa in een reactor te bepalen, omdat geen representatieve monsters genomen kunnen worden. In dit hoofdstuk wordt een aantal methoden van indirecte biomassabepaling onderling vergeleken. De methode waarbij de geleidbaarheid van het medium (een maat voor de hoeveelheid aanwezige zouten) wordt gemeten, bleek het eenvoudigst om uit te voeren en voldeed ook goed.

In Hoofdstuk 5 wordt de reaktie van de hairy roots beschreven op de aanwezigheid van verschillende

oplosmiddelen. Ook hier bleek weer de hydrofobiciteit de belangrijkste parameter te zijn. Het solvent hexadecaan kwam naar voren als één van de gunstigste voor dit systeem en hiermee zijn dan ook reactorexperimenten uitgevoerd, tot een schaal van 6 liter. Het bleek zeer goed mogelijk om gedurende een maand de cellen in dit tweefasen systeem te laten groeien. Frappant was hierbij dat, terwijl normaal in een éénfase systeem met alleen medium de uitscheiding onder de 1% ligt van de totale produktie, er nu tot ± 70% van de totale produktie werd uitgescheiden.

In Hoofdstuk 6 wordt beschreven hoe de hairy roots zich gedragen in een zogenaamd waterig tweefasen systeem. Ook hierin groeien ze goed, ook in reactoren. In dit systeem gaat de excretie van < 1% naar ± 10%.

Hoofdstuk 7 is een verslag van de effekten van het toevoegen van verschillende elicitoren aan de hairy root culturen. Het bleek mogelijk om op deze manier de produktie met 85% te verhogen.

Hoofdstuk 8 beschrijft wat er gebeurt wanneer als tweede fase een vaste stof wordt gebruikt, XAD-7, al of niet in combinatie met een elicitor. Het bleek dat XAD-7 de totale produktie niet beïnvloedde, maar dat de uitscheiding van de thiofenen ook nu weer van 0 to ± 50% werd verhoogd. In tegenstelling tot eerdere experimenten had nu het toevoegen van een elicitor geen effect. Het feit dat zelfs dezelfde plantecellijn wisselend reageert op dezelfde veranderingen, is nog steeds een algemeen kenmerk van de plantecelbiotechnologie.

In het laatste hoofdstuk (general discussion) wordt tenslotte het geheel nog eens geëvalueerd.

CURRICULUM VITAE

Reinetta Margaretha (Nettie) Buitelaar werd geboren in Eindhoven op 30 juli 1959. Na het behalen van het Atheneum-B diploma aan het Willem de Zwijger College te Bussum in mei 1977, begon ze in september 1977 aan de studie Huishoudwetenschappen aan de toenmalige Landbouwhogeschool in Wageningen. Na twee jaar bleek dit niet de juiste keus, zodat ze in september 1979 begon met de studierichting Levensmiddelentechnologie. Als specialisatie koos zij de Proceskunde, met als doctoraalvakken: Proceskunde, Industriële Microbiologie, Meet-, regel- en systeemtechniek en als extra vak Informatica. Op 28 november 1986 studeerde ze af, om op 1 december 1986 te beginnen aan een vierjarig promotie-onderzoek aan de Landbouwuniversiteit, bij de Bioprocestechnologie-groep. Het resultaat van dit onderzoek staat beschreven in dit proefschrift.

Vanaf 1 december 1990 is zij werkzaam als wetenschappelijk medewerkster bij de afdeling Biotechnologie van TNO-Voeding te Zeist.