Plant cells:
immobilization and oxygen transfer

Promotoren: dr. ir. J. Tramper,
hoogleraar in de bioprocestechnologie

dr. ir. K. van 't Riet,
hoogleraar in de levensmiddelenproceskunde

A. C. Hulst

Plant cells: immobilization and oxygen transfer

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STELLINGEN

1. Het vergelijken van zuurstofopname door vrije cellen met die van geïmmobiliseerde cellen in het kader van zuurstof diffusielimitatie is fundamenteel onjuist.
(Gosmann & Rehm, Appl. Microbiol. Biotechnol. 23 (1986), 163-167.)

2. Het bepalen van effectieve diffusiecoëfficiënten van substraat in geïmmobiliseerde plantecelsystemen of callusweefsel met behulp van een mathematisch model waarin geen celkinetiek is verwerkt, is principieel onjuist wanneer deze experimenten met levende cellen worden uitgevoerd.
(Mavituna et al, Chem. Eng. J. 34 (1987), B1-B5.)

3. Het vermelden van de termen 'groot' en 'klein' als maat voor aggregaatgrootte is niet eenduidig en moet worden vermeden, aangezien de ene auteur (Kinnersley & Dougall, 1980) sommige aggregaten als groot omschrijft terwijl een andere auteur (dit proefschrift) dezelfde aggregaten hooguit als een 'fijne celsuspensie' zou bestempelen.
(Kinnersley & Dougall, Planta 149 (1980), 200-204.)

4. Bij het hanteren van een methode voor het bepalen van diffusiecoëfficienten in gelen dient men op zijn minst de diffusieweerstand in de stagnante grenslaag aan het geloppervlak te beschouwen.
(Sato & Toda, J. Ferment. Technol. 61 (1983), 239-245.)

5. Het telkens herhalen van de eigenschap dat plantecellen zeer gevoelig zijn voor afschuifkrachten lijkt verdacht veel op een 'brainwash' van de onderzoekers binnen de plantecelbiotechnologie.

6. De toe te kennen maximale duur van een promotieonderzoek waarin het werken met biologisch celmateriaal centraal staat, zou recht evenredig moeten zijn met de verdubbelingstijd van deze organismen.

7. Door de uitzonderlijk hoge bedragen die tegenwoordig zijn gemoeid met het adopteren van een buitenlands kind, lijkt dit zozeer een elitezaak te worden, dat de pedagogische doelstelling 'het belang van het kind' enigszins uit het oog wordt verloren.

8. De bijbelse uitdrukking 'Onderzoekt alles en behoudt het goede' zou voor de wetenschap in het kader van het natuurbehoud moeten luiden: 'Onderzoekt het goede en behoudt alles'.

9. Een proefschrift dient een zodanige kwaliteit en kwantiteit te hebben dat men geen aanleiding ziet om het onderdeel 'telefoonboek scheuren' in de titelstrijd 'Sterkste Man van Nederland' te vervangen door 'proefschrift scheuren'.

Stellingen behorende bij het proefschrift 'Plant cells: immobilization and oxygen transfer' van A.C. Hulst. Wageningen, 14 oktober 1987.

VOORWOORD

De produktiviteit van een enkele plantecel binnen een cluster van andere plantecellen is meestal vele malen groter dan die van een eenzame soortgenoot buiten zo'n cluster. Zo is het ook binnen het wetenschappelijk onderzoek: wanneer men enig zichtbaar resultaat binnen afzienbare tijd wil verkrijgen, is de hulp en inzet van andere personen onontbeerlijk. Velen hebben bijgedragen aan de totstandkoming van dit proefschrift en daarvoor ben ik hen veel dank verschuldigd. De meest betrokkenen wil ik echter op deze plaats 'persoonlijk' bedanken.

- Hans Tramper, jouw deskundige en enthousiaste begeleiding tijdens dit onderzoek, de waardevolle adviezen en de kritische noten die in dit proefschrift zijn verwerkt, waren onmisbare elementen in de wording van dit boekje. Jouw stimulerende en inspirerende manier van samenwerken, zelfs rond de bridgetafel, heb ik als zeer prettig en enorm leerzaam ervaren.
- Klaas van 't Riet, jouw 'pep-talks', adviezen en ideeën hebben telkens weer nieuwe impulsen aan dit onderzoek gegeven.
- De ex-doctoraal studenten Hans Westerbeek, Harry Gruppen, Greetje Meyer, Ron Ogg en Harrie Hens en de stagiaires Angela Joosten en Jos Sewalt, het zijn jullie bijdragen die letterlijk tastbaar zijn geworden.
- De medewerkers van het Ital, in het bijzonder Hans Breteler en David Ketel, het was met jullie zeer prettig samenwerken in de afgelopen jaren.
- Nettie Buitelaar, door jouw bereidwillige aard zijn, vooral tijdens het hektische eindstadium van de onderzoeksperiode, de laatste puntjes op de 'i' gezet.
- De mensen van de Centrale Dienst Biotechnion (werkplaats, tekenkamer, magazijnen, beheer, bibliotheek, fotolokatie), mede door jullie ontelbare diensten in de afgelopen jaren kon dit onderzoek 'gladjes' verlopen.
- De medewerkers/collega's van de sektie Proceskunde, door jullie collegialiteit was een prima werksfeer gewaarborgd, wat voor mij een absolute basis vormt voor werkplezier. Met name kamergenootje Paul Verlaan, jouw zeer nabije aanwezigheid heeft in positieve zin de afgelopen periode tot een onvergetelijke gemaakt.
- De organisatie van Cacao de Zaan, die mij in staat stelde om dit werk af te kunnen ronden.

Thea, jouw steun was voor mij van onschatbare waarde.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

In biotechnological research much attention is given to the production of fine chemicals. Culturing plant cells, which produce high value compounds as secondary metabolites, in liquid media, is a promising production technique. This can be performed in a bioreactor, where growth and production can take place. Because of inherent advantages, like for instance continuous or repeated utilization, the use of immobilized plant cells in a bioreactor can raise the feasibility for industrial applications in some cases.
The subject of this thesis concerns such investigations on the production of secondary metabolites by immobilized plant cell species. The immobilization of plant cells, its consequences on oxygen supply to the immobilized plant cells, and the possible effects on the secondary metabolite production were investigated. This study was in close cooperation with the Research Institute Ital in Wageningen, within the framework of NOVAPLANT, which provided the thiophene producing cell lines of the Tagetes species.

The organization of this thesis is as follows. A general literature review on immobilized plant cells is presented in Chapter 2. Here, the advantages of the use of immobilized plant cells, the immobilization techniques, the consequences of immobilization, the bioreactors, and the future prospects concerning the immobilized plant cells are discussed.

Chapters 3 and 4 deal with the upscaling of immobilization techniques for plant cells in view of application of immobilized (plant) cells in processes on pilot-plant or even industrial scale.

In the literature, some papers have been published which reported enhanced secondary metabolite production for alginate immobilized plant cells, which was not observed at plant cells immobilized in other gel supports. In Chapter 5 comparisons are made between the respiration rates of plant cells immobilized in alginate, κ-carrageenan and agarose in order to explain this phenomenon.

The effective diffusion coefficient of oxygen in gel supports is an important parameter in order to predict the occurrence of oxygen diffusion limitation for immobilized plant cells by model calculations. Chapter 6 deals with

the experimental determination of the effective diffusion coefficients of oxygen in widely-used support materials like agarose, agar, gellan gum, κ-carrageenan, and calcium alginate.

In Chapter 7 experiments are described in order to test the validity of a mathematical model for calculating oxygen concentration profiles in gel beads containing viable plant cells by experimental measurements of these profiles in the beads with the aid of oxygen micro-electrodes.

In Chapter 8, this model was used to calculate the oxygen concentration profiles in plant cell aggregates in order to establish the effect of oxygen diffusion limitation on secondary metabolite production.

In Chapter 9 the investigations are generally discussed.

CHAPTER 2

IMMOBILIZED PLANT CELLS: A LITERATURE SURVEY.

A.C. Hulst, J. Tramper

Department of Food Science, Food and Bioengineering Group,
Agricultural University,
De Dreijen 12, 6703 BC Wageningen, The Netherlands.

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INTRODUCTION

Immobilization techniques have had a great impact on biotechnology nowadays. A review article on the subject of immobilized cells in general has recently been written by Scott (1987). Plant cells have been investigated for the production of high-value compounds, usually so-called secondary metabolites. In the last decade, especially immobilized plant cells made their entry in this research field and recently much has been published on immobilized plant cell cultures (Brodelius & Mosbach, 1982; Brodelius, 1983; Rhodes, 1986). This review will give a literature survey on the usage of immobilized plant cells without pretending to be complete. Historical perspectives, production processes, immobilization techniques, bioreactor design and future prospects, concerning immobilized plant cells, are discussed in this review.

HISTORICAL PERSPECTIVES (Gautheret, 1983; Staba, 1985; Street, 1977; Pierik, 1985; Fowler, 1984).

Plants always have played an important role for mankind. Not only as a source of food, but plants were also used as rough materials for all kinds of products (Brown, 1985). For centuries, nothing principly changed in the use of plants for these purposes. It was in the 19^{th} century that Schleiden and Schwann postulated their plant cell theory. Their theory originates in 1838 and implies that a plant cell is capable of autonomy and in principle is totipotent. With that theory in fact the fundamentals were given for the cultivation of plant tissue and plant cells. It was about 60 years later, when Haberlandt in 1902 was successful in cultivating single plant cells in nutrient solutions. Although serious efforts were made by him, the cells were not able to divide and grow. After Haberlandt much work has been done by several researchers in the field of plant tissue and cell culture. In order to avoid an endless list of historical details, only the milestone events will be mentioned in the following paragraph, showing the acceleration of this kind of research.

It was in 1934 when Kögl isolated and identified auxin (Indole Acetic Acid, IAA) and the importance of this hormone was recognized in the control of plant growth by Went and Thimann in 1937. This knowledge was applied by White & Gautheret, in 1939, who were successful in their work to establish a

viable subculture of callus plant cells. Van Overbeek, in 1944, discovered the positive influence on cell growth of coconut milk on in vitro cultures of Datura embryo's. Later on, in 1955, the growth hormone kinetin, a coconut milk component, was discovered by Miller & Skoog. It were Murashige & Skoog who developed in 1962 a chemically well-defined growth medium (MS-medium, Murashige & Skoog, 1962). It was this finding which offered prospects for the usage of plant cells for secondary metabolite production purposes amongst numerous other techniques like extraction of natural raw materials, because plant cells were able to grow and possibly produce secondary metabolites in liquid media under defined, controllable conditions.

In the following years, much work has been done to improve plant cell culture techniques and more insight was gained into the morphological, physiological and genetic mechanisms of plant cells. During the 70's, plant cells were more and more investigated with the aim to be applied for the production of several chemical compounds. It was in 1979 when Brodelius (Brodelius et al, 1979) introduced the use of immobilized plant cells for this purpose. A new area seemed to be opened.

Most publications on the subject of immobilized plant cells concern the use of whole cells, occurring as single cells as well as little cell aggregates. An overview of used cell species is given by Shuler et al (1983) and Lindsey & Yeoman (1983). In general one can say that only those cells are used which are able to produce some secondary metabolite either for industrial and economical production of these compounds or for model studies in research.

For investigations on aspects of plant cell biochemistry and physiology, protoplasts (wall-less plant cells) of Vicia faba have been immobilized in calcium alginate (Scheurich et al, 1980; Schnabl et al, 1983). Bornman & Zachrisson (1982) anchored protoplasts of Datura innoxia to a microcarrier (Cytodex 1) as an alternative type of protoplast immobilization. Linsefors & Brodelius (1985) studied the viability of protoplasts of Daucus carota and Catharanthus roseus immobilized in κ-carrageenan, agarose and alginate. They found that immobilized protoplasts were more viable after 14 days compared to free protoplasts under the same conditions. Also an increased tolerance to osmotic shocks compared with free protoplasts was shown (Linse & Brodelius, 1984). Warren et al (1984) was successful in reversibly binding protoplasts on agarose beads. No reports have been published in which protoplasts are

applied for production of secondary metabolites.

Pfitzner & Zenk (1982) immobilized the enzyme strictosidine synthase , purified from Catharanthus roseus cell cultures, to CNBr-activated Sepharose. However, this is beyond the framework of 'plant cell immobilization' but it is more concerning 'enzyme immobilization'. Here, the plant cells can be seen as producers of a specific enzyme as product.

ADVANTAGES OF IMMOBILIZED PLANT CELLS

At the time when plant cells were immobilized for the first time, immobilization of biocatalysts in general was not a new area in the biotechnological field. It was already widely applied in fermentation processes for enzymes and microbial cells for a relative long period (Chibata, 1978; Mattiasson, 1983). The experience learned that immobilization of biocatalysts in case of production could bring along some important advantages (Bucke, 1983; Rosevear, 1984), especially the improved possibility of continuous application. The first step to immobilize plant cells, which was taken by Brodelius et al (1979), was a logical one in view of the successes of application of immobilized enzymes and microbes.

Brodelius (Brodelius et al, 1979) mentioned that the more general advantages of immobilization such as lower cost because of the possibility of reusing the biocatalyst, the ease of separation of product from the biocatalyst and the suitability for continuous flow-through processes, were also valid for immobilization of plant cells. In addition, for plant cells as a more specific advantage, he mentioned the more efficient exploitation of the chemical potential of the slow growing plant cells as result of immobilization. Later on, Brodelius and his group (Brodelius et al, 1982; Brodelius, 1985a) mentioned as additional advantages the better control of processes based on immobilized plant cells and the increased metabolic stability of the plant cells. Shuler et al (1983) mentioned the physical protection from shear forces as an advantage. However, they emphasized that the most important advantages of the use of immobilized plant cells were on the level of 'processing', i.e. the possibility to maintain high cell densities in the reactor which results in a high volumetric productivity and shorter residence times (Lambe & Rosevear, 1983), the use of flow rates well in excess of washout conditions, prevention of wall attachment of cells and plugging of outflow lines, and finally the minor risk of contamination in the reactor. Prenosil

(Prenosil & Pederson, 1983) gave as additional advantage the prevention of cell aggregation, in many cases an undesirable general characteristic of plant cells. In some cases however, the occurrence of aggregates is a prerequisite for the production of secondary metabolites by the plant cells (see Chapter 8). In a recent review, Rosevaer and Lambe (1986) reported that the uncoupling of growth and production phases is probably the most important benefit of plant cell immobilization.

Specifically, for plant cells Brodelius (1984) and Wichers (1983) found that immobilization of the cells stimulated the secondary metabolite production compared to a free cell suspension. According to Lindsey and Yeoman (1985) this could be due to a high cell to cell contact resulting in limitation of the rate of cell division and establishment of gradients of nutrients, among other things. In contrast to free cells where the product is often accumulated within the cell, the product can be secreted by the cell as a consequence of immobilization, a necessary phenomenon for application in continuous processes (Lambe & Rosevear, 1983). Another specific feature for plant cells was discovered by Morris and Fowler (1981) and later also observed by Hamilton et al (1984). They used immobilization of plant cells as a means for production of fine cell suspensions, c.q. absence of cell aggregates.

In many articles, published on the subject of immobilized plant cells, authors mention a number of the before-mentioned advantages to support their particular use of immobilized plant cells.

REQUIREMENTS FOR PLANT CELL IMMOBILIZATION TECHNIQUES

Due to some specific characteristics of plant cells, the number of available immobilization techniques which are suitable for plant cells is severely reduced. Slow growth rate of the cells (doubling times in the range of 24 hours to several weeks) makes processing with plant cells very sensitive to bacterial contamination. This knowledge demands that the total immobilization procedure has to be done under strictly axenical conditions. Because plant cells have large dimensions in relation to microorganisms and possess a rigid, thick cell wall, the cells are sensitive to shear forces. It brings along that generation of shear forces has to be avoided as much as possible during the immobilization procedure. It is obvious that plant cells, once immobilized, should have a high retention of viability. In order to

maintain the viability of the cells, one should avoid aggressive chemical reagents, which are hazardous to the cells, in the immobilization procedure. If they are necessary for a particular immobilization technique, the effect of reagents on the viability of the plant cells has to be investigated in advance. For research purposes, working with spherical particles is profitable, yielding a simpler approach for mathematical modelling of bead-shaped particles. Running a bioreactor with uniform particles will be advantageous with respect to homogenization of the reactor contents and efficient use of the immobilized plant cells.

If reactors on pilot-plant scale or bigger geometries have to be provided with a filling of immobilized plant cells, the technique of immobilization must have a high production capacity. Otherwise it will take too long to supply that reactor with immobilized cells. Plant cell immobilization techniques, on large scale also, should comply to the before-mentioned requirements as much as possible if plant cell immobilization is employed.

IMMOBILIZATION TECHNIQUES AND SUPPORT MATERIALS

Since immobilization of plant cells was introduced as beneficial aid for the production of secondary metabolites, gel entrapment has been the most widely used type of immobilization method because it is a cheap, simple and reproducible technique with mild conditions during the immobilization. Later on, efforts have been made to use other immobilization methods, some of them with reasonable success. In the next parts, plant cell immobilization techniques reported until now will be discussed and the consequences afterwards.

Gel-entrapment techniques.

Gel-entrapment techniques used to immobilize plant cells have recently been discussed by Rosevear and Lambe (1986). The principle of gel entrapment is that cells are mixed with a pregel solution. After gelification the plant cells are enclosed in the gel material. When applied in a bioreactor the water-soluble substrates and nutrients 'freely' can pass the gel to provide the cells its necessaries of life.

Immobilization in **alginate gel** is the most common method. A suspension of plant cells is mixed with a sodium alginate solution. The alginate con-

centration depends on the type of alginate used (2-8%), but has to be sufficient to get a firm gel (McNeely & Pettitt, 1973). The mixture of cells and alginate solution is dropwise extruded through an orifice or hollow needle. The formed drops are caught in a salt solution with bivalent cations, usually calcium chloride (50-300 mM) or in medium with the addition of calcium chloride (Majerus & Pareilleux, 1986). As soon as a drop falls in the calcium chloride solution, sodium and calcium ions begin to exchange and a water insoluble calcium alginate gel is formed at the outer surface of the drop resulting in a spherical particle (Figure 1). The bead is further hardened by remaining in the salt solution, so that the calcium ions can be transported to the centre of the bead till an equilibrium state has been reached. The hardening times reported in the literature show a great variety, ranging from 15 minutes to 12 hours or even longer. The minimum hardening time is dependent on experimental conditions such as temperature, salt concentration, bead diameter and type of alginate. During the hardening procedure the gelvolume shrinks upto 40% of the original dropvolume, dependent on the type of gel, the gel concentration and the salt concentration.

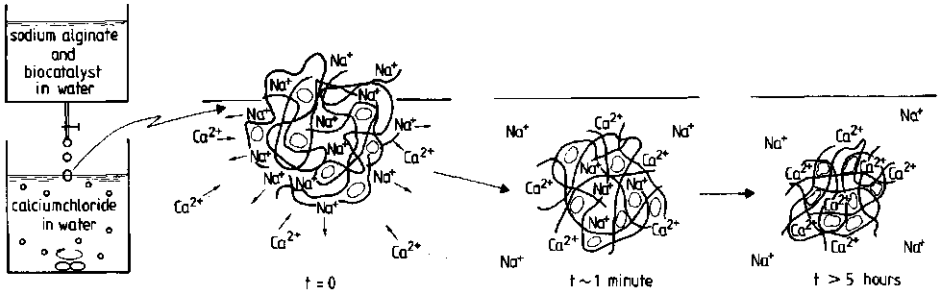

Figure 1. Schematic representation of the exchange of Na^+ and Ca^{2+} ions during the hardening process of calcium alginate gel beads.

Immobilization in κ-carrageenan is carried out in a similar way as alginate (Brodelius & Nilsson, 1980; Nakajima et al, 1985). Gelification of κ-carrageenan takes place when the solution is cooled to the appropriate gelification temperature, but only in the presence of cations, usually K^+ ions. The immobilization procedure is as follows: A mixture of 5% κ-carrageenan solution and plant cells is extruded dropwise in a 300 mM KCl solution at a

temperature of 50°C. After gelification, the formed particles remain approximately 1 hour in the KCl solution for hardening. The use of a purer κ-carrageenan (i.e. containing less cations) with a lower gelification temperature (about 20°C) for immobilization of plant cells has not yet been found in the literature. Mosbach & Nilsson (1983) patented a technique by dispersing the κ-carrageenan-cell mixture in soy oil with subsequent cooling, identical to the technique used with agar(ose), which is described below. Moritz et al (1982) used locust bean gum to improve the κ-carrageenan gel structure.

Immobilization in **agarose** and **agar** is carried out by adding a plant cell suspension to a solution of agarose or agar (3-5%) at the temperature just above the gelling temperature, i.e. 50°C for agar and 30-50°C for agarose depending on the type of agarose. The mixture can be moulded into beads with subsequent cooling (Brodelius & Nilsson, 1980). Another method consists of pouring the mixture in a petridish, cooling and after gelification cut into small pieces (Nakajima et al, 1985) or dispersion of the mixture into a hydrophobic phase (soy, parrafin, silicon oil, tri-n-butylphosphate, dibutylphtalate) by mechanical forces. After cooling the dispersion, beads are formed and can be separated from the hydrophobic phase (Nilsson et al, 1983).

Gelatin immobilization is performed by adding a suspension of plant cells to a mixture of 10-20% gelatin solution. The gelatin is crosslinked by use of the crosslinking agent glutaraldehyde with a concentration in the range of 1.5-2.0%. Beads can be obtained by the moulding technique (Brodelius & Nilsson, 1980), or other-shaped particles by fragmentation of the obtained crosslinked gelatin gel structure (Felix & Mosbach, 1982).

Brodelius and Nilsson (1980) also immobilized plant cells in a mixture of **alginate and gelatin**. Therefore, plant cells were suspended in a mixture of 5% sodium alginate and 20% gelatin in the volume ratio of 3:1. Beads were obtained by dripping the suspension in 50 mM calcium chloride solution with subsequent crosslinking the gelatin in 2% glutaraldehyde for 30 minutes. In this technique alginate is used for obtaining spherical particles and gelatin as the main support material.

Similarly, immobilization in a mixture of **agarose and gelatin** was carried

out by Brodelius and Nilsson (1980). Here, the beads are shaped by agarose with the moulding technique and gelatin forms the firmness of the bead.

Moritz et al (Moritz et al, 1982) immobilized plant cells in **chitosan**. The used method is described by Vorlop and Klein (1981). Plant cells are mixed with about 2% acidified chitosan solution. The mixture was immediately added dropwise to a stirred 1.5% sodium-tri-polyphosphate solution (pH < 6). In this case polyvalent anions induce crosslinking and gel formation. Hardening of the beads takes place in a sodium-tri-polyphosphate solution (pH > 7.5) during 3 hours. During the hardening procedure the gel shrinks.

Immobilization of plant cells in **polyacrylamide** has been performed by Brodelius and Nilsson (1980) by adding plant cells to a solution of the monomers acrylamide and N,N'-methylenebis-acrylamide in Tris-HCl buffer. By adding the polymerization initiator ammoniumpersulphate and N-N-N'-N'-tetramethylethylenediamine to the suspension, beads can be obtained by the moulding technique.

Galun et al (1983) immobilized plant cells in **polyacrylamide-hydrazide** (PAAH) by mixing plant cells with a 3% aqueous PAAH solution. Instantaneous polymerization takes place after adding 0.5% glyoxal to the mixture. After cutting the gel into cubes, spontaneous self-hardening and shrinkage will take place during about 1 hour. Afterwards the gel pieces are fragmented.

A rather new polymer suitable for plant cell immobilization is **gellan gum** (see Chapter 3). The characteristics are comparable to those of κ-carrageenan. Plant cells are mixed with a 1% gellan gum solution in 0.03 g dm^{-3} $MgSO_4.7H_2O$ at a temperature of 40°C. From this mixture beads can be formed in a mould after cooling and subsequent hardening in 20 mM $MgSO_4$, or the mixture can be dispersed in a hydrophobic phase in the same way as described for agar(ose).

Other techniques.

Adsorption and covalent linkage of plant cells to activated **polyphenyleneoxide** (PPO) was described by Jirku et al (1981). Activated PPO gel was prepared by suspending it in a 5% glutaraldehyde solution during 48 hours under stirring. Afterwards the activated gel is contacted with the plant cell

suspension at room temperature for 60 minutes. In this period adsorption and covalent coupling occurs.

Another method based on adsorption was introduced by Lindsey et al (1983). They found that plant cells strongly adhere to reticulate **polyurethane** foam particles. When cubes of this material are added to a plant cell suspension, plant cells spontaneously intrude into the foam material.

Another spontaneous immobilization by cell adsorption is described by Rhodes et al (1985). They used **nylon** discs (15 mm diameter x 6 mm height), which were added to a plant cell suspension. After 3-5 days of culturing, cells are adsorbed to the nylon material.

Aggregation, the natural characteristic of plant cells in suspension, can also be seen as spontaneous self-immobilization of plant cells. Fuller & Bartlett (1983) call such aggregates Naturally Immobilized Cell Systems, abbreviated by NICS. Dainty et al (1985) critically discussed the 'artificial' immobilization methods like immobilization in gel supports. When they are compared to the 'free cell' suspensions, they concluded that the measure of aggregation in these suspensions should always be reported for a proper comparison.

Immobilization of plant cells in **hollow fibre** reactors was firstly reported by Shuler (1981). Prenosil & Pederson (1983) applied this concept in their experiments on secondary metabolite production. The plant cells are introduced via ports to the shell side of the reactor module. In fact, in this way the immobilization step is realized by putting cells directly in some type of membrane bioreactor, and will be discussed under that subject in this paper.

Black (1984) divided immobilization techniques into two categories: 'passive' and 'active' techniques. Plant cell immobilization in polyurethane foam and cell aggregation are regarded as 'passive' techniques and the other described techniques are covered by the term 'active' techniques.

It has to be noted that plant cell immobilization is very difficult or in some cases even impossible if these cells consist of big aggregates (clogging

problems), so fine cell suspensions are preferable. Methods to obtain these fine cell suspensions are described in the literature (Nakajima et al, 1985; Morris & Fowler, 1981; Morris et al, 1983).

CONSEQUENCES OF IMMOBILIZATION

It is obvious that immobilization of plant cells will have its impact on several aspects concerning the manner of secondary metabolite production. The consequences of plant cell immobilization discussed below are split up into three main categories:

1. Practical consequences of immobilization methods and techniques.
2. Growth and production.
3. Mass transfer and diffusional limitations.

Practical consequences of immobilization.

The immobilization in calcium alginate has the advantages of being cheap, simple, reproducible and mild, while uniform beads are produced. However, calcium alginate also has its drawbacks. In practice, the mechanical gel stability can reduce in time. One of the major causes for disruption of the alginate gel is the chelation of Ca^{2+} ions with other compounds in the medium. It is mainly by the phosphate ions, which are of frequent occurrence in media (Robinson et al, 1985), including the polyphosphate ATP. Moreover, the chelation of phosphates with Ca^{2+} ions, makes them unusable in the metabolic reactions in the cells itself (Prenosil & Pederson, 1983). In order to prevent this type of gel disruption, the gel is stabilized by maintaining a sufficient Ca^{2+} concentration in the medium (Jones & Veliky, 1981a; Wichers et al, 1983) or by using other bivalent cations inducing gelification. Tamponnet et al (1985) used Ca^{2+}, Ba^{2+}, Cd^{2+} and Zn^{2+} for algae cell immobilization resulting in good mechanical properties of the obtained gels, which was not the case with Mg^{2+}. However cell viability only remained when Ca^{2+} and Ba^{2+} were used. One has to be careful by using Ca^{2+} because it is known as an important trigger for many key metabolic processes (Rosevear, 1984).

Rochefort et al (1986) used the trivalent Al^{3+} cation for extra mechanical stabilization of the calcium alginate gel, but did not use plant cells in their experiments.

For research purposes, the effect of solving the alginate gel by chelation of Ca^{2+} with chelating agents like phosphate (Nakajima et al, 1985), citrate or EDTA (Brodelius & Mosbach, 1982) can be advantageous, because cells, which have to be used for further investigations, can be released from the gel without any harm. Wichers et al (1983) used 2-(N-morpholino)ethane sulfonic acid (MES) for this purpose.

Another disadvantageous consequence of alginate entrapped cells of Catharanthus roseus was mentioned by Felix & Mosbach (1982). They found a lack of activity of the enzymes isocitrate dehydrogenase and cathenamine reductase, due to the sequestering of positively charged $NADP^+$ cofactors by the gel.

Especially for immobilizing plant cells, sterilization of the sodium alginate solution before the immobilization step is a first demand. If autoclaving is applied, a permanent reduction of the viscosity of the alginate solution occurs due to partial hydrolysis of the polymer (Rosevear & Lambe, 1986; Brodelius, 1985b) with reduction or even total loss of gel formation potency. These features are probably dependent on the type of alginate because the problem is not always mentioned in the literature. Shorter autoclaving times or lower autoclaving temperature can be the solution to this problem, but increases the risk of microbial contamination. From one's own experience we also had to contend with the above-mentioned problem with immobilized plant cells in calcium alginate. Our solution was the application of a maximum autoclaving temperature of 115°C instead of 121°C resulted in firm alginate gel beads without the occurrence of microbial infection afterwards. Other sterilization methods for alginate in case of plant cell immobilization have not yet been described.

Although alginate immobilization is the most popular method for plant cells, Rosevear and Lambe (1986) stated that alginate immobilization is lacking in bead size c.q. droplet size control. Our experience is that alginate is an outstanding example of a gel support which results in beads of a very uniform size. Brodelius (1985b) mentioned that bead size in the range of 2-4 mm diameter can be controlled by using different needle diameters. Smaller beads in the range of 0.2 to 1 mm diameter can be obtained by using an air stream along the needle. The size of the drops which are blown off from the needle is controlled by adjusting the air stream velocity. From

one's own experience with the latter technique it was not possible to obtain beads (without cells) smaller than 0.5 mm. In case of immobilization of plant cells this limit can not even be reached because of the inevitable clogging problems in the needle caused by the large plant cells.

The capacity of most in literature described immobilization procedures is sufficient for use in laboratory experiments (reactor volumes up to 10 dm^3). However, if experiments with immobilized plant cells reach the 'pilot-plant level', the conventional needle technique has one big shortcoming: the production capacity is low. For one needle it is in the order of 100-500 cm^3 per hour. Brodelius and Mosbach (1982) designed a device with six needles with simultaneous dripping. Here, control of the bead size by applying the air stream is difficult. Moreover, by using six needles instead of one, a statistical deviation in droplet (c.q. bead) size will be promoted. Another technique for upscaling the alginate immobilization technique has been introduced by Hulst et al (1985a). They made use of a vibration nozzle. The principle of this device consists of breaking up a jet of the cell-alginate mixture into uniform droplets by mechanical vibrations which are transferred to the jet. The production capacity of the vibration nozzle is two orders of magnitude larger than the conventional needle technique. The drop size can be controlled by varying the jet diameter, vibration frequency and jet velocity. Cell viability was very well preserved by this technique, also plant cell viability.

Rehg et al (1986) used a system with six needles combined with the air stream along each needle, which he called an atomizer, for producing small uniform beads (0.5-2 mm diameter). However, no cells were immobilized and the production capacity was not stated.

Matulovic et al (1986) designed a device with a capacity in the order of magnitude of the vibration nozzle. They constructed an apparatus with a high-speed, rotating, nozzle ring. Experiments for testing this device with alginate, chitosan and κ-carrageenan, were done with microorganisms, but no plant cells were used. No data were reported of cell viability after immobilization.

Immobilization in κ-carrageenan is a good alternative if alginate can not be applied in practice. κ-carrageenan is less sensitive to chelating reagents in media. In contrast to alginate, κ-carrageenan particles produced by the

dripping technique are less spherical and uniform. So, if spherical particles are desired, the mixture of κ-carrageenan solution and plant cells has to be poured into a spherical mould and cooled until a weak gel is formed. After removal from the mould, the beads are hardened in KCl solution. When κ-carrageenan of high purity (less contamination with K^+ ions) is used the moulding technique is useless, because temperature gelification does not take place. In case of κ-carrageenan of high purity, the dripping technique is simpler because it can be performed at room temperature.

Upscaling of κ-carrageenan immobilization is possible with the vibration nozzle technique (see Chapter 4).

The drawback of immobilization in agar or agarose is the necessity of a relative high temperature (≈ 50°C) which can be deleterious for plant cells in some cases (Lindsey & Yeoman, 1983). Once immobilized, the gel beads of agar(ose) are inert to medium constituents. However, Nakajima et al (1985) found that the mechanical strength of agar gels was insufficient for repeated use of the entrapped cells. Felix & Mosbach (1982) have increased the mechanical stability of agarose beads by cross-linking with hexamethylene diamine and glutaraldehyde. However, this did have a deleterious effect on key enzyme activities in the cells.

By immobilizing in low-gelling agarose high temperatures can be avoided, but the raw material is very expensive which is not favourable for application as support material in industrial processes.

The dispersion technique with agar(ose) in hydrophobic phases has in principle a high production capacity. The bead size can be controlled by varying the stirrer speed (Nilsson et al, 1983), resulting in finer dispersed droplets. Nevertheless, the control is very rough and uniform sized dispersion drops (c.q. beads) can hardly be obtained with this technique without fractionation afterwards. Another drawback of the dispersion method is that after separation from the hydrophobic phase the removal of the hydrophobic liquid film at the bead surface is very difficult to perform. Methods to do this are not described in the literature.

The immobilization methods concerning gelatin are not very attractive to immobilize plant cells because the crosslinking reagent glutaraldehyde is deleterious to plant cells, resulting in non-viable immobilized cells.

To our knowledge, no reports have been published in which plant cells did survive the immobilization procedure with polyacrylamide. Chemical reagents used in this procedure seem to be totally deleterious to plant cells.

Entrapment in polyacrylamide-hydrazide with retained cell viability is only reported by one research group (Galun et al, 1983; Galun et al, 1985). Despite their successful results, no use of this method has been reported in the literature by other researchers.

The same comment is valid for adsorption of plant cells on activated polyphenyleneoxide. To our knowledge, only one publication shows some experience with this type of immobilization but not many data are given (Jirku et al, 1981).

The most important advantage of plant cell immobilization in polyurethane foam is the minimization of the incidence of microbial contamination because it is performed as a single step process (Lindsey et al, 1983). The method is natural, easy, and harmless to cells, but the 'immobilization time', in the order of 10-24 days, is extremely long.

The common form of a polyurethane foam is a block. Formation of polyurethane beads is described by Klein & Kluge (1981), but the immobilization procedure, based on polycondensation of monomers, is different and plant cells very likely do not survive.

In order to establish a more effective invasion of cells into the foam matrix, Mavituna & Park (1985) keep the foam fixed with respect to the surrounding medium, resulting in a better filtration effect of the cells. However, if the fixed blocks with cells immobilized in this way are to be collected, this alternative method would degradate to a multi-step process again.

As a forerunner to immobilization in polyurethane foam, Lindsey & Yeoman (1983) used nylon pan scrubbers in combination with agar or alginate as support material. However, these techniques are very cumbersome and were not applied later on.

The formation of plant cell aggregates always takes place to a certain extent in 'free cell' suspensions. However, the size of the aggregates is very dependent on the type of plant cells which can vary in the range up to 30 mm. Even in the same cell suspension a great variety exists in aggregate

diameters. Controllability of the aggregate diameters by external measures is hardly possible until now. Due to this fact, in addition with the fact that aggregates in most cases are of varying shapes, aggregates are not very suitable for usage in a bioreactor (Prenosil & Pederson, 1983).

Growth and production

Production of biochemicals by immobilized plant cells can be classified into three categories (Brodelius et al, 1979; Brodelius & Mosbach, 1982). First the 'de novo' synthesis (e.g. anthraquinones by Morinda citrifolia cells). This type of production is called 'de novo' because the product is formed in a multistep synthesis out of simple carbon and nitrogen sources as the basic materials. The second type is the formation of products from precursors (e.g. indole alkaloid ajmalicine from tryptamine and secologanine by Catharanthus roseus). Here, the precursors are transformed into the product by a limited number of metabolic reaction steps. The third one is called the biotransformation, where a substrate is transformed into the product by one step within the cell metabolism (e.g. digitoxin to digoxin by Digitalis lanata). All cases of production by plant cells described in the literature can be classified under one of these three types of product formation.

After immobilization of plant cells it is important to know if they have survived the immobilization procedure and if so, can the plant cells stay alive in the period after immobilization. The latter item is mainly dependent on the possibility of substrate transfer from the medium. This will be discussed later in this paper.

The way to express the cell viability is open for discussion (Lindsey & Yeoman, 1984a). Brodelius & Nilsson (1980) gave three distinct methods to test the cell viability. First, the intactness of the cell membrane, which is tested by staining techniques. Other staining techniques are based upon the presence of specific enzymes in the cell, for instance esterases by fluorescin diacetate (Galun et al, 1983). A second indication of cell viability is measuring the retained cell respiration after immobilization by means of an oxygen electrode. The third test is determination of the capability of cell growth and cell division (indicated by mitotic index, (Brodelius, 1984)) after immobilization. From experiments by Brodelius & Nilsson (1979) it can be concluded that membrane intactness is not a good criterion for cell viabi-

lity because they found that glutaraldehyde used as an crosslinking agent for gelification does not destruct the membrane, but respiration and cell growth after immobilization did not take place. Lindsey & Yeoman (1984a) used determination of enzyme activity (cell staining) and nutrient uptake by the immobilized cells as a measure for cell viability beyond the respiration of cells. ^{31}P NMR spectra, which can be used as a measure for intracellular pH-shift, were also successfully used by Brodelius and his group (Brodelius & Vogel, 1984; Brodelius, 1985) as an indication for cell viability of immobilized cells. In most studies cell viability was tested by respiration measurements (Nakajima et al, 1985). Nakajima et al (1985) also confirmed cell growth by determination of increase of cell number and chlorophyll content. Another obvious method is the microscopic observation of the state of the immobilized plant cells, something that could be done as a first indication of immobilization survival.

In general, alginate entrapped cells show a high retention of viability. The support material has been used in most studies on immobilized plant cells after it appeared to give the best results compared with other supports (Brodelius & Nilsson, 1980). Veliky & Jones (1981) used alginate entrapped Daucus carota cells for the biotransformation of gitoxigenin to 5β-hydroxy-gitoxigenin. Cell viability was determined by measuring the respiration rate which was directly correlated with the rate of biotransformation (Jones & Veliky, 1981b). The biotransformation of β-methyldigitoxin to β-methyldigoxin with alginate entrapped Digitalis lanata was studied by Alfermann et al (1980). The biotransformation activity of the immobilized cells corresponded to one-half of that of free cells under the same conditions and remained constant for more than 60 days, much longer than that of free cells. After this period the alginate gel started to disintegrate. Later on, Alfermann et al (1983) showed that the constant production rate could be prolonged to at least 170 days with alginate immobilized cells. In contrast, the production by free cells parallels cell growth, reaching a maximum after 13 days. Free as well as immobilized cells excreted more than 90% of the product into the medium. No explanation was given why disintegration of beads did not occur in this case. The growth of alginate immobilized cells of Daucus carota and Petunia hybrida was investigated by Hamilton et al (1984) and compared to free cells and appeared to be quite similar. They also investigated phenolics production by immobilized D.carota cells at different temperatures and found

an optimum range between 22 and 23°C. Alginate entrapped D.carota cells were chosen by Jones & Veliky (1981a) as a model system mainly for studies of the effects of a number of medium constituents on the cell viability by measuring the respiration activity, which resulted in the formulation of a simple buffer mixture without loss of the production capacity of the cells. Nakajima et al (1985) used agar, κ-carrageenan and alginate to immobilize Lavandula vera cells for production of blue pigments. Alginate entrapped cells for pigment production were used for over 7 months by alternating growth and production phases. The total amount of pigment production of immobilized cells was similar to that of free cells. In both cases the blue pigments were excreted into the medium. Alginate entrapped cells of Mucuna pruriens were used by Wichers et al (1983) for the biotransformation of L-tyrosine into L-DOPA. In the experiments they did not observe cell release from the beads. In contrast to a free cell suspension of M.pruriens, where 90-95% of the L-DOPA was stored intracellularly, immobilized cells excreted 90% of the total amount of L-DOPA produced. Additional Ca^{2+} in the medium to stabilize the beads severely inhibited the synthesis of L-DOPA. Alginate entrapped Papaver somniferum cells were used by Furuya et al (1984) in order to perform the biotransformation of (-)codeinone to (-)codein. In shake flasks, 88% of the codeine converted was excreted in the medium and the immobilized cells were biologically active for 6 months. Robinson et al (1985) experimented with algal cells (Chlorella emersonii) in alginate beads. They mainly investigated cell growth in the beads and cell leakage from the beads. Occurence of cell leakage with alginate was mentioned as a serious problem. Cell leakage was stopped by drastic decrease of the phosphate concentration in the medium from 66.7 mM to 1 µM without significant affecting the respiration activity and growth of the cells. Also Tamponnet et al (1985) used alginate immobilized algal cells (Euglena gracilis). They studied the long term storage of immobilized cells, which could be more than 2 years in case of Ca-alginate. The effects of immobilization (agar, agarose, κ-carrageenan and alginate) and environmental stress (low temperature, periodical absence of oxygen and carbohydrate-free medium) on growth and production of non-polar metabolites with Tagetes minuta cells were investigated by Ketel et al (1987). As a result, they observed a significant inhibition of cell growth in case of alginate compared to the other gel supports. They also observed that the effect of low temperature (10°C) is prompting the immobilized cells to excrete the non-polar metabolites into the medium.

Lindsey et al (1983) tested the immobilization method with polyurethane foam for Capsicum frutescens and Daucus carota. They investigated cell retention and viability: 95-99% of the cells remained in the foam particles after 4 days of cultivation and 70-80% of the viability was left of both C.frutescens and D.carota cell species after 21 days of cultivation. It was found that the capsaicin production by immobilized cells of C.frutescens was two orders of magnitude higher than in freely suspended cells. In both cases the capsaicin was excreted into the medium. Addition of precursors gave a 50-60 fold higher yield in polyurethane immobilized cell cultures (Lindsey & Yeoman, 1984b). Cold storage at 3°C of C.frutescens (for 4 weeks) and D.carota (for 6 weeks) immobilized in polyurethane foam did not affect the cell viability (Lindsey & Yeoman, 1984a). Mavituna & Park (1985) showed with their experiments that the growth pattern of freely suspended cells and polyurethane immobilized cells of C.frutescens are comparable.

Mentha cells, immobilized in polyacrylamide-hydrazide, were used by Galun et al (1983) for biotransformation of (-)menthone to (+)neomenthol and (+)pulegone to (+)isomenthone. If compared to free cells, the immobilized cells show a similar rate of biotransformation. Repeated use of immobilized Mentha cells did not affect the biotransformation capability. Later on, Galun et al (1985) used γ-irradiated Mentha cells, resulting in non-dividing cells, to suit the purpose of biotransformation of monoterpenes in a continuous process without growth of cells.

Jirku et al (1981) investigated the production of steroid glycoalkaloids with Solanum aviculare cells on polyphenyleneoxide. Adding the substrate sucrose semicontinuously to the immobilized cells, the glycoalkaloids were released into the medium with significant regular oscillation in time, varying from 0.10 to 0.30 mg per gram dry cell weight per day.

Mass transfer and diffusional limitations

One of the objectives to immobilize plant cells is the improvement of the secondary metabolite production rate. It has been observed that immobilized plant cells have in some cases a higher production rate than free cells in suspension under otherwise the same conditions (Brodelius & Mosbach, 1982). No satisfying explanation has been found for this phenomenon. However,

Mattiasson & Hahn-Hägerdal (1982) hypothesized that microenvironmental effects, which apply for immobilized cells, could change the metabolic behaviour of the immobilized cells. In general, for plant cells, the production takes place when the cells are not able to grow (Yeoman et al, 1980). This 'non-growth' condition is fulfilled when plant cells have reached their stationary phase in their growth cycle. In case of free cell suspensions it is here where in general the secondary metabolite production starts (Lindsey & Yeoman, 1983). Immobilization of plant cells is one of the means to meet this 'non-growth' condition. Although it is not clear what the real mechanisms are for this so called 'stress', one hypothesis is the occurrence of a diffusional barrier of substrates and products to and from the plant cells in immobilized state or in big cell aggregates (Shuler et al, 1984). The latter is stated by Shuler (1981) in relation to cell growth. He mentioned the occurrence of substrate diffusion limitation which results in partially differentiated cells in the centre of the aggregates named as 'feeder' cells. These cells seem to excrete products which are thought to be stimulatory to peripheral cells.

In studies of substrate diffusion limitation of substrate consuming biocatalysts in gel beads, the importance of knowledge of the value of the diffusion coefficient of the concerning substrate in the gel material is evident. Tanaka et al (1984) measured glucose diffusion coefficients in 2% Ca-alginate gel beads ($6.83*10^{-10}$ m^2 s^{-1}) and found that this was equal to that in pure water ($6.80*10^{-10}$ m^2 s^{-1}) at 30°C. Nguyen & Luong (1986) did the same for 3% κ-carrageenan and found a diffusion coefficient of $4.8*10^{-10}$ m^2 s^{-1} at 30°C, which is 70% of the value in water. Mavituna et al (1987) determined the effective diffusion coefficient of glucose in callus of Capsicum frutescens, which was supposed to be approximating the value of the diffusion coefficient in polyurethane foam immobilized cells. Their results gave a range of $0.028-0.28*10^{-9}$ m^2 s^{-1} for the diffusion coefficient at 25°C.

Not only the type of gel is important, also gel concentration and cell loading within the gel affects the diffusion rate of substrates (Klein & Manecke, 1982). To this purpose, Hannoun & Stephanopoulos (1986) determined the influence of yeast cells on the effective diffusion coefficients of glucose and ethanol in Ca-alginate. At a cell concentration of 20% (w/w), no effect of the cells on the diffusion coefficients could be determined by them. Other workers (Rhodes et al, 1985) attach much value to the formation

of a thin mucilaginous film covering the plant cells immobilized in polyurethane in relation to diffusion-limited oxygen uptake kinetics. This film is formed by secretion of polysaccharides by the plant cells in immobilized state.

Especially exhaustion of oxygen, because of its relative low solubility in water or aqueous media, can play an important role in connection to growth and production of plant cells (Veliky & Jones, 1981; Jones & Veliky, 1981; Adlercreutz, 1985). Brodelius (1984) observed that growth of plant cells entrapped in alginate only occurred at the periphery of the beads. He reported that this was possibly caused by an oxygen diffusion barrier in the alginate matrix. Hulst et al (1985b) investigated the diffusional limitation of oxygen for plant cells immobilized in agar, agarose, κ-carrageenan and alginate by respiration measurements in order to explain the higher production rate of ajmalicine by Catharanthus roseus cells when immobilized in alginate as compared to other supports (Brodelius & Nilsson, 1980). Assuming an equal diffusion coefficient for oxygen in these gel materials, a higher oxygen diffusion limitation in the centre of the alginate beads with respect to the other gels, could not be determined.

Although some work on diffusion of oxygen in beads with immobilized plant cells or cell aggregates has thus been done, it still needs further research to obtain a better understanding of the influence of oxygen on immobilized plant cells.

REACTORS

Experiments with immobilized plant cells which are described in the literature are in most cases executed in the most simple 'reactor' configuration: batch cultivation in an Erlenmeyer flask, varying in volume from 50 to 500 cm^3, on a rotary shaker. It implies that to date the use of more advanced bioreactors for immobilized plant cells is still in its infancy in contrast to free cell suspensions. Free plant cell suspensions have been studied in airlift loop reactors (Schmauder et al, 1983; Fowler, 1981; Smart & Fowler, 1984; Breuling et al, 1985), stirred tank reactors (Spieler et al, 1985) and bubble columns (Kato et al, 1975). Nevertheless, some work has been done with immobilized plant cell bioreactors.

Majerus & Pareilleux (1986) used calcium alginate entrapped cells of

Catharanthus roseus for the continuous production of indole alkaloids in a simple 1 dm^3 Erlenmeyer reactor which was placed on an orbital rotary shaker. Their system was functional for more than two months, but only a small amount of the alkaloids was excreted into the medium. Enhanced excretion of the products was obtained by lowering the pH value in the medium from 6 to 5.

Plant cells of Daucus carota immobilized in Ca-alginate beads (2.5-3.0 mm diameter) were used by Veliky & Jones (1981) in a column reactor with a rather small volume of approximately 0.20 dm^3 (Figure 2). The reactor was operated by two different ways of air supply. Firstly, 'external' aeration of the medium with subsequent flow through a packed bed of the beads and secondly, 'internal' aeration with the bioreactor operated as a bubble column. The reactor was run in a semi-continuous way and was functional for more than 30 days. The way of oxygen supply had significant effect on the rate of biotransformation of gitoxigenin to 5β-hydroxygitoxigenin by the cells: with 'internal' aeration a conversion of 75-80% could be measured and 60-65% in case of 'external' aeration.

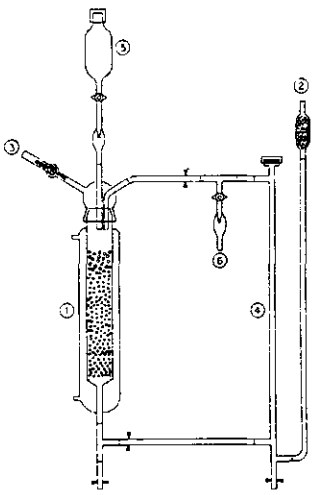

Figure 2.
Column bioreactor: 1) column with immobilized cells in Ca-alginate beads; 2)air inlet; 3) air outlet; 4) airlift pump; 5) medium-buffer reservoir; 6) sampling outlet; (from Veliky & Jones, 1981).

A 0.140 dm^3 bubble column was used by Furuya et al (1984) in which they investigated the effects of temperature and the aeration rate on the observed biotransformation of (-)-codeinone to (-)-codeine by alginate immobilized Papaver somniferum cells. A packed-bed reactor was used by Brodelius et al

(1980) (Figure 3). They used alginate immobilized cells of Catharanthus roseus for the production of ajmalicine. The medium was continuously circulating through an external vessel containing chloroform, which was used for the extraction of the product. Traces of chloroform in the circulating medium did result in a pronounced release of the product from the cells to the surrounding medium. Morris et al (1983) made use of a fluidized bed reactor with a working volume of 0.857 dm^3. In their experiment, alginate immobilized cells were used for the continuous production of a fine plant cell suspension. But the reactor can also be used for production of secondary metabolites by immobilized plant cells. According to the researchers, upscaling of this reactor to 30 dm^3 will give no problems except for the energy input to keep the bed fluidized.

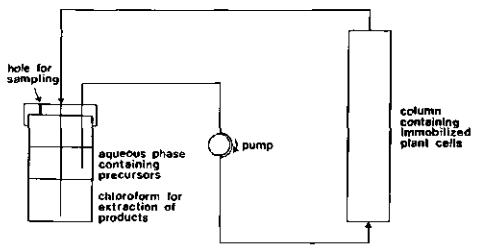

Figure 3.
Schematic diagram of the experimental setup used for continuous extraction of lipophilic products; (from Brodelius et al., 1980).

Hamilton & Pedersen (1984) also used a fluidized bed reactor (0.400 dm^3 medium volume, 0.075 dm^3 total alginate bead volume) in which sugar conversion and phenolics production were studied. The way they aerated the reactor is rather notable: air was sparged into the medium in an external reservoir just below the inlet port to the reactor itself so that in this way medium and air bubbles were pumped into the reactor.

Lindsey & Yeoman (1983) used a batch reactor based on percolation of the liquid medium through a bed of plant cells immobilized in nylon pan scrubbers and gel material. The medium was continuously circulated through the bed.

Large aggregates (up to 2 cm diameter) of Tagetes patula were cultured in a 165 dm^3 airlift-loop reactor by Hulst et al (1987). The aggregate size did not change and the cells stayed viable during the experiment. However, after one week the experiment was terminated due to microbial infection. Within that time no secondary metabolites (thiophenes) could be detected in the medium.

Another type of immobilized plant cell reactors, known as membrane reactors, was divided by Shuler et al (1984) in five possible configurations namely hollow fiber units, flat plate systems, spiral wound units, tubular membrane reactors and multimembrane reactors (Figure 4). The principle of a membrane reactor is that the plant cells are physically separated from the liquid production medium by a membrane, allowing substrates, nutrients and cell products freely to pass. In comparison to gel immobilized plant cells Shuler et al (1984) mentioned better control of fluid dynamics and flow distribution, a direct access to the cells during actual operation, and an easier scale-up as advantages of the membrane reactors. However, membrane reactors are expensive, liable to fouling, and the occurrence of more severe problems with gas transfer may be considered as drawbacks. An additional problem for the tubular membrane reactor is the difficult manual loading of the cell solutions into the dialysis tubing while still maintaining sterility (Shuler et al, 1986).

A preliminary experiment (phenolics production by tobacco cells in a flat plate system) showed good prospects for a continuous production process, but also demonstrated that the membrane resistance to mass transfer and the thickness of the cell layer are important variables in the design of a membrane reactor.

Other workers (Prenosil & Pedersen, 1983; Jose et al, 1983) used hollow fiber reactors and studied the production of phenolics with Daucus carota cells and the invertase activity of the cells by measuring the sucrose conversion to glucose.

An inherent advantage to the usage of membranes is that they give an extra prevention against microbial contamination in the plant cell compartment (Prenosil & Pederson, 1983).

Several aspects of immobilized plant cell reactors and reactor operation have been recently reviewed by Rosevear & Lambe (1986).

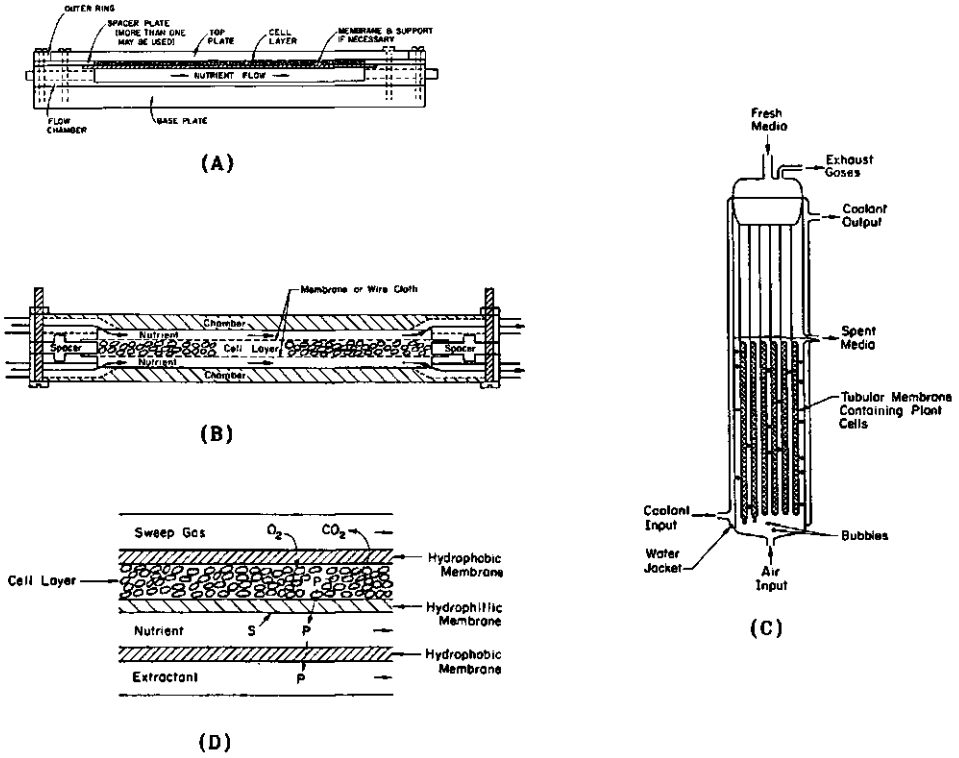

Figure 4. Configurations of membrane reactors: A) one-sided flow reactor; B) two-sided flow reactor; C) tubular membrane reactor; D) multi-membrane reactor; (from Shuler et al., 1984; Shuler et al., 1986).

PRODUCT RELEASE

A continuous process with immobilized plant cells is only feasible if the product is released by the cells. However, secondary products are often stored in the vacuole of the cultured cells. According to Lindsey & Yeoman (1985), inducing the release of intracellularly retained products is perhaps the most obvious potential difficulty. However, in order to obtain release of the products, some techniques are described in the literature.

It was observed that products were excreted by the cells in response to

immobilization per sé for some unexplained reason (Lambe & Rosevear, 1983). Better results could be achieved by using chemical agents to alter the membrane structure of the cells. Brodelius & Mosbach (1982) reported that permeabilization of the plant cells can be carried out with organic solvents, such as ether, toluene or dimethylsulfoxide (DMSO), with proteins such as cytochrome C or protamine, or with lipophilic compounds such as nystatin or lysolecithin. Fuller & Bartlett (1985) reported the successful use of the detergent cetyl trimethyl ammonium bromide (CTAB) as permeabilization agent for plant cells. It was also observed by Brodelius et al (1980) that traces of chloroform in the medium probably permeabilized the cell membranes of Catharanthus roseus.

It has been shown by Brodelius & Nilsson (1983) that it is possible to release products by permeabilization of the cell membranes with DMSO without affecting the viability or biosynthetic capacity of the immobilized cells. Majerus & Pareilleux (1986) observed a pronounced product release from plant cells by decreasing the pH of the medium (from 6 to 5) in their experiments.

One can see that there are a number of possibilities to release secondary products from the immobilized plant cells. In contrast to the opinion of Lindsey & Yeoman (1985), it was Shuler et al (1986) who optimistically stated that product accumulation in the cells is not an insurmountable barrier to the use of immobilized cells.

FUTURE PROSPECTS

Immobilized plant cells used for secondary metabolite production can be applied in two different ways, which are schematically given in Figure 5. This figure roughly outlines the process from plant to product. The first application concerns the production of fine cell suspensions under growth conditions (Morris & Fowler, 1981) and the second application is the use of cells in order to produce secondary metabolites under optimum conditions for production. Despite all successful research that has been done in the past few years and all the hopeful expectations within the field of immobilized plant cells, no announcements of economically benificial industrial processes with immobilized plant cells have been reported. This development, to our opinion, could partly be ascribed to one or more causes which will be mentioned below.

- The research on immobilized plant cells has only been started in 1979.
- The types of optional products from immobilized plant cells are limited to only high-value compounds with a small market.
- If suitable cell lines have been found, the overall costs of producing secondary metabolites by immobilized plant cells are too high with respect to conventional production processes (e.g. chemical synthesis, extraction from whole plants).
- Improvements of production by immobilized plant cells are often marginal with respect to already existing processes.
- The immobilization procedure is an extra risk for contamination and requires extra investments.
- General characteristics of plant cells like slow growth and cell aggregation do not encourage industrial application.
- Most of the secondary pathways in plant cells are very complex and not well understood.
- Plant cells generally show genetic instability after subculturing for a long time.
- The consequences of 'stress' conditions such as immobilization of cells, which can trigger secondary metabolism in the cells, are not very well understood.
- Products are often stored in the cells, which makes them unattractive for use in continuous processes.

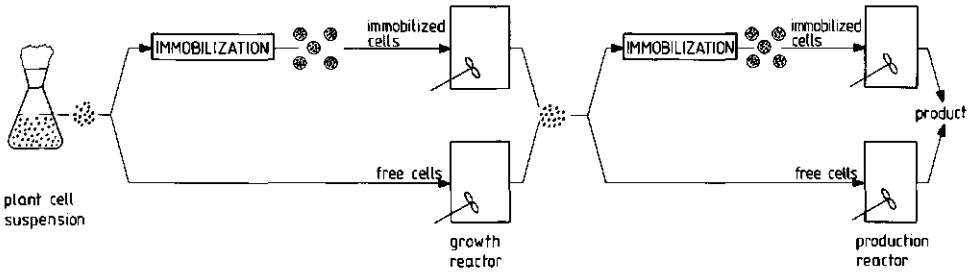

Figure 5. Schematic diagram of the possible applications of immobilized plant cells in view of secondary metabolite production.

It can be expected that future research will mainly be aimed at obtaining

more insight in these problems. According to Rosevear & Lambe (1986a), immobilized plant cell technology is likely to have its greatest impact in the so-called 'new product area', like new bitter flavours and anticancer agents. They also see immobilized plant cells as producers of phytoallexines as natural antiviral and antimicrobial agents, if elicitors are supplied. Scott (1987) expects a continuation of study of new approaches and materials for immobilizing plant cells but emphasizes that the research trend will probably be directed towards the more effective utilization of existing immobilization techniques. In contrast, Rosevear & Lambe (1986a) emphasized that there is still further scope for new polymer matrices with low toxicity, high rigidity and high porosity. Further investigations are required in the biochemistry of some secondary metabolic pathways and the factors involved in its control (Lindsey & Yeoman, 1985; Rhodes et al, 1986). As future prospects Fuller & Bartlett (1985) reported that there will be a need for more productive cells to immobilize rather than more ways of immobilizing relatively poor producing cells. The conception that immobilization of plant cells is now sufficiently well developed will shift the major emphasis to process development, including operation of pilot plants (Scott, 1987). Fuller & Bartlett (1985) extend this view by integration of downstream processing of the product in the process.

Apart from research aimed at processing of products, immobilized plant cells can serve as a valuable tool for fundamental physiological studies like interrelation of cells in whole organisms (Fowler, 1985; Rosevear & Lambe, 1986).

In our opinion, it can be concluded that the research area on immobilized plant cells is a relatively young one and many unanswered questions will need more research in the future. However, it is obvious that immobilized plant cells offer potentials to both industrial production of high value compounds (Kennedy, 1982) as well as a tool in scientific research, but on the short term no one should expect spectacular developments. This is also the view of some other workers in the research area of plant cell biotechnology (Brodelius, 1985; Fuller & Bartlett, 1985).

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CHAPTER 3

A NEW TECHNIQUE FOR THE PRODUCTION OF IMMOBILIZED BIOCATALYST IN LARGE QUANTITIES

A.C. Hulst, J. Tramper, K. van 't Riet, J.M.M. Westerbeek

Department of Food Science, Food and Bioengineering Group,
Agricultural University Wageningen,
De Dreijen 12, 6703 BC Wageningen, The Netherlands.

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SUMMARY

A new technique is presented for the production of immobilized biocatalysts in large quantities. It consists of breaking up a jet of the biocatalyst/pre-support mixture in uniform droplets by means of a resonance technique. Entrapment of yeast and plant cells in calcium alginate has been used as the model. The production capacity of the nozzles used (0.5, 0.8 and 1.1 mm exit diameters) is two orders of magnitude larger than the production capacity of the conventional techniques (maximum capacity with 1.1 mm nozzle diameter is 24 $dm^3\ h^{-1}$). Depending on frequency, nozzle diameter and volumetric flow rate, the bead size varies between 1 and 2 mm, with standard deviations of 3 - 5 % for yeast immobilization and 10 - 15 % for plant cells. The deactivation of both yeast and plant cells is small and comparable to that found in the corresponding conventional procedures.

INTRODUCTION

Immobilization of biocatalysts by entrapment in hydrogels has been reported frequently. Typical examples are entrapment in calcium alginate (Brodelius & Mosbach, 1982), κ-carrageenan (Brodelius & Nilsson, 1980; Tosa et al, 1979) and chitosan (Vorlop & Klein, 1981). These authors commonly obtain droplet formation by pumping the biocatalyst/pre-support mixture through a needle at a speed slow enough to prevent a jet. Consequently, the production capacity is poor and can only be improved by increasing the number of needles. However, for application on industrial scale, or even on 10 dm^3 scale, it is inadequate.

Small spherical biocatalyst beads of uniform size are desirable for several reasons. When the beads are too large, the reaction rate by diffusion of the substrate(s) and/or product(s) is likely to be limited. On the other hand, too small and irregularly shaped particles with a wide size distribution bring along problems such as large pressure drops over, channeling in, or even clogging of packed-bed reactors, and abrasion and wash-out in stirred vessels. For industrial applications, an immobilization procedure should at least preferably satisfy the following two demands: High production capacity and formation of beads with a uniform, optimum size.

It is obvious that the conventional dripping method does not satisfy the first demand. In this article, a technique is described which satisfies the

first demand better than the conventional method while yielding biocatalyst beads having equal good properties. It is based on the breakup of a jet of the biocatalyst/pre-support mixture in uniform droplets by means of mechanical vibration. Immobilization of yeast (Saccharomyces cerevisieae) and plant cells (Haplopappus gracilis) in calcium alginate has been used as the model.

THEORETICAL BACKGROUND

Much has been published about the mechanism of breakup of a liquid jet (Rayleigh, 1878; Dabora, 1967; Hinze, 1979; Schmidt & Walzel, 1980). In the late 19th century, Rayleigh (1878) theoretically analyzed the instability of capillary jets and found relations which predicted the operational conditions to cause breakup of a Newtonian liquid (e.g. water). This theory was not applicable to non-Newtonian liquids. Weber (1931) extended the analysis to include the effect of liquid viscosity. The optimum wavelength for breakup, according to Weber, is given by:

$$\frac{\lambda_{opt}}{d_j} = 4.44\ (1 + 3Vi)^{\frac{1}{2}} \qquad (1)$$

with:

$$Vi = \frac{\mu_v}{(\rho_v \sigma d_j)^{\frac{1}{2}}} \qquad (2)$$

where Vi is the viscosity number (dimensionless); μ_v is the dynamic viscosity (Ns m^{-2}); ρ_v is the density (kg m^{-3}); and σ is the surface tension (N m^{-1}).

The frequency (f) is related to the jet velocity (u_j) and the wavelength by:

$$f = \frac{u_j}{\lambda} \qquad (3)$$

When a jet is mechanically vibrated at such a frequency, drops of uniform size are formed. According to Schneider and Hendricks (1964), however, a range of wavelengths (related to frequency by equation (3)) exists where uniform size of drops still occurs. As a range they give:

$$3.5\ d_j < \lambda < 7d_j \qquad (4)$$

Considering that one liquid cylinder with length λ breaks up in one droplet with diameter d_p, it can be shown that (as a result of conservation of mass):

$$\frac{1}{4}\pi d_j^2 \lambda = \frac{1}{6}\pi d_p^3 \qquad (5)$$

When the volumetric flow rate is expressed as:

$$\phi_v = u_j \frac{1}{4}\pi d_j^2 \qquad (6)$$

then the droplet diameter (d_p), after substituting eqs. (3) and (6) into eq. (5), can be calculated by:

$$d_p = \left(\frac{6\phi_v}{\pi f}\right)^{1/3} \qquad (7)$$

Substitution of eq. (5) into eq. (1) gives:

$$\frac{d_p}{d_j} = 1.89\ (1 + 3Vi)^{1/6} \qquad (8)$$

Figures 1 and 2 represent eqs. (1) and (8), which show that the influence of the viscosity is negligible when $Vi < 1$.

Recently, the breakup of a liquid jet has been investigated for viscoelastic liquids such as polymer melts and polymer solutions (Schümmer & Tebel, 1983). In particular, the influence of the elastic behaviour has been studied on the breakup characteristics of the jet. It is clear that the elastic properties of the liquid have an influence on the rheological behaviour during the very small time lapse in which the breakup takes place (less than 30 ms). The viscoelastic approach (elongational flow behaviour) has not been applied for this article. This is not only because of the complexity of this approach, but also because the rheological behaviour of the sodium alginate solutions used in this study was found to be pseudoplastic. Therefore, since the only objective was to obtain a rough idea of the operational conditions in advance, Weber's theory could be used satisfactorily.

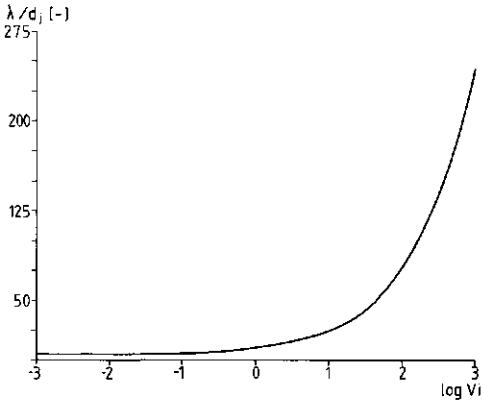

Figure 1.
Optimum wavelength for breakup of a jet as a function of the viscosity number.

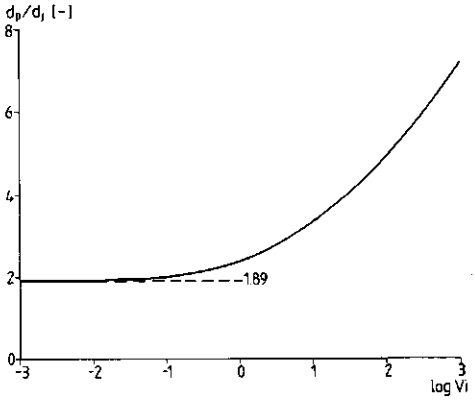

Figure 2.
Obtained droplet diameter at the optimum wavelength as a function of the viscosity number.

MATERIALS AND METHODS

Biocatalysts and preparation for immobilization.

Yeast (normal trade mark, Gist-Brocades) was prepared in the following manner. A clump of wet cells was dispersed in water [(1:1 (w/w)] and 3 cm^3 of this suspension was used for the dry weight determination. Part (100 cm^3) of this suspension was mixed with 200 cm^3 3 % (w/v) sodium alginate (Alginate Industries Ltd., England; type Manucol DM) solution.

Plant cells used were Haplopappus gracilis. Culture conditions consisted of growth in the dark in 50 cm^3 B5 medium (Gamborg et al, 1968) in 250 cm^3 flasks on a rotary shaker (120 rpm, 28°C). The inoculum was 7 cm^3 from the previous culture. Harvesting was performed after ca. 10 days.

The collected suspension was filtered on a Büchner unit and, to eliminate the growth medium, washed with a 0.05 M NaCl solution. The washed cells were resuspended in 50 cm^3 NaCl solution and sieved (grid diameter 0.5 mm) to eliminate large aggregates. After settling of the cells, the liquid was decanted and 10 cm^3 of the cell suspension used for dry weight determination. Part (100 cm^3) of the suspension was mixed with 200 cm^3 3 % (w/v) sodium alginate.

Assay procedures.

Respiration of the free and immobilized cells was measured in a Biological Oxygen Monitor (YSI Inc., model 53) under the conditions given in Table 1. After saturation of the medium with pure oxygen, the (immobilized) biocatalyst was added and the consumption of oxygen recorded as a function of time. The rate of consumption was determined from the slope of the curve at 80% of saturation conditions and expressed in moles per second per kilogram (dry weight) of biocatalyst.

The viscosity of the alginate(-cell) suspensions was measured with a viscosymeter (Haake Rotovisko, type RV), at 20°C.

Determination of the dry weight was as follows: 3 cm^3 of yeast suspension or 10 cm^3 of plant cell suspension were pipetted in a preweighted weighing flask, the suspension evaporated to dryness on a waterbath (90°C), and dried in an oven at 110°C for 1 h. After cooling in a desiccator the dry weight was determined and expressed as g dm^{-3} suspension. The determination was done three times.

	Yeast cells	Plant cells
Substrate 1	pure oxygen	pure oxygen
Substrate 2	glucose (8.33 g/dm^3)	saccharose (20 g/dm^3)
Assay volume	3 mL	3–8 mL[a]
Medium	distilled water	B5 medium
Temperature	30°C	30°C

[a] Volume depended on the amount of beads introduced.

Table 1. Conditions for the respiration measurements.

Immobilization with the vibration nozzle.

The principle of the technique used, and originally developed and studied for other purposes (Aarts, 1976), is as follows: A stock vessel (Figure 3) is filled with alginate-cell suspension and closed. By applying air pressure, the suspension is transferred to the nozzle and the liquid velocity in the formed jet adjusted by means of the air pressure controller. The signal of the sinus generator is transformed by the vibrator into a mechanical movement of the pin, which is connected to the rubber membrane of the nozzle (Figure 4). The signal is transmitted to the liquid by the rubber membrane and causes the vibration in the jet, which results in the breakup into droplets. The

nozzle diameter can be changed by using another capillary. The droplets are collected in a 0.2 M $CaCl_2$ solution, situated 0.5 m below the nozzle, and hardened for 2 h.

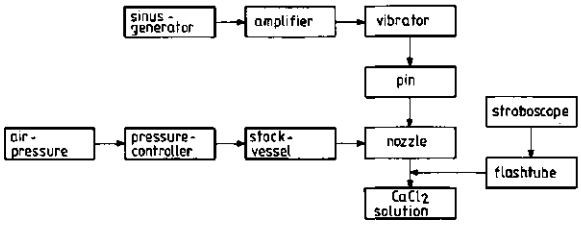

Figure 3. Schematic representation of the immobilization set up using the vibration nozzle.

The following measurements have been done during or after the immobilization procedure:

The production capacity under certain conditions was measured by interception of the produced drops and measuring the volume during a time interval.

The visual judgment of the breakup was done with the aid of a stroboscope.

The determination of the bead uniformity was done by measuring with a binocular the mean diameter of the beads (after shrinkage) in a sample and calculating the standard deviation. Each sample contained 20 beads, taken aselectically from a population.

The droplet diameter after breakup was calculated with eq. (7).

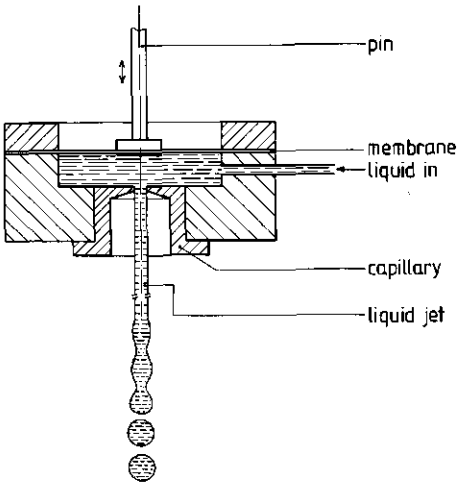

Figure 4.
Schematic representation of the vibration nozzle.

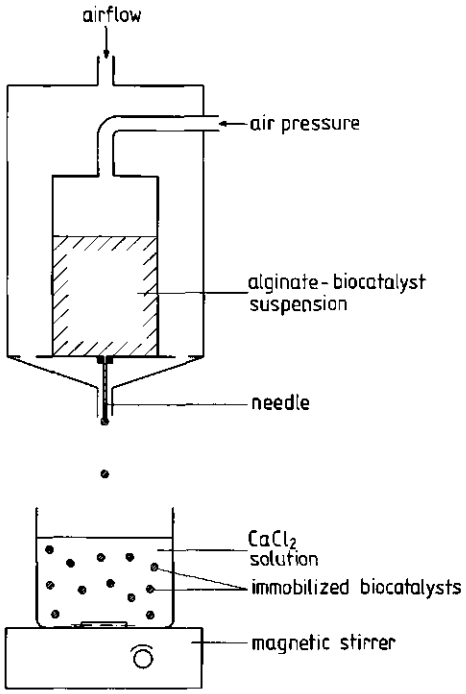

Figure 5.
Schematic representation of the immobilization set up with the dripping method.

Immobilization with the dripping method.

The apparatus is schematically given in Figure 5. The same alginate-cell suspension as previously mentioned was pressed through the needle by means of air pressure. The formed droplets were collected in the 0.2 M $CaCl_2$ solution and hardened for 2 h. The size of the droplets was adjusted by means of a longitudinal airflow.

RESULTS AND DISCUSSION

Influence of frequency on uniformity.

At fixed capillary diameter and liquid velocity, the frequency was varied around the optimum value calculated from Weber's theory. The uniformity of the formed beads was determined. The results are given in Table 2. From the visual judgment, an optimum at 400 s^{-1} for breakup was seen. A picture of the breakup process is given in Figure 6.

A suitable frequency range (300 - 450 s^{-1}) can be observed, which agrees with the findings of Schneider and Hendricks (1964). At smaller frequencies (less than 300 s^{-1}), the breakup was irregular, and nonuniform beads and satellites were formed. At a frequency of 200 s^{-1} and less, no breakup at all was observed. With increasing frequency values above 450 s^{-1}, similar results were obtained. The existence of an optimum frequency value of 400 s^{-1} is also clear from the spread in the diameters (Table 2). Minimal standard deviation was found at the optimum frequency.

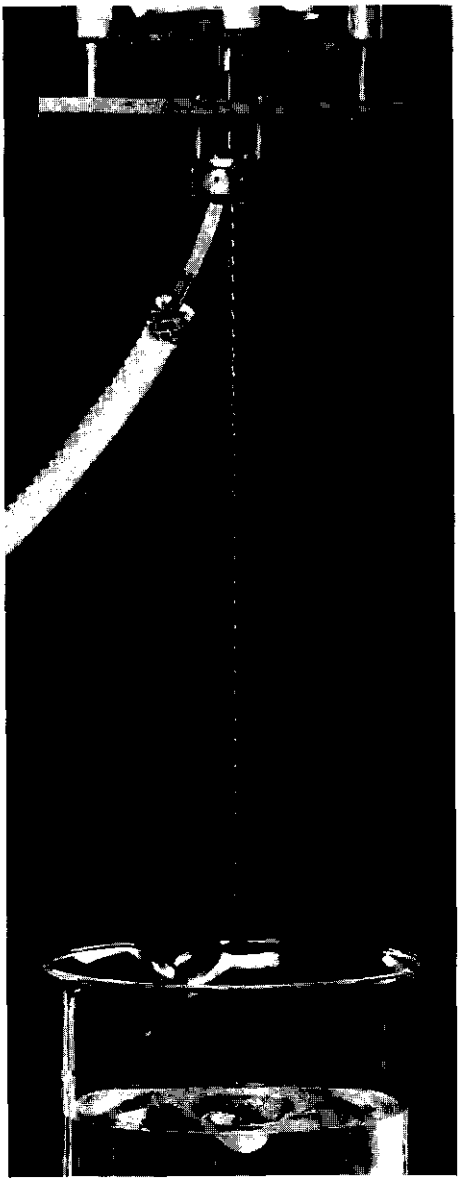

Figure 6.
Photograph of the breakup process of a 2% alginate solution taken at a flash time of $3*10^{-6}$ s.

f (s^{-1})	Visual judgment	$d_{p,s}$ (mm)	Standard deviation (mm)	Standard deviation (%)
200	–	—	—	—
250	±	2.30	0.24	10.4
300	+ +	2.07	0.11	5.3
350	+ +	1.91	0.09	4.7
400	+ + +	1.87	0.07	3.7
450	+ +	1.83	0.10	5.5
500	±	1.85	0.18	9.7
550	–	—	—	—

Table 2. Determination of the optimum breakup frequency of a 2% alginate solution for liquid velocity (u_j) of 3.9 m s^{-1} and capillary diameter (d_j) of 1.1 mm. Optimum frequency from Weber (1931) is 390 s^{-1}, at μ_V = 250 mPa s.

Influence of jet velocity on uniformity of beads.

For two capillary diameters (0.5 and 0.8 mm) the liquid velocity was varied in order to determine the influence on uniformity of the beads. During the experiments the frequency was visually adjusted to its optimum. The results are given in Table 3.

The uniformity of the beads is rather independent of the velocity at d_0 = 0.5 mm. For d_0 = 0.8 mm, on the other hand, the standard deviation slightly increases as the velocity increases. This, however, is not caused by nonuniformity in size, but is the result of deviation from a bead shape. At liquid velocities greater than 8 and 7 m s^{-1} at nozzle diameters 0.5 and 0.8 mm, respectively, there is no question of a bead shape, rather an irregular-shaped particle is found in this case. The maximum volumetric flow rate at nozzle diameter of 1.1 mm, while still obtaining uniform droplets, was 24 dm^3 h^{-1}.

	u_j (m/s)	f (s^{-1})	λ (cm)	$d_{p,s}$ (mm)	Standard deviation (mm)	(%)	ϕ_c (dm^3/h)
	3.6	350	1.03	1.13	0.07	6.2	2.6
	5.1	400	1.28	1.21	0.06	5.0	3.7
d_0 = 0.5 mm	6.4	400	1.60	1.26	0.05	4.0	4.5
	7.7	400	1.93	1.32	0.07	5.3	5.4
	8.7	no breakup in uniform droplets					
	4.5	450	1.00	1.60	0.07	4.4	8.2
	5.0	450	1.11	1.66	0.09	5.4	9.0
d_0 = 0.8 mm	5.6	450	1.24	1.67	0.10	6.0	10.1
	6.1	450	1.36	1.78	0.13	7.3	11.1
	6.6	450	1.47	1.87	0.18	9.6	12.0
	7.0	no breakup in uniform droplets					

Table 3. The influence of the liquid velocity of a 2% alginate solution on the breakup frequency and the uniformity of the beads for capillary diameters of 0.5 and 0.8 mm.

Influence of cells on the uniformity of beads.

The uniformity of the beads was determined for four loads of yeast cells and at three visually determined 'optimum' frequencies. This was also done for one experiment with plant cells in duplo. The results are given in Table 4.

	Cell load (dry wt) (g/g)	u_j (m/s)	f (s^{-1})	$d_{p,s}$ (mm)	Standard deviation (mm)	(%)
	0.51	5.4	450	1.63	0.07	4.3
	0.51	5.4	400	1.67	0.09	5.4
	0.51	5.4	350	1.80	0.09	5.0
	1.12	4.9	400	1.65	0.07	4.2
	1.12	4.9	350	1.74	0.07	4.0
Yeast cells	1.12	4.9	300	1.82	0.09	4.9
	1.54	4.5	400	1.60	0.06	3.8
	1.54	4.5	350	1.70	0.06	3.5
	1.54	4.5	300	1.75	0.07	4.0
	2.23	5.0	400	1.72	0.09	5.2
	2.23	5.0	350	1.83	0.07	3.8
	2.23	5.0	300	1.96	0.13	6.6
Plant cells	0.092	5.0	350	1.84	0.24	13.0
	0.092	5.0	350	1.87	0.20	10.7

Table 4. Influence of cell load on breakup frequency and uniformity of beads. The capillary diameter was 0.8 mm.

From Table 4 can be derived that the load of yeast cells does not

influence the uniformity of the beads. There is no significant difference in uniformity between loaded beads (Table 4) and unloaded beads (Table 3).

Preliminary experiments showed that jet formation did not occur at all with unsieved plant cell suspensions. So it is very important that fine plant cell suspensions are available for immobilization when beads are required smaller than 3 mm diameter. In theory, the bead diameter is not limited to a minimum value, but practically, it is dependent on the dimension of the cells or aggregates, for successful immobilization. The diameter of plant cells is 50 - 150 µm and plant cell aggregates are multiples of this range. These aggregates will clogg the nozzle hole when it is too small, and the jet formation is thus obstructed. The same will happen, however, with the needle technique.

The results in Table 4 show less uniform beads for plant cells, compared with those of unloaded and yeast-loaded beads. This was caused by the cell or aggregate dimensions (100 - 500 µm) disturbing the breakup process.

Comparison of dripping technique and vibration nozzle technique.

Comparison of these two methods was performed by the immobilization of an alginate-cell suspension with both techniques with subsequent determination of the respiration. The techniques were carried out under such conditions that the formed beads were of the same size within 5%. In consequence, these experimental conditions were not optimum for the production capacity for both a single needle and a single nozzle. Under the experimental conditions, the capacity of the nozzle was ca. 10 $dm^3\ h^{-1}$ and for the needle this was ca. 0.1 $dm^3\ h^{-1}$. The choice of the used bead diameter in this comparison was arbitrary.

It has to be noted that the production capacity of one nozzle can easily be increased by adaptation of the nozzle design: it is possible to apply more than one exit in one nozzle without the need to multiply the number of single nozzles. The production capacity of the needle technique is limited by the fact that drop and no jet formation should occur. It can only be increased by multiplying the number of needles (Klein et al., 1983).

The results of the respiration measurements for the yeast cells and plant cells are given in Table 5. It shows that there is no difference between nozzle and dripping method as far as the respiration is concerned. The mean percentages for yeast cells were in both cases ca. 80% of the value of free

cells and for plant cells ca. 70%. The loss of activity of 20% for yeast and 30% for plant cells, as compared to free cells, is probably due to inactivion before or during the immobilization procedure. The conclusion is that the vibration nozzle can be used advantageously for both microbial and plant cells instead of the dripping technique.

	Immobilization technique	Cell load (dry wt) (g/g)	u_j (m/s)	$d_{p,s}$ (mm)	V (mol O_2/kg s)	η (%)	$\bar{\eta}$ (%)
Yeast cells	Vibration nozzle	0.53	5.2	1.61	6.4×10^{-4}	78	78
		1.04	5.3	1.60	6.6×10^{-4}	80	
	d_0 = 0.8 mm	1.54	5.2	1.63	6.3×10^{-4}	77	
	f = 400 s^{-1}	2.04	5.0	1.69	6.0×10^{-4}	73	
	Dripping method	0.53	—	1.61	7.4×10^{-4}	90	80
		1.04	—	1.62	6.0×10^{-4}	73	
	d_0 = 0.3 mm	1.54	—	1.67	5.8×10^{-4}	71	
		2.04	—	1.63	6.9×10^{-4}	84	
	Free cells	—	—	—	8.2×10^{-4}	100	
Plant cells	Vibration nozzle	0.11	5.7	1.68	1.3×10^{-4}	77	72
	d_0 = 0.8 mm	0.11	5.7	1.72	1.1×10^{-4}	67	
	f = 350 s^{-1}						
	Dripping method	0.11	—	1.76	1.1×10^{-4}	65	68
	d_0 = 0.3 mm	0.11	—	1.77	1.2×10^{-4}	70	
	Free cells	—	—	—	1.7×10^{-4}	100	

Table 5. Comparison of immobilization techniques.

Cell damage during immobilization with vibration nozzle.

An experiment was done to investigate if the loss of activity (20-30% related to free cells) is due to the nozzle technique itself. Therefore, the cells were passed through the vibration nozzle a number of times. During each passage some droplets were collected in $CaCl_2$ solution and after hardening the respiration was measured. The experiment has been done twice for plant cells. In Table 6 the results are given for both yeast and plant cells.

Organism	Number of treatments	f (s^{-1})	u_j (m/s)	V (mol O_2/kg s)	$\bar{\eta}$ (%)
Yeast cells	1	400	4.9	8.4×10^{-4}	74
	2	400	5.0	8.5×10^{-4}	75
	3	400	5.1	8.6×10^{-4}	76
	Free cells	—	—	11.3×10^{-4}	100
Plant cells I	1	350	4.8	6.1×10^{-5}	85
	2	350	4.8	7.4×10^{-5}	103
	Free cells	—	—	7.2×10^{-5}	100
Plant cells II	1	350		1.0×10^{-4}	70
	2	350		1.8×10^{-4}	129
	4	350		1.7×10^{-4}	121
	Free cells	—		1.4×10^{-4}	100

Table 6. Activity loss of immobilized cells as a consequence of the vibration nozzle technique. Capillary diameter was 0.8 mm. The respiration measurements have been done in duplicate for yeast cells and plant cells I and in triplicate for plant cells II.

Table 6 clearly shows that yeast cells are not damaged in the vibration nozzle. It is clear that the yeast cells are not sensitive to shear forces nor to the mechanical vibration of the membrane in the nozzle, since the activity of 75% stayed constant after even three treatments. For plant cells, a remarkable feature appeared. The results of the experiments with plant cells show that after two treatments the activity has increased above the level of free cells and then remains constant after more treatments. An explanation could be that the aggregates are broken up by means of the actual shear forces in the nozzle, and the cells occur as single cells in the alginate matrix. The diffusional barrier caused by aggregation is lost in this way.

CONCLUSIONS

We have shown that the vibration nozzle technique can be applied for the immobilization of microorganisms and plant cells in calcium alginate beads. This technique yields uniform beads and has a high production capacity compared to the conventional dripping method.

The operational conditions of this technique for non-Newtonian liquids can be roughly predicted by the theory of Weber. Fine adjustment of the conditions has to be made by observation. Fine cell suspensions do not disturb the breakup process, but cell clumps, which usually occur with plant cells,

cause less uniform beads. The activity of cells is not influenced by the technique.

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NOMENCLATURE

d_j	= diameter liquid jet	[m]
d_p	= diameter droplet	[m]
$d_{p,g}$	= mean diameter, after shrinkage	[m]
d_0	= nozzle diameter	[m]
η	= relative activity of immobilized cells to free cells	[%]
$\overline{\eta}$	= mean relative activity of immobilized cells to free cells	[%]
f	= frequency	[s^{-1}]
f_{opt}	= optimum frequency	[s^{-1}]
μ_v	= liquid viscosity	[Pa s]
λ	= wavelenght of vibration	[m]
λ_{opt}	= optimum wavelenght	[m]
σ	= liquid surface tension	[N m^{-1}]
u_j	= jet velocity	[m s^{-1}]
Vi	= viscosity number	[dimensionless]
V	= maximum respiration rate	[mol kg^{-1} s^{-1}]
ϕ_v	= volumetric flow rate	[m^3 s^{-1}]

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CHAPTER 4

CELL IMMOBILIZATION IN THERMOGELS USING A RESONANCE NOZZLE FOR RAPID DROP FORMATION AND A COLD WATER-IMMISCIBLE SOLVENT FOR GELIFICATION

A.C. Hulst, R.M. Buitelaar, J. Tramper

Department of Food Science, Food and Bioengineering Group,
Agricultural University,
De Dreijen 12, 6703 BC Wageningen, The Netherlands.

SUMMARY

The resonance nozzle immobilization technique was tested with thermo-hardening hydrogels (agar, gellan gum, κ-carrageenan). After nozzling the 'warm' gel solution (both with and without cells) the formed droplets were caught in an ice-cold two-phase system consisting of an organic solvent (n-butyl acetate, n-hexane or n-nonane) as top phase, in which gelification occurs, and medium as bottom phase. The cells used were insect cells (Spodoptera frugiperda), plant cells (Tagetes minuta), yeast cells (Saccharomyces cereviseae) and bacterial cells (Pseudomonas stutzeri). The mechanical strength and shape of the obtained particles were dependent on the combination of the type of organic solvent and the type of gel. Retention of respiration activity of the immobilized plant cells decreased in the order of nonane, hexane and butyl acetate, which corresponds to the general relation of the log P value of the organic solvent and the retention of activity of cells. Bacterial cells show the same trend as plant cells. The retention of respiration activity of yeast cells did not show such relation. Insect cells did not survive the immobilization procedure.

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 is executed by dripping the biocatalyst/-presupport mixture through a needle. Dependent on the type of gel material, the formed droplets are induced to gelify in different ways. For instance, alginate will gelify 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 prohibitive. 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 increased droplet production. Rehg et al (1986) made use of 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 dripping technique with one needle.

We have applied a resonance nozzle in an immobilization technique for production of immobilized biocatalysts in large quantities (see Chapter 3). 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 biocatalyst (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. Immobilization of plant cells (Tagetes minuta), insect cells (Spodoptera frugiperda), yeast cells (Saccharomyces cereviseae) and bacterial cells (Pseudomonas stutzeri) have been used as model systems. The influence of the organic solvent on the respiration of the cells, the mechanical characteristics and the shape of the formed particles are used as criteria for suitability.

MATERIALS AND METHODS

Media

The medium used for growth of plant cells contained 4.72 g dm^{-3} Murashige-Skoog mixture (Murashige & Skoog, 1962) in addition to 100 mg dm^{-3} myo-inositol, 5 mg dm^{-3} N-benzyladenine, 0.5 mg dm^{-3} naphtylacetate, and 20 g dm^{-3} sucrose. The pH of the medium was adjusted to 5.8 with KOH solution. The medium used for the respiration assays contained 4.72 g dm^{-3} MS mixture and 20 g dm^{-3} sucrose.

The medium used throughout for insect cells was Hink's medium (Hink, 1982)

with 10% Foetal Bovine Serum (FBS).

The medium used throughout for yeast cells contained per dm^3 demineralized water: 120 g glucose, 5.19 g $(NH_4)_2SO_4$, 1.53 g KH_2PO_4, 0.55 g $MgSO_4.7H_2O$, 1 g yeast extract, 3.4 g $NaCH_3COO.3H_2O$ and 2.1 cm^3 17.5 M acetic acid.

The medium used throughout for bacterial cells contained per dm^3 demineralized water: 2 g NH_4Cl, 0.1 g $(NH_4)_2SO_4$, 0.075 g $MgCl_2.6H_2O$, 2 cm^3 Vishniac & Santer spore solution, 2.03 g $K_2HPO_4.3H_2O$ and 0.75 g $NaH_2PO_4.H_2O$. The pH was 7.

Cultivation of cells

Plant cells (Tagetes minuta) were obtained from the stock cell line, which was maintained since 4 years in MS-medium at 24°C in 1 dm^3 flasks containing 500 cm^3 medium on a rotary shaker (Gallenkamp Orbital Shaker, 120 rpm) under light conditions (5 W m^{-2}), with subculturing every week (Ketel, 1987). The harvested cells were filtered on a Büchner funnel until adhered water on the cells had disappeared, then weighed and used in the experiment.

Insect cells (Spodoptera frugiperda) were cultivated in 0.5 dm^3 culture flasks on a rotary shaker in the appropriate medium at 28°C. After centrifugation (10 min, 12000*g), the pellet was resuspended in the medium and used in the experiment.

Yeast cells (Saccharomyces cereviseae) were obtained from a local bakery (normal trade mark, Gist Brocades).

Bacterial cells (Pseudomonas stutzeri) were obtained from a frozen stock culture. After thawing, the culture was suspended in medium and used in the experiment.

Assay procedures.

Respiration of the free and immobilized cells was measured in a Biological Oxygen Monitor (own design) which was calibrated in advance. After oxygen saturation of 4 cm^3 MS medium with air, a known amount of the (immobilized) cells was added and the consumption of oxygen recorded as a function of time. The rate of consumption was determined from the slope of the curve at 90% of saturation conditions and expressed in moles per second per kilogram (fresh

weight) of the cells. The temperature was set at 24°C.

Preparation of presupport.

Fifty grams of agar (Difco Laboratories, Detroit, USA) were dissolved in 1 dm^3 demineralized water under continuous magnetic stirring at 100°C on a boiling plate. The agar solution was cooled to 55°C in a temperature controlled cabinet.

Twenty grams of gellan gum (Kelco, Chicago, USA) were suspended in 1 dm^3 $MgSO_4$ solution (0.3 g dm^{-3} $MgSO_4.7H_2O$ in water) with an ultraturrax mixer (IKA, type TP 18/10). Afterwards, the mixture was heated to boiling point in a magnetron (Philips 2010 C) and subsequently cooled to 55°C in a temperature controlled cabinet.

Twenty-eight grams of κ-carrageenan (Genugel type X0828, The Copenhagen Pectin Factory, Denmark) were suspended in 1 dm^3 KCl solution (0.75 g dm^{-3} in water) with an ultraturrax mixer. The mixture was heated to boiling point in a magnetron and subsequently cooled to 40°C in a temperature controlled cabinet.

Experimental procedure.

The respiration rates of samples of the free cells were measured in the Biological Oxygen Monitor.

For each support material, the procedure was as follows. After adjustment to the optimum nozzling conditions (Hulst et al, 1985), 0.5 dm^3 of the presupport solution was nozzled into ice-cold 2 dm^3 Erlenmeyer flasks, each containing 200 cm^3 aqueous medium and 1 dm^3 of an ice-cold organic solvent, creating a two-phase system with the organic solvent on top. The used organic solvents were n-hexane (HPLC-grade, Rathburn Chemicals, Walkerburn, Scotland), n-butyl acetate (GR, Merck, Darmstadt, FRG) and n-nonane (Merck, Schuchardt, FRG). The obtained particles were sieved out of the aqueous medium, washed with destilled water, and visually judged under a microscope in order to qualify shape and uniformity of the particles.

The same procedure was performed with a mixture of 0.5 dm^3 presupport and an accurately weighed amount of cells. The used combinations of cells and gel materials are given in Table 1. Additionally, the respiration kinetics of the immobilized cells were measured and compared to each other. As a blanc, gel

material including the cells, was intercepted after passing the nozzle and was solidified at room temperature without organic solvent. After gelification, the gel layer was cut into small cubes (about 1 mm^3) and the respiration was measured.

cell type \ support type	κ-carrageenan	gellan gum	agar
insect cells	X		
plant cells	X		
yeast cells	X	X	X
bacterial cells	X		

Table 1. Tested combinations of cell type and support type.

RESULTS AND DISCUSSION

Beads without cells

The results of the experiment are given in Table 2. From Table 2 it is obvious that bead formation with 5% (w/v) agar was successful in butyl acetate. Both use of hexane and nonane resulted in variably sized particles. It was observed that non-solidified agar accumulated at the interface between the two phases, resulting in the occurrence of some flat-shaped particles.

	organic solvent								
	butyl acetate			hexane			nonane		
support type	shape[1]	firmness[2]	uniformity[3]	shape[1]	firmness[2]	uniformity[3]	shape[1]	firmness[2]	uniformity[3]
agar	+++	+++	++	--	++	--	---	++	--
gellan gum	---	---	---	++	+++	-	++	+++	-
κ-carrageenan	++	++	+	+++	++	++	+/-	++	--

Table 2. Qualitative judgement of the obtained beads without cells after nozzling in different organic solvents (temperature = 0°C).
+++ very good, ++ good, +/- reasonable, -- bad, ---very bad.
1) judged on spherical shape.
2) subjective 'finger' test.
3) judged on uniformity in particle size.

In case of 2% (w/v) gellan gum, solidification did, for unexplained reason, not occur at all in butyl acetate. In case of hexane and nonane spherical particles were produced, albeit with a great variety in bead diameter. The occurrence of this variety in bead size was partly due to the used amount of gel solution (0.5 dm^3) which limited the total time for the nozzling procedure to about 3 minutes. Within this short time lapse the formation of the jet, setting of the breakup frequency and collection of the formed droplets should occur, so that fine adjustment of the optimum breakup frequency was hardly possible.

Because of a higher viscosity as compared to the gel solutions of agar and gellan gum, 2.8% (w/v) κ-carrageenan solution resulted in easier breakup of the jet in uniform droplets. Spherical particles were obtained with butyl acetate and hexane. The use of nonane resulted in irregular-shaped particles. The beads which were produced by nozzling in butyl acetate consisted of two types with a volume ratio of 2:1. This was probably due to systematical conglomeration of two droplets just after falling in the butyl acetate. The beads produced in hexane were quite uniform. The non-spherical particles produced in nonane partly consisted of conglomerates.

Beads with cells

The break-up properties of gel jets containing the plant cells do result in less uniform droplets if compared to pure gel solution or gel solution containing the other cells. This is due to the relative large size of plant cells which moreover are tending to aggregate to cell clumps, resulting in severe disturbance of jet breakup. The mechanical characteristics and the shape of the obtained beads were identical to those of the unloaded beads (see Table 2).

A fraction of the beads, obtained by sieving (1.0-2.0 mm diameter) was used for the respiration assay. The results of the respiration measurements are given in Figure 1. All values are related to free cells (= 100%).

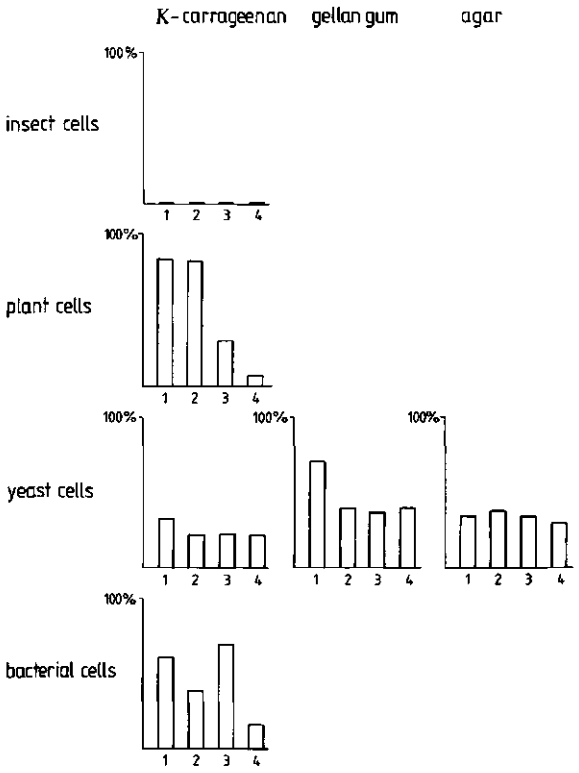

Figure 1. Results of the respiration measurements of immobilized cells by the nozzle technique in organic solvents, according to the experimental set-up in Table 1. All data are referred to free cells (=100%). 1 = blanc, 2 = nonane, 3 = hexane, 4 = butyl acetate.

For the plant cells immobilized in κ-carrageenan a trend of decreasing respiration activity could be observed for respectively nonane, hexane and butylacetate. These results agree with the findings of Laane et al (1987), who related the retention of biocatalyst activity with the log P value of the organic solvent, which was in contact with the biocatalyst. The log P value is a quantitative measure of the polarity of the organic solvent. Laane et al (1987) found no biocatalyst activity for log P less than 2, an increment of activity at increasing log P values between 2 and 4, and high activity for log P values exceeding 4. The log P values of nonane, hexane and butyl ace-

tate are 5.1, 3.5 and 1.7 respectively.

The immobilized insect cells don't have any retention of respiration activity. Immobilization of insect cells by the more gentle needle technique also resulted in non-respiring cells. From this can be concluded that insect cells are not able to survive the immobilization in κ-carrageenan sec.

No significant differences in retention of respiration activity could be observed for the immobilized yeast cells with respect to the used organic solvent. Probably yeast cells are not sensitive for the type of the used organic solvents. For agar and κ-carrageenan the exposure to the organic solvents did not result in a decreasing cell activity. This was the case for gellan gum where about 40% of the yeast cell respiration activity was lost.

The retention of activity for the bacterial cells show the same trend as for the plant cells, except for the use of hexane. In this case, no explanation for the high respiration activity could be found.

CONCLUSIONS

From the described experiments it is obvious that immobilization of cells in temperature solidifying gel materials can be performed successfully by nozzling through water immiscible solvents. The choice of the organic solvent in order to obtain spherical particles is inherent to the type of gel and in case, should be found empirically. In order to obtain immobilized plant cells with a high retention of activity, the log P value of the organic solvent should be considered (preferably higher than 4). This is not the case for yeast cells, which appear to be insensible to the type of organic solvent used in the immobilization procedure.

ACKNOWLEDGEMENTS

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CHAPTER 5

IMMOBILIZED PLANT CELLS: RESPIRATION AND OXYGEN TRANSFER

A.C. Hulst, J. Tramper, P. Brodelius[*], L.J.C. Eijkenboom[+], K.Ch.A.M. Luyben[+]

Department of Food Science, Food and Bioengineering Group,
Agricultural University Wageningen,
De Dreijen 12, 6703 BC Wageningen, The Netherlands.

[*]Institute of Biotechnology,
Swiss Federal Institute of Technology,
Hönggerberg, CH-8093 Zürich, Switzerland.

[+]Laboratory of Bioengineering,
Department of Chemistry and Chemical Engineering,
Delft University of Technology,
Julianalaan 67, 2628 BC Delft, The Netherlands.

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SUMMARY

The influence of support material (calcium alginate, κ-carrageenan and agarose), cell loading and, in case of alginate, bead diameter on the rate of respiration of immobilized plant cells (Daucus carota) was investigated. No significant differences were observed between the three supports and no loss of respiration activity occurred as result of immobilization per se. The results show further that above a critical combination of cell loading and bead diameter limitations of the rate of respiration by diffusion of oxygen increases with increasing loading and diameter.

INTRODUCTION

The production of secondary metabolites by plant cells in suspension is a subject of current interest (Böhm, 1981; Zenk, 1978). Immobilization of plant cells sometimes induces and/or increases secondary metabolite formation and may even stimulate excretion of the metabolites (Brodelius & Mosbach, 1982). The explanation usually given for these phenomena is that enclosure in a support exerts a certain stress on the plant cells leading to restricted growth, the condition generally considered as essential for secondary metabolite production (Yeoman et al, 1980). This restricted growth may simply be due to lack of space in the support or, as a result of relatively slow mass-transfer processes, caused by depletion of substrate and/or accumulation of growth-inhibiting compounds.

Oxygen is a critical substrate for cell metabolism. It is also the compound which is likely to deplete in the support because of the poor solubility in aqueous solutions. Studying the respiration kinetics of immobilized plant cells is therefore an obvious first goal.

A method very suitable for immobilization of plant cells is entrapment in gel matrices such as alginate, carrageenan, and agarose. It has been observed (Brodelius & Nilsson, 1980) that of the three mentioned supports, alginate especially stimulates secondary metabolite formation. In order to establish if this phenomenon could be due to a more severe oxygen diffusion limitation in alginate, Daucus carota cells were immobilized in the three supports and the influence of bead diameter, cell loading and support material on the respiration kinetics determined. In addition, the three support matrices were studied in order to establish a possible effect of the

mechanisms, (ionotropic, ionotropic/thermal, and thermal-gelation (Klein, 1978)) involved in the gelation of the support material.

EXPERIMENTAL

Growth of plant cells.

Daucus carota cells were grown in 250 cm^3 shake flasks on MS-medium (Murashige & Skoog, 1962) containing in addition 2,4-dichlorophenoxy acetic acid (0.4 mg dm^{-3}), kinetine (0.04 mg dm^{-3}) and saccharose (20 g dm^{-3}). The pH was 5.8 and the temperature 28°C. After 5 days of growth the cells were harvested by filtration through a Büchner funnel.

Immobilization in alginate.

Cells collected on the Büchner filter were mixed with water and 3% (w/v) sodium alginate (Manucol DM, Alginate Industries, London) solution in the weight ratios 12:0:24, 3:9:24, and 1:11:24 (cells : water : alginate). Beads were made by dropwise extrusion of the resulting suspensions through a needle into MS-medium containing calcium chloride (50 mM). The size of the beads was controlled by adjustment of a longitudinal airflow along the needle. The beads were hardened in the magnetically stirred and aerated $CaCl_2$/MS-medium for 2 h, washed with MS-medium containing 5 mM calcium chloride, and used in the assays.

Immobilization in carrageenan.

Cells (2.5 g) collected on the Büchner filter were suspended in 22.5 g of a 3% (w/v) carrageenan (FMC) solution containing 9 g dm^{-3} sodium chloride. As with the alginate suspensions, the carrageenan suspension was extruded into MS-medium but in this case containing potassium chloride (0.3 M); hardened for 1 h, washed with MS-medium with no additives and used immediately.

Immobilization in agarose.

This was done as described previously (Nilsson et al, 1983). Cells (2.5 g) collected on the Büchner filter were suspended in 22.5 g of a 5 % (w/v)

agarose (Sigma, type VII) at 40°C. After dropwise extrusion of this suspension into magnetically stirred paraffin oil of 40°C, the dispersion formed was cooled in an ice/water bath. The hardened beads were washed with MS-medium, fractionated by sieving and three fractions (1.7-2.0, 2.4-2.8, 3.4-4.0 mm diameter) used immediately.

Assays.

The rate of respiration of free and immobilized cells was measured in duplicate using two types of biological oxygen monitors (a Model 53, Yellow Springs Instrument Co. Inc. Yellow Springs, Ohio, USA, and an Orbisphere, Orbisphere Laboratories, Geneva, Switzerland). The procedure for each assay was as follows. Four cm^3 of MS-medium in a magnetically-stirred glass vessel (maximum volume 20 cm^3, 2 cm i.d.) having a water jacket thermostatted at 30°C were saturated with air and a sample of free or immobilized cells was added. The vessel was closed with a plunger containing the O_2-electrode (Clark type) and the oxygen concentration recorded as a function of time. By fitting the integrated Michaelis-Menten equation to these experimental data, the concentration-time curves were reduced to apparent kinetic constants (V_m', K_m').

RESULTS AND DISCUSSION

Alginate as support matrix.

The respiration kinetics of cells immobilized by entrapment in calcium alginate are given in Figure 1 in the form of Lineweaver-Burk plots. The figure shows that as cell loading and diameter of the bead increase the slope of the plots becomes steeper, indicating increasing limitation of the respiration rate by diffusion of oxygen. In agreement with the theory on heterogeneous catalysis (Froment & Bischoff, 1979) the effect of the diameter is more pronounced than that of the cell loading. According to this theory the internal effectiveness factor of a catalyst particle is a function of the Thiele-modulus which in turn, for a spherical particle, is proportional to the particle diameter and only a square-root function of the reaction rate per unit volume of support (see equation 2), i.e. in this particular case a square-root function of cell loading.

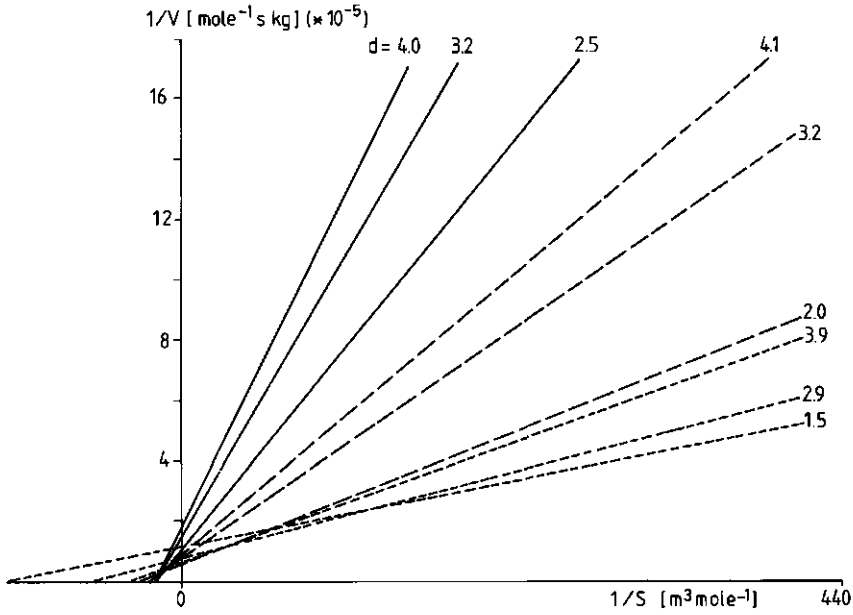

Figure 1. Lineweaver-Burk plots for alginate with different cell loadings (——— = 50.6%,— — — = 14.1%, ---- = 5.6%) and bead diameters (values in mm units).

In Figure 2 the slopes of the LB-plots (K_m'/V_m') are plotted as a function of the bead diameter for the various cell loadings. In this figure theoretical curves of K_m'/V_m' against bead diameter are also plotted. The curves were obtained by a computer simulation where the mean values of the intrinsic kinetic constants V_m and K_m, together with the effective diffusion coefficient ID_e and values of the experimental conditions (cell loading, bead diameter) result in values for apparent kinetic constants V_m' and K_m'. The required intrinsic constants were estimated by an iterative procedure in which the effectiveness factor, correcting the Michaelis-Menten equation, is a weighted average of the first and zero-order effectiveness factors (Eijkenboom & Luyben, unpublished). The effective diffusion coefficient ID_e was assumed to be 80% of the value in pure water, in all three cases. This is probably an incorrect assumption, since the cell loading affects ID_e, but is perhaps reasonable as determination of ID_e, both theoretically (Radovich, 1985) and by measurement, is not accurate for oxygen. Furthermore, only the results obtained with the middle cell loading allowed estimation of

reliable intrinsic kinetic parameters (corresponding with a ID_e of 80% of that in water). The deviation of the theoretical curves at high and low cell loadings can be explained on the grounds that at high cell loadings ID_e is very likely lower than 80% of the value in pure water and at low cell loadings, higher.

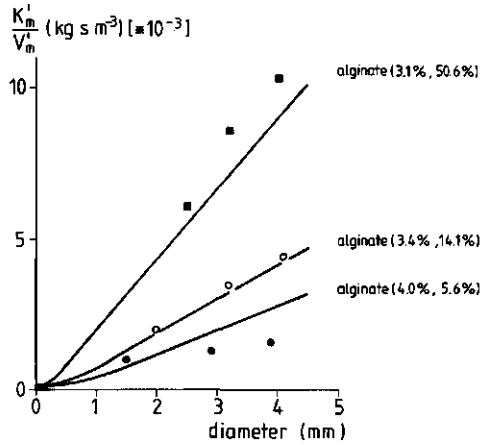

Figure 2. Slopes of the Lineweaver-Burk plots for alginate with different gel concentration (1st value in parentheses in kg dry wt. kg^{-1} total*100%) and different cell loading (2nd value in parentheses in kg wet wt. kg^{-1} total*100%). —— = theoretical curves at experimental conditions (ID_e=1.9*10^{-9} m^2 s^{-1}, mean values used for intrinsic parameters: V_m = 1.23*10^{-5} mol kg^{-1} s^{-1}, K_m = 0.202*10^{-2} mol m^{-3}).

The maximum bead diameter of the biocatalyst for unlimited respiration (d_{crit}) can be derived from the generalized Thiele-modulus (Froment & Bisschoff, 1979):

$$\phi = \frac{V_p}{A_p} \frac{r_s}{\sqrt{2}} \left\{ \int_{C_{eq}}^{C_s} ID_e r(c) dc \right\}^{-\frac{1}{2}} \qquad (1)$$

Assuming spherical geometry and zero order kinetics (unlimited respiration) leads to:

$$\phi = \frac{R}{3} \frac{r_s}{\sqrt{2}} \left(\frac{1}{ID_e r_s (C_s - C_{eq})} \right)^{\frac{1}{2}} = R \left(\frac{r_s}{18\ ID_e (C_s - C_{eq})} \right)^{\frac{1}{2}} \qquad (2)$$

The effectiveness for zero order kinetics is < 1 if the generalized Thiele-modulus Φ is > $1/3\sqrt{3}$ (according to Moo-Young & Kobayashi (1972) with introduction of an appropriate shape factor: 1/3). So for the critical diameter (d_{crit}) this yields:

$$\frac{1}{3}\sqrt{3} = \frac{d_{crit}}{2} \left(\frac{r_s}{18\ ID_e(C_s - C_{eq})}\right)^{\frac{1}{2}} \qquad (3)$$

hence,

$$d_{crit} = \left(24\ ID_e \frac{(c_s - c_{eq})}{r_s}\right)^{\frac{1}{2}} \qquad (4)$$

with C_s the concentration of oxygen at the surface of the aggregates, C_{eq} the concentration of oxygen after an infinite reaction time (here assumed to be approaching zero) and ID_e the effective diffusion coefficient of oxygen in the aggregate. Substitution of this study's data (C_{eq}=0.0 M, V_m = $1.0*10^{-5}$ mol kg^{-1} s^{-1}) in this equation yields the results given in Table 1. Wuhrmann (1964) also gives a similar equation for diffusion of metabolites into aggregates of living cells. Instead of the substrate concentration C_{eq} (equation 4) at equilibrium, he used the substrate concentration around a cell in the centre of the aggregate. Taking C in the centre $10*K_m$ (unlimited respiration) yields similar values to those given in Table 1.

Cell loading (%)	c_s (% saturation)				
	2	20	50	75	95
5.6	0.55	1.74	2.76	3.38	3.80
14.1	0.39	1.23	1.95	2.39	2.69
50.6	0.20	0.64	1.02	1.25	1.40

Table 1. Critical bead diameters, calculated with equation (4) with experimental data for alginate immobilized plant cells; effective diffusion coefficient is $1.9*10^{-9}$ m^2 s^{-1} (80% of the value in pure water); concentration range 2-20 % contains K_m' of the immobilized plant cells.

In concentration regions close to the K_m' of the immobilized plant cells (experimental values are in the range of 2-20 % of air saturation), the low effectiveness of the beads starts to influence the K_m'/V_m' ratio. Therefore the critical diameters at values of 2 and 20 % air saturation correspond well with the diameter where the theoretical K_m'/V_m' curves start to level-off from the intrinsic K_m/V_m ratio.

Assuming that in production situations the oxygen concentration will be about 95% saturation, one can see from Table 1 that for the support to have good diffusion characteristics the diameter of the beads can be as high as 1.40 mm for high cell loading to up to 3.80 mm for the low cell loading.

Similarly, the agreement with critical diameters and cell loadings obtained from calculated oxygen concentration profiles (Figure 3) agree well. These profiles were calculated by means of the numerical solution of a differential mass balance over the beads with internal oxygen diffusion and consumption. Calculations using film theory have shown that oxygen is mainly limited in the support material and not in the liquid film around the particle. These calculations yield a surface concentration of 95% of the bulk concentration (assumed to be saturated), which was used in the calculation of the profiles.

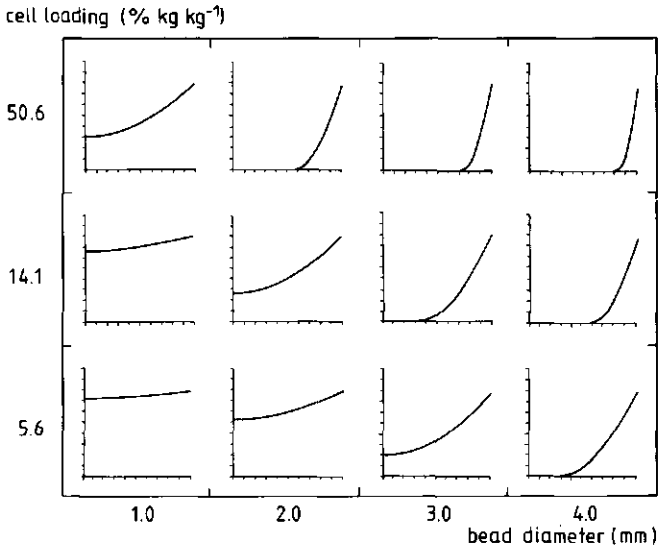

Figure 3. Calculated oxygen concentration profiles in the biocatalyst bead as a function of bead diameter and cell loading (V_m = $1.23*10^{-5}$ mol kg^{-1} s^{-1}, K_m = $0.202*10^{-2}$ mol m^{-3}, C_s = 0.2375 mol m^{-3}, ID_e = $1.9*10^{-9}$ m^2 s^{-1}).
Range x-axis: from centre (left) to surface (right) of the bead.
Range y-axis: from zero (under) to 0.30 mol m^{-3} (above).

Carrageenan as support matrix.

For this matrix only the influence of bead diameter on the extent of limitation of the cell respiration by oxygen diffusion was investigated and the results are given in Figure 4. As expected the slope of the Lineweaver-Burk plot is steeper as the diameter increases.

Agarose as support matrix.

As with carrageenan, only the influence of bead diameter on oxygen diffusion limitation of cell respiration was investigated. The results (Figure 4) show the same trends as the other matrices.

Comparison of the supports.

The V_m of the free cells for respiration was found to be about $1.0*10^{-5}$ moles of oxygen consumed per kg of wet cells per second and is of the same order of magnitude as the V_m'-values of the immobilized cells, irrespective of support material. This means that there were no considerable activity losses associated with the immobilization procedures.

The characteristics of the various gel beads are summarized in Table 2. The data are corrected for shrinkage, which is especially pertinent in case of alginate. Evaluation of the data in this Table and in Figure 4 leads to the conclusion that only minor differences exist between the various support materials. Consequently, a more severe diffusion limitation of oxygen in alginate is not likely to be the explanation for the stimulated secondary metabolite formation in cells immobilized in this support.

	Gel load (%, w/w)	Cell load (%, w/v)	Bead diameter (mm)			
			1	2	3	4
Alginate[a] (low)	4.0	5.6	1.5	2.9	3.9	–
(medium)	3.4	14.1	2.0	3.2	4.1	–
(high)	3.1	50.6	2.5	3.2	4.0	–
Agarose	4.5	10.0	2.0	2.4	3.7	4.3
Carrageenan	2.7	10.0	2.9	3.3	4.7	–

[a]The data for alginate are given after correction for measured shrinkage.

Table 2. Values of experimental variables.

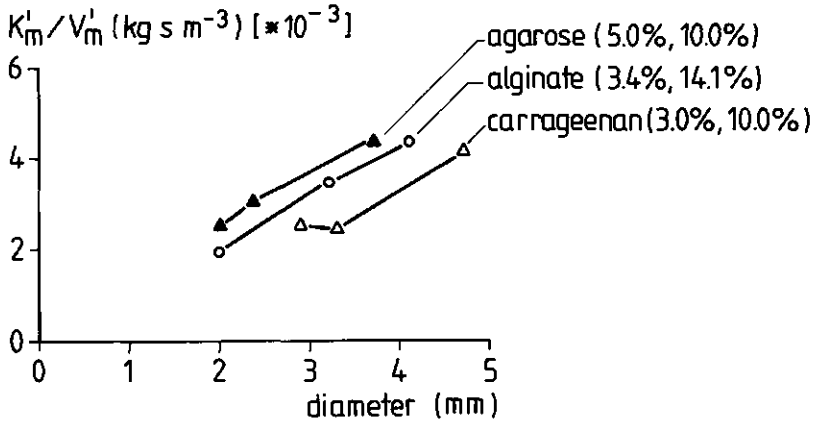

Figure 4. Slopes of the Lineweaver-Burk plots for different gel types each with different gel concentration (1st value in parentheses in kg dry wt. kg^{-1} total*100%) and different cell loading (2nd value in parentheses in kg wet wt. kg^{-1} total*100%).

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NOMENCLATURE

A	=	surface of bead	[m^2]
V	=	volume of bead	[m^3]
r_s	=	consumption rate	[mol m^{-3} s^{-1}]
ID_e	=	effective diffusion coefficient	[m^2 s^{-1}]
Φ	=	generalized Thiele modulus	[-]
R	=	radius of bead	[m]
C_s	=	substrate concentration at surface	[mol m^{-3}]
C_{eq}	=	substrate concentration at equilibrium	[mol m^{-3}]
d_{crit}	=	critical bead diameter	[m]
V_m	=	intrinsic maximum consumption rate	[mol kg^{-1} s^{-1}]
K_m	=	intrinsic Michaelis-Menten constant	[mol m^{-3}]
V_m'	=	apparent maximum consumption rate	[mol kg^{-1} s^{-1}]

K_m' = apparent Michaelis-Menten constant [mol m^{-3}]

* = multiplication sign

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CHAPTER 6

DETERMINATION OF THE EFFECTIVE DIFFUSION COEFFICIENT OF OXYGEN IN GEL MATERIALS IN RELATION TO GEL CONCENTRATION

A.C. Hulst, H.J.H. Hens, J. Tramper

Department of Food Science, Food and Bioengineering Group,
Agricultural University Wageningen,
De Dreijen 12, 6703 BC Wageningen, The Netherlands.

Submitted for publication

SUMMARY

The effective diffusion coefficient of oxygen, ID_e, was determined in different gel support materials (calcium alginate, κ-carrageenan, gellan gum, agar and agarose) which are generally used for immobilization of cells. The method used was based upon fitting Crank's model on the experimental data. The model describes the solute diffusion from a well-stirred solution into gel beads which are initially free of solute. The effect of the gel concentration on ID_e of oxygen in the gel was investigated at a temperature of 30°C. The results showed a decreasing ID_e for both agar and agarose from about $2.5*10^{-9}$ to $1.6*10^{-9}$ m^2 s^{-1} at increasing gel concentration from 2 to 8% (w/v) respectively. In case of calcium alginate and gellan gum, a maximum range in ID_e was observed. It is hypothesized that this phenomenon is due to a changing gelpore structure at increasing gel concentrations. The ID_e of oxygen in calcium alginate, κ-carrageenan and gellan gum varied from $1.5*10^{-9}$ to $2.1*10^{-9}$ m^2 s^{-1} (54.2 and 75.8% of the value in pure water, resp.) in the gel concentration range of 0.5 to 5% (w/v).

INTRODUCTION

In the biotechnological research field, modelling of immobilized biocatalysts in gels could be used as a valuable tool for studying the behaviour of the immobilized biocatalyst (Radovich, 1985). Within this scope, mass transfer of products and substrates from and to the immobilized biocatalyst is important. The effective diffusion coefficient in the support is the parameter to mathematically describe mass transfer of substances in immobilized biocatalyst systems. However, due to a lack of experimentally determined diffusion coefficients, estimations of the value of the effective diffusion coefficient are often used and in most cases this suffices. But in case of a more quantitative study of the mass transfer of the substrate in immobilized biocatalysts, the use of an accurate value of the effective diffusion coefficient is a prerequisite.

In the framework of our research, especially oxygen as a substrate of immobilized plant cells or plant cell aggregates, is subject of research. In the centre of the gel beads containing the plant cells, depletion of oxygen due to oxygen consumption by the cells and hampered transport through the gel material, could be regarded as a 'stress' condition to the immobilized plant

cells (see Chapter 8). A hypothesis was that this 'stress' condition resulted in increased secondary metabolite production. In order to determine the occurrence of oxygen diffusion limitation in gel immobilized plant cells, an accurate value of the diffusion coefficient of oxygen in the gel material is needed. In the literature, some data of oxygen diffusion coefficients in gels are available. Sato & Toda (1983) measured the effective diffusion coefficient of oxygen in 2% (w/v) agar. They calculated ID_e from an oxygen balance over a flat agar layer which was located between two liquid phases with different oxygen concentrations in equilibrium state. At 30°C they found a value of $1.94*10^{-9}$ m^2 s^{-1} (70% of that in pure water). Hiemstra et al (1983) calculated the diffusion coefficient in 2% barium alginate containing yeast cells, which was $0.7*10^{-9}$ m^2 s^{-1} at a temperature of 37°C (25% of that in pure water). Adlercreutz (1986) found a value of $2.11*10^{-9}$ m^2 s^{-1} for the diffusion coefficient of oxygen in 2% calcium alginate (77% of that in pure water).

In this paper, the effective diffusion coefficient of oxygen is experimentally determined in 5 types of gel support materials (agar, agarose, alginate, κ-carrageenan, and gellan gum). The determination method is based upon diffusion of oxygen from a well stirred aqueous bulk solution into gel beads, which are initially free of oxygen. From the course of the decreasing oxygen concentration in the bulk solution, the effective diffusion coefficient was determined. The effect of the gel concentration on the effective diffusion coefficient was investigated.

THEORY

The theoretical background for the determination of the effective diffusion coefficient was obtained from Crank (1973). He formulated a mathematical model of the diffusion of solutes from a well-stirred solution of limited volume into a sphere. The concentration of solute in the solution is assumed to be always uniform and is initially C_0. The sphere (radius a) is initially free from solute. The total amount of solute, M_t, in the sphere after time t is expressed as a fraction of the corresponding quantity after infinite time, M_∞, by equation (1).

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=1}^{n=\infty} \frac{6\alpha\,(\alpha+1)\; e^{-IDq_n^2 t/a}}{9+9\alpha+q_n^2\alpha^2} \qquad (n=1,2,\ldots) \quad (1)$$

where the q_n's are the nonzero roots of

$$\tan q_n = \frac{3\, q_n}{3+\alpha q_n^2} \qquad (2)$$

The capacity ratio, α, can be expressed as

$$\alpha = \frac{V_f}{\frac{4}{3}\pi a^3 K} \qquad (3)$$

K is the partition factor of the solute between the sphere and the solution and is defined as the solute concentration ratio in the sphere and in the solution in equilibrium. V_f is the volume of the liquid. The parameter α can be expressed in terms of the final fractional uptake of solute by the sphere:

$$\frac{M_\infty}{V_f C_0} = \frac{1}{1+\alpha} \qquad (4)$$

with $M_\infty = (C_0 - C_\infty) * V_f$, equation (4) yields:

$$\alpha = \frac{C_0}{C_0 - C_\infty} - 1 \qquad (5)$$

with C_∞ is the solute concentration in the solution after infinite time. Combination of eqs. (3) and (5) yields:

$$K = \frac{V_f}{\frac{4}{3}\pi a^3 \left(\frac{C_0}{C_0 - C_\infty} - 1\right)} \qquad (6)$$

With the eqn's (1), (2), (5), and (6) the solute concentration in the solution on time t, C_t, can be expressed as:

$$C_t = C_0 - \frac{M_t}{V_f} \qquad (7)$$

MATERIALS AND METHODS

Preparation of gel beads

The used solutions for dissolving the gel material and for hardening the beads are given in Table 1.

gel	dissolving in	hardening in
agar 2/5/8 %	demi water	-
agarose 2/5/8 %	demi water	-
Ca-alginate 1/2/3 %	demi water	0.2 M $CaCl_2$
κ-carrageenan 1%	10 mM KCl	0.1 M KCl
κ-carrageenan 3%	2 mM KCl	0.1 M KCl
κ-carrageenan 5%	demi water	0.1 M KCl
gellan gum 0.5/1/2 %	5% $MgSO_4.7H_2O$	0.1 M $MgSO_4.7H_2O$

Table 1. Solutions used in the experiments.

Calcium alginate beads were prepared by dissolving an amount of sodium alginate (Manucol DM, batch ref. 473454, Kelco/Ail Int. Ltd., Girvan Agrshire, Scotland) in demineralized water to the pertinent concentration. After homogenization of the solution with an ultra-turrax mixer and subsequent deaeration, the solution was dripped into 0.2 M $CaCl_2$ solution via a rubber tube (8 mm internal diameter). After hardening in this solution for 2 h, the beads were equilibrated in saline solution (7.35 g dm^{-3} $CaCl_2$ and 6.08 g dm^{-3} NaCl in water). The obtained bead diameters were 4.5, 5.0 and 5.4 mm at the gel concentrations of 1, 2 and 3%, respectively.

Beads of the other gels were prepared by use of a perspex mould (1 cm diameter). For this, a gel solution in the belonging liquid (Table 1) was prepared by heating on a boiling plate. The warm solution was injected into the mould by a syringe via an injection port. The mould was cooled in ice and after (partial) thermal gelification the beads were removed from the mould and hardened for 2 h in the appropriate salt solution (Table 1). Hardening of the agar and agarose beads was not necessary. Afterwards, the beads were equilibrated in the saline solution for at least 24 h, and used in the experiments.

Experimental set-up

The equipment used for the measurements of the effective diffusion coef-

ficient is schematically given in Figure 1. Two vessels A and B were situated in a thermostatted water bath. Vessel A (glass material) contained oxygen saturated saline solution. Vessel B (perspex material) contained saline solution with a number of gel beads. The contents of vessel B contained no oxygen. Both vessels were closed with a perspex gas tight plunger. Vessel B was provided with 4 standard-sized baffles and a magnetic stirrer. The oxygen concentration in the bulk liquid was measured by a Clark-type oxygen electrode (Yellow Springs Instruments, Model 5331) and a Biological Oxygen Monitor (BOM) (Yellow Springs Instruments Co, Ohio, USA, Model 53). In order to register the oxygen concentration in time, the BOM was connected with a recorder and a HP-85 microcomputer via a datalogger (HP3421A).

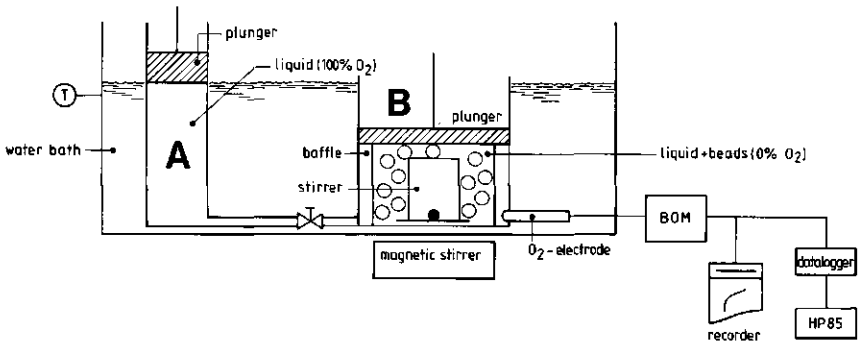

Figure 1. Schematic representation of the experimental set-up.

Experimental procedure

The oxygen electrode was calibrated with oxygen-saturated water (100% level) and nitrogen-saturated water (0% level). Saline solution in vessel A was saturated with oxygen by injecting pure oxygen gas for at least 15 minutes. Saline solution with a number of the gel beads in vessel B was saturated with nitrogen by injecting pure nitrogen gas for at least 15 minutes. Afterwards, the vessels were quickly closed with the plungers. A known amount of liquid from vessel A was instantaneously pushed into vessel B via the connection tube (t=0). As a result of the oxygen diffusion into the beads, the oxygen concentration in the bulk liquid decreased in time, which was registered with the recorder and the microcomputer. The experimental data (volumes of liquid, initial oxygen concentration, number of beads, bead

diameter) and the experimental curve were input to a computer program which fitted Crank's diffusion model on the experimental data with 'fit'-parameter ID_e.

The amount of oxygen loss during the experiments due to electrode consumption and leakage through the vessel wall was negligible with respect to the measurement.

The Biot numbers, as a measure for the ratio of the internal and external mass transfer resistance, were estimated to be 70 in case of alginate beads and at least 100 in case of the other gels under the experimental conditions. In view of the minimum Biot value of 50 for negligible external mass transfer resistance (Luikov, 1968), it is assumed that in the experiments the external mass transfer resistance in the stagnant film layer around the beads could be neglected with respect to the internal mass transfer resistance.

RESULTS AND DISCUSSION

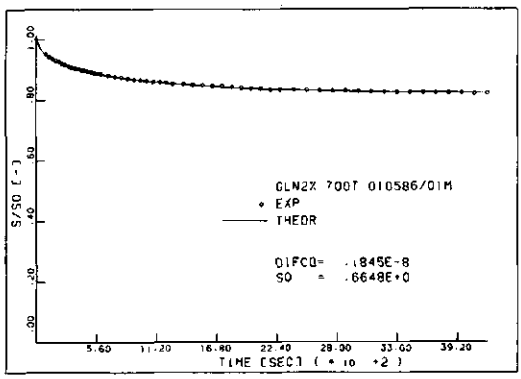

Figure 2. Example of a measurement for determination of the effective diffusion coefficient of oxygen in gellan gum (o = experimental data, —— = theoretical curve).

The model-fits in the experimental data were good. An example of an experiment is given in Figure 2. The results of the determined effective diffusion coefficients (ID_e) of oxygen in the different gels as a function of gel concentration are given in Table 2 and Figure 3.

gel	concentration (w/v)	number of duplicates	mean ID_e ($*10^{-8}$ m^2 s^{-1})	standard deviation ($*10^{-8}$ m^2 s^{-1})
Ca-alginate	1%	7	0.175	0.018
	2%	2	0.198	0.002
	3%	3	0.179	0.004
gellan gum	0.5%	2	0.151	0.024
	1%	3	0.210	0.006
	2%	6	0.185	0.015
κ-carrageenan	1%	4	0.158	0.015
	3%	4	0.158	0.014
	5%	3	0.187	0.013
agarose	2%	3	0.256	0.017
	5%	2	0.199	0.016
	8%	2	0.168	0.011
agar	2%	4	0.235	0.016
	5%	3	0.204	0.002
	8%	4	0.164	0.006

Table 2. Results of the determination of the diffusion coefficients of oxygen in different gel materials.

In case of agar and agarose, ID_e is decreasing at increasing gel concentration due to increasing internal mass transfer resistance, as expected. The experimental values of ID_e for agar and agarose are quite the same which could be expected because both gelification mechanisms are the same. Compared with ID_e of oxygen in water at the temperature of 30°C (=100%), the value in agarose and agar varies between 92.4 and 59.2 %, dependent on the gel concentration. Sato & Toda (1983) found for the ID_e of oxygen in 2% agar a value of $0.194*10^{-8}$ m^2 s^{-1} at 30°C which is lower than the value from our experiments ($0.235*10^{-8}$ m^2 s^{-1}).

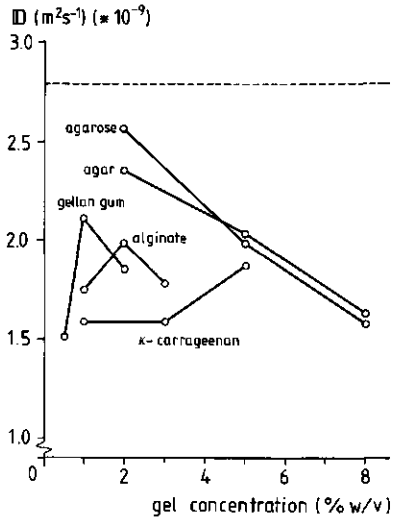

Figure 3.
Determined diffusion coefficients of oxygen in different types of gels as a function of gel concentration (temperature is 30°C).

Gellan gum and alginate show a remarkable maximum value for the ID_e with respect to the gel concentration. This feature has to our knowledge not been reported before for oxygen in gels. Mashelkar & Soylu (1974) reported a similar behaviour of the ID_e of CO_2 in polymeric solutions. No explanations of these observations were given by them. Niebelschuetz & Reuss (1984) also reported an optimum value of the ID_e of oxygen at low concentrations of xanthan gum (< 1 g dm^{-3}) solutions, which they ascribed to a possible appearance of undesired convective transport processes in their experiments. In our case, a possible explanation of the observed phenomenon can be derived from the 'egg-box' model which is assumed to be the case for alginate and other ionic-hardening gels like κ-carrageenan and gellan gum (Morris, 1985). At a low gel concentration 'egg-box' regions in the gel possibly are not formed (Muhr & Blanshard, 1982), resulting in a more homogeneous distribution of the gel material (see Figure 4) with a higher tortuosity. In contrast, higher gel concentrations show more open pores in the gel which results in a lower diffusional barrier, i.e. a higher diffusion coefficient. κ-carrageenan possibly can have a maximum value at a higher concentration with respect to alginate and gellan gum. However, due to practical limitations in preparing κ-carrageenan gels with a concentration exceeding 5% (w/v), these measurements could not be performed.

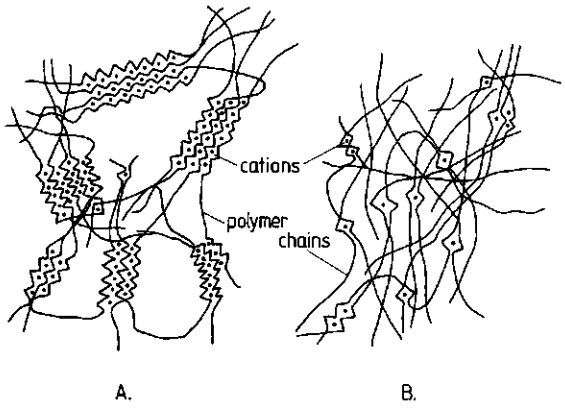

Figure 4. Schematic representation of possible gel structures at high (A) and low (B) concentrations.

The ID_e's of oxygen for κ-carrageenan, alginate and gellan gum are all in the range of $1.5*10^{-9}$ to $2.1*10^{-9}$ $m^2\ s^{-1}$ (54.2 to 75.8 % of the value in pure water). The values of ID_e of oxygen in gels, available from the literature, are given in Table 3. As can be seen, these values correspond quite well with the values from our experiments.

gel	diffusion coefficient ($*10^{-8}\ m^2\ s^{-1}$)	temperature (°C)	reference
Ca-alginate 2%	0.211	30	Adlercreutz (1986)
Ba-alginate 2%	0.07	37	Hiemstra et al. (1983)
agar 2%	0.194	30	Sato & Toda (1983)

Table 3. Literature data of diffusion coefficients of oxygen in gel supports.

CONCLUSIONS

Crank's mathematical model could be used as a basis for determination of effective diffusion coefficients of oxygen in gel beads by fitting it to

experimental data from diffusion experiments. The fits were good. The effective diffusion coefficients of oxygen in agar and agarose gels decreased with increasing gel concentration. The diffusion coefficients of oxygen in alginate and gellan gum appear to have a maximum value with respect to the gel concentration. Probably, this was due to different gel structures at different concentrations. The values of the effective diffusion coefficient of oxygen in the gels were in all cases lower than that in pure water.

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CHAPTER 7

THEORETICAL AND EXPERIMENTAL DETERMINATION OF OXYGEN CONCENTRATION PROFILES IN AGAROSE BEADS WITH IMMOBILIZED PLANT CELLS

A.C. Hulst, R.M. Buitelaar, C.M. Hooijmans[+)], J. Tramper

Department of Food Science, Food and Bioengineering Group,
Agricultural University,
De Dreijen 12, 6703 BC Wageningen, The Netherlands.

[+)] Laboratory of Bioengineering,
Department of Chemistry and Chemical Engineering,
Delft University of Technology,
Julianalaan 67, 2628 BC Delft, The Netherlands.

SUMMARY

Oxygen concentration profiles in equilibrium state have been measured with an oxygen micro-electrode in 5% (w/w) agarose beads containing viable plant cells of Tagetes minuta. The results were compared with mathematical model calculations of the oxygen concentration profiles, based on cell respiration kinetics and mass transfer properties of oxygen in the support material, in order to test the model validity. Several plant cell loads in the range of 200-555 kg fresh weight per m^3 total bead volume, and several bead diameters in the range of 4-15 mm, were tested. The measured and calculated oxygen concentration profiles in the beads correspond well.

INTRODUCTION

In recent years, many researchers have been investigating plant cell cultures in order to produce secondary metabolites. It was shown that the secondary metabolism of suspended plant cells in liquid media is more pronounced when the cells are subjected to so called 'stress' conditions, for instance by immobilization of the plant cells (Lindsey & Yeoman, 1983). This externally exposed 'stress' condition generally reduces the cell growth rate and triggers the secondary metabolism of the cells (Mattiasson & Hahn-Hägerdal, 1982). Examples of the increased secondary metabolite production of immobilized plant cells have been reported by several researchers (Brodelius & Mosbach, 1982; Wichers et al, 1983) An explanation of this phenomenon could be that the immobilized cells are subject to a lack of substrates due to a diffusional barrier for the substrates formed by the gel material (Shuler et al, 1984). Especially molecular oxygen is a substrate of interest (Jones & Veliky, 1981), because of its low solubility in water compared to most other substrates. The measure of oxygen diffusion limitation for the immobilized cells can be determined by calculation of oxygen consumption efficiencies for instance via the Thiele modulus (Froment & Bischoff, 1979). Another more 'visual' way to show the occurrence of oxygen diffusion limitation of immobilized cells is the calculation of oxygen concentration profiles inside the particles, which are containing the cells. In equilibrium state such time-independent profiles can be calculated with a rather simple mathematical model based on cell respiration kinetics and mass transfer properties of oxygen in the support material (see Chapter 6). The use of these

kinds of model calculations can be very useful for design of biotechnological processes. However, due to the fact that mathematical models generally are based on assumptions, the validity of such a model should always be experimentally tested.

In this paper, calculated concentration profiles in agarose beads containing plant cells are compared with experimentally determined concentration profiles using an oxygen micro-electrode for the measurements.

MATERIALS AND METHODS

Four distinct experimental runs were performed. For each run the general experimental procedure was as follows.

Preparation of beads and respiration measurements

Plant cells (Tagetes minuta) were obtained from a stock cell suspension culture. This stock culture was maintained since 4 years by weekly subculturing in 500 cm^3 MS-medium (Murashige & Skoog, 1962) in 1 dm^3 Erlenmeyer flasks. The flasks were incubated at 24°C on a rotary shaker (Gallenkamp Orbital Shaker, 120 min^{-1}) under light conditions (5 W m^{-2}) (Ketel, 1987). The harvested cells were filtered on a Büchner funnel until adhered water on the cells had visulally disappeared. A weighed sample of these free cells was used for respiration measurements in a Biological Oxygen Monitor (Orbisphere Laboratories, Geneva, Switzerland). Fifteen cm^3 of MS medium were air saturated in a magnetically-stirred vessel (maximum volume 20 cm^3, 2 cm i.d.) having a water jacket with a thermostat at 24°C. The sample was added and the vessel was closed with a plunger containing the O_2-electrode (Clark type, Orbisphere Laboratories, Geneva, Switzerland). The oxygen concentration was recorded as a function of time. By fitting the integrated Michaelis-Menten equation to these experimental data, the kinetic constants (V_m, K_m) were derived from the concentration-time curves.

Agarose (type VII, Sigma, St.Louis, USA) was suspended in water in a concentration of 5% (w/w). The suspension was heated in a magnetron until the agarose was completely dissolved and subsequently the agarose solution was cooled to 50°C in a temperature controlled cabinet.

A weighed amount of fresh plant cells was added to a known amount of agarose solution and stirred until the cells were homogeneously suspended in the agarose solution. This suspension was immediately injected with a syringe into a spherical mould of perspex material via an injection port. Afterwards, the mould was cooled in ice until the agarose was gelified.

The weight ratio of the cells and agarose solution in each run was different, resulting in different cell loads ranging from 200-555 kg per m^3 total bead volume. Also different mould sizes were used resulting in different bead sizes ranging from 4-15 mm diameter.

Measurements of oxygen concentration profiles with a micro-electrode

A Clarck type oxygen microelectrode, constructed according to Revsbech (1986) at the laboratory of the Department of Biochemical Engineering, Delft University of Technology, was used to measure the oxygen concentration profiles in the agarose beads. The signal of the microelectrode in water (20°C) was about 2.4 nA. The electrode showed no dependence on stirring, the signal was linear with the oxygen concentration and the 90% response time was about 3 seconds. The glass tip of the electrode had a size of 15 µm.

The electrode was put in a specially constructed holder and moved by means of a micromanipulator into the gel bead, which was submerged in MS-medium (Murashige & Skoog, 1962) and fixed in a flowchamber, thermostatted at 24°C. The signals were parallelly sent to a computer and a strip-chart recorder and reduced to oxygen concentration profiles in the bead.

Calculation of oxygen concentration profiles

The oxygen concentration profiles were calculated by means of a mathematical model based on the numerical solution of a differential mass balance over the beads with internal oxygen diffusion and consumption. In the Appendix the calculation procedure is outlined.

RESULTS AND DISCUSSION

The experimental data and results of the respiration measurements are given in Table 1. The results of the measured and calculated oxygen concentration profiles are given in Figure 1.

run number	cell load (kg/m^3)	bead diameter (mm)	V_m (mol/kg s)	K_m (mol/m^3)
1	200	4	$1.35*10^{-6}$	$3*10^{-3}$
2	430	4	,,	,,
3	555	4	,,	,,
4	400	6, 8, 15	$1.30*10^{-6}$	$5*10^{-2}$

Table 1. Experimental data.

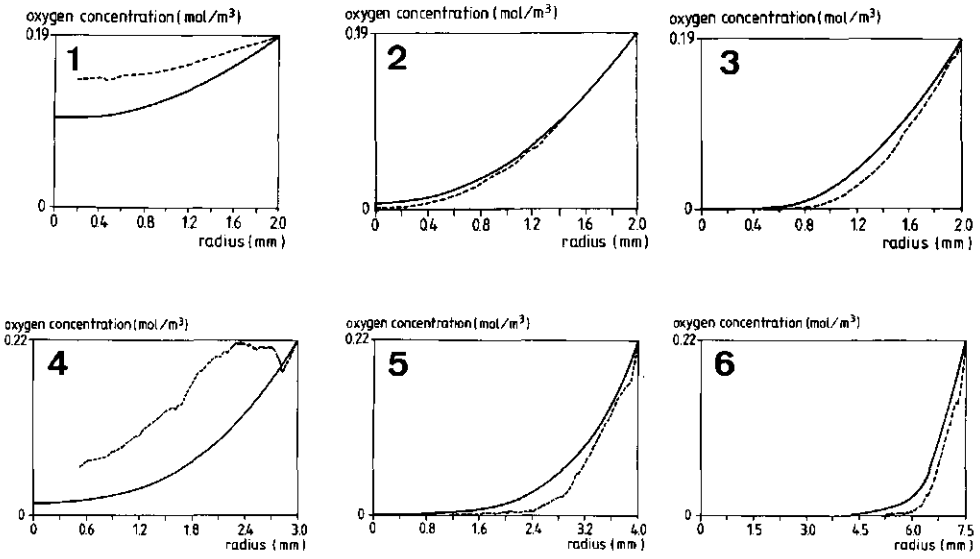

Figure 1. Calculated (—) and measured (- -) oxygen concentration profiles in agarose beads containing Tagetes minuta plant cells for different cell loads and bead diameters.

As can be seen in Figure 1 the measured and calculated profiles generally correspond quite well. As was observed in preliminary experiments by microscopic observations (results not published), the 'dips' which appear in some measured curves were due to local plant cell aggregates where a relatively higher oxygen consumption took place resulting in local lower oxygen concentration.

Measurements 2, 3, 5 and 6 clearly show that in the centre of the beads the oxygen concentration is about zero, which means that in these cases cells in

the centre are not able to respire to a significant extent.

CONCLUSIONS

The experiments have shown that the used mathematical model is valid for calculation of oxygen concentration profiles within agarose gel beads containing plant cells. Predictions can be made for the experimental conditions (cell load, bead diameter) where oxygen diffusion becomes limiting. The results showed that the mathematical model can be a useful tool in studies concerning immobilized plant cells with respect to oxygen transfer and consumption.

NOMENCLATURE

ID_e	= effective diffusioncoefficient	[$m^2\ s^{-1}$]
C_s	= substrate concentration at surface	[$mol\ m^{-3}$]
$C_{s,exp}$	= experimental value of C_s	[$mol\ m^{-3}$]
V_m	= maximum consumption rate	[$mol\ kg^{-1}\ s^{-1}$]
K_m	= Michaelis Menten constant	[$mol\ m^{-3}$]
V_c	= convection velocity	[$m\ s^{-1}$]
c	= oxygen concentration	[$mol\ m^{-3}$]
t	= time	[s]
r	= distance from centre	[m]
R	= radius of bead	[m]
R(c)	= oxygen consumption	[$mol\ m^{-3}\ s^{-1}$]
*	= multiplication sign	

APPENDIX

A differential mass balance over a bead (containing plant cells) in which diffusion and consumption of oxygen occurs simultaneously yields:

$$\frac{dc}{dt} + V_c \frac{dc}{dr} = ID_e \left(\frac{1}{r^2} \frac{d}{dr}\left(r^2 \frac{dc}{dr}\right)\right) - R(c) \qquad (I)$$

where c is the oxygen concentration, t is the time, r is the distance from the centre of the bead, V_c is the convection velocity, ID_e is the effective diffusion coefficient of oxygen in the support material and R(c) the oxygen

consumption as a function of the oxygen concentration.

With the following assumptions,

1) all plant cells in the bead are identical.
2) respiration of the cells can be described by Michaelis-Menten kinetics,
3) the distribution of plant cells throughout the bead is homogeneous,
4) oxygen concentration gradients only occur in radial direction,
5) the effective oxygen diffusion coefficient (here $1.9*10^{-9}$ m^2 s^{-1}) is independent of the oxygen concentration, and
6) oxygen transport occurs only by diffusion (no convective transport),

this differential equation, on steady state conditions, can be reduced to:

$$ID_e\left(\frac{1}{r^2}\frac{d}{dr}\left(r^2\frac{dc}{dr}\right)\right) = \frac{V_m * c}{K_m + c} \qquad \text{(II)}$$

with the boundary conditions:

(IIA) r=0 ==> dc/dr=0 (centre of the bead)

(IIB) r=R ==> $c=c_{surf}$ (surface of the bead)

where V_m and K_m represent the Michaelis-Menten constants, R is the radius of the bead and c_{surf} is the oxygen concentration at the surface of the bead. Partial differentiation yields:

$$\frac{d^2c}{dr^2} = \left(\frac{V_m * c}{K_m + c}\right)/ID_e - \frac{2}{r} * \frac{dc}{dr} \qquad \text{(III)}$$

Equation (III) can only be solved by numerical integration, finally resulting in an oxygen concentration profile in the bead. This was done by a computer program on a DEC-10 mainframe computer (Digital). For the numerical integration of equation (III) the IMSL-routine DREBS was applied, using a Rebson algorithm with variable steps. This routine needs a 'starting point' for correct integration (see Figure 2). Initially this point is set to the centre of the bead ($r_{start}=0$) with an oxygen concentration of $c_{start}\approx 0$. From this point the subroutine calculates the appropriate concentration profile in the bead. The obtained oxygen concentration at the bead surface c_s is com-

pared with the known concentration $c_{s,exp}$ (program input parameter). If $c_{s,exp} > c_s$ then r_{start} will be set to zero and c_{start} is variable. On the other hand, if $c_{s,exp} < c_s$ then r_{start} will be variable and c_{start} will be set to a value approximating zero. This process will iterate until $c_s = c_{s,exp}$ and the appropriate oxygen concentration profile is plotted.

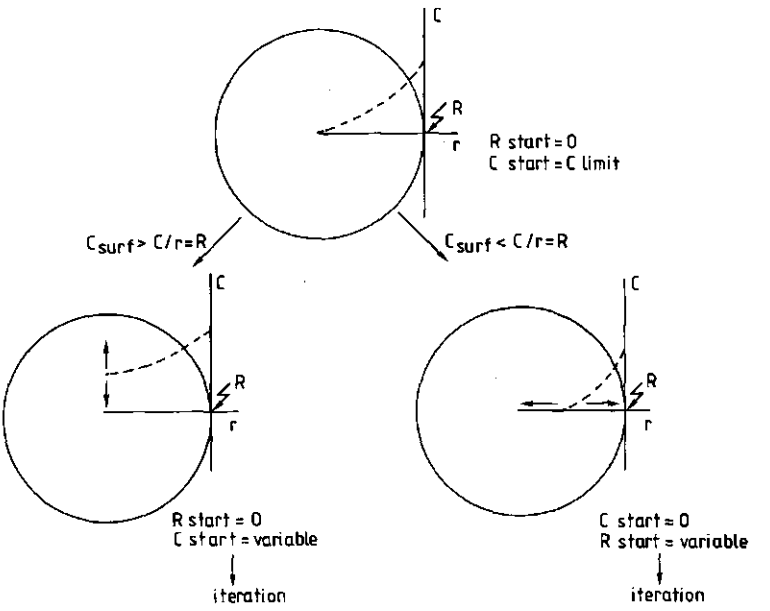

Figure 2. Schematic representation of the iterative procedure of the calculation of the oxygen concentration profile in the bead.

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CHAPTER 8

EFFECT OF AGGREGATE SIZE IN CELL CULTURES OF TAGETES PATULA ON THIOPHENE PRODUCTION AND CELL GROWTH

A.C. Hulst, M.M.T. Meyer, H. Breteler*), J. Tramper

Department of Food Science, Food and Bioengineering Group,
Agricultural University,
De Dreijen 12, 6703 BC Wageningen, The Netherlands.

*)Research Institute Ital,
Keijenbergseweg 6, 6704 PJ Wageningen, The Netherlands.

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SUMMARY

The effect of the size of Tagetes patula (marigolds) cell aggregates on growth and thiophene production in MS-medium was studied. A heterogeneous aggregate suspension was aseptically divided into 7 fractions, each with a defined aggregate diameter range, with subsequent inoculation of the fractions into MS growth medium. Growth occurred in all aggregate fractions and thiophene production increased with increasing aggregate diameter starting at about 3 mm, an effect possibly due to an increasing lack of oxygen in the aggregate centre. Calculations of oxygen concentration profiles in the aggregates showed namely, that the critical aggregate diameter where the oxygen concentration in the aggregate centre becomes very low, is about 3 mm. Aggregates with a diameter exceeding 1.2 cm showed a decreased thiophene production, however, these aggregates were hollow. The thiophenes produced mainly consisted of 5-(4-hydroxy-1-butenyl)1-2,2'-bithienyl, which was excreted into the medium.

INTRODUCTION

Tagetes species (marigolds, e.g. T.erecta, T.minuta and T.patula) are capable of producing thiophenes (Chan, 1975; Hartough, 1952). Thiophenes are naturally-occurring biocides. Particularly against nematodes, thiophenes have a high biocidal activity (Gommers, 1981). The general structure of thiophenes and of some thiophenes prevailing in higher plants are given in Figure 1.

Thiophenes may be obtained by extraction of plant material by non-polar solvents like hexane. As an alternative, a biotechnological approach is investigated in our group: the production of thiophenes with aggregate cultures of Tagetes species in a bioreactor. The aim of this research is to develop high producing Tagetes cell lines and to study the optimum conditions for thiophene production in a bioreactor. One aspect of our research concerns the stress on cells as result of immobilization in support materials like agar(ose), alginate and κ-carrageenan (Ketel et al, 1987). In particular, the occurrence of oxygen diffusion limitation in immobilized plant cell particles and its effect on growth and on the production of secondary metabolites is considered a factor of high biotechnological potential. Within this scope, aggregation of plant cells can be seen as a 'natural' way of immobilization with the highest possible density of cells (Fuller, 1984).

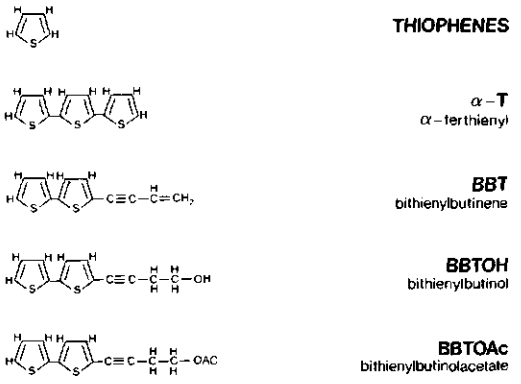

Figure 1. Basic structure of a thiophene molecule and of some thiophenes prevailing in plants and cell cultures of Tagetes patula.

Preliminary experiments showed that cell suspensions of T.minuta and T.erecta were not able to produce thiophenes, however T.patula showed a high thiophene production (Ketel, 1987). Furthermore, it was noticed that the cultures of T.patula consisted of large cell aggregates (diameter up to 20 mm) in contrast to T.erecta and T.minuta (diameter up to 2 mm). However, T.minuta cells immobilized in polyurethane showed thiophene production in contrast to a free cell suspension (results not published), indicating that cell aggregation probably is a prerequisite for thiophene producion (Helsper et al, 1987).

In this paper, experiments are described in which the influence of the aggregate size on growth and thiophene production of the cells was investigated. In addition, the occurrence of oxygen diffusion limitation in cell aggregates was investigated as a possible explanation for the observed positive correlation between thiophene production and aggregate size.

THEORY

In order to quantify the level of oxygen diffusion limitation in the cell aggregates, two methods have been used:

1) Calculation of the oxygen concentration profile in the aggregates.
2) Determination of the critical aggregate diameter.

ad 1) Oxygen concentration profiles in the aggregates were calculated by means of the numerical solution of a differential mass balance over the aggregates with internal oxygen diffusion and consumption, as explained in the Appendix of Chapter 7. In this case in the Appendix the word 'bead' should be replaced by 'aggregate'.

ad 2) The critical diameter of an aggregate is defined as the maximum diameter for unlimited respiration throughout the aggregate (Hulst et al, 1985; Wuhrmann, 1964). The critical diameter (d_{crit}) can be calculated with equation 1:

$$d_{crit} = (24\ ID_e \frac{(c_s - c_{eq})}{r_s})^{\frac{1}{2}} \qquad (1)$$

In this equation ID_e is the effective diffusion coefficient of oxygen in the aggregate in $m^2\ s^{-1}$, c_s is the oxygen concentration at the aggregate surface in mol m^{-3}, c_{eq} is the oxygen concentration after an infinite reaction time (here assumed to be approaching zero) in mol m^{-3}, and r_s the oxygen consumption rate in the aggregate in mol $m^{-3}\ s^{-1}$.

The equation is valid under the same assumptions as mentioned under ad. 1, with the additional assumption that only zero-order kinetics are appropriate (i.e. concentrations >> K_m).

MATERIALS AND METHODS

Cell line.

Tagetes patula cell cultures were started from callus tissue (Ketel, 1987). This stock cell line was already grown for 9 months in MS-medium at 24°C in 1 dm^3 flasks on a rotary shaker (Gallenkamp Orbital Shaker, 120 rpm) under dim light conditions (2 klux), and subcultured every 2 weeks, before the experiments described here were started.

MS-medium.

The medium contained 4.72 g dm^{-3} MS-mixture (Murashige & Skoog, 1962) supplemented with 100 mg dm^{-3} myo-inositol, 5 mg dm^{-3} benzyladenine, 0.5 mg dm^{-3} naphthalene acetic acid, 20 g dm^{-3} saccharose and 1 g dm^{-3} polyvinyl

pyrrolidone-40. Before autoclaving the pH was adjusted to 5.85 with KOH-solution.

Rinsing medium, used in the sieving procedure, consisted of MS-medium without the hormones benzyladenine and naphthalene acetic acid.

Fractionation

Fractionation of the cell culture was achieved by sieving the cells on a sieve-set (Retsch, grid diameters 0.1, 0.5, 1.0, 1.4, 2.0, 2.8 and 4.0 mm) under sterile conditions in a laminar flow cabinet. The cells were poured on the upper sieve and after flushing with rinsing medium, the aggregate fractions were separated, collected and used immediately in the experiments.

The aggregate fractions larger than 6 mm were obtained by manual selection of the aggregates.

Assays

Growth of the small cell aggregates (diameter < 5 mm) was estimated by the change of the packed cell volume (PCV, Gilissen et al, 1983) in the Erlenmeyer flasks. The Erlenmeyer flasks were tilted and after 10 minutes in each flask the length of the chord formed by the surface of the packed cells across the bottom of the flask was measured. The PCV was determined with a calibration curve.

Large aggregates (> 5 mm) were weighed on a balance. The cell volume was calculated with a conversion factor of 0.893 cm^3 per g of fresh weight, which was experimentally determined in advance by measuring the PCV of a larger amount of aggregates and weighing them afterwards.

The dry weight of the aggregates was determined by drying an accurately determined volume of aggregates during 48 h in a freeze dryer. Afterwards, the dry cell material was kept in an desiccator for conditioning to room temperature, and then weighed.

Aggregate dimensions were measured by microscopic determination of the length (L) and the width (W) of an aggregate. The diameter of a corresponding spherical aggregate (d_{agg}) was calculated by equation 2:

$$d_{agg} = \left(\frac{L * W^2}{1/6*\pi}\right)^{1/3} \qquad (2)$$

The mean aggregate diameter for each fraction was determined by measuring 10 randomly taken aggregates..

The rate of respiration of the aggregates was measured in duplicate using a biological oxygen monitor (own design). The procedure for each assay was as follows. An accurately-weighed amount of MS-medium in a magnetically-stirred vessel (maximum volume 20 cm^3, 2 cm i.d.) having a water jacket with a thermostat at 24°C was saturated with air and a sample of the cell aggregates was added. The vessel was closed with a plunger containing the O_2-electrode (Clark type, Orbisphere Laboratories, Geneva, Switzerland) and the oxygen concentration recorded as a function of time. By fitting the integrated Michaelis-Menten equation to these experimental data, the apparent kinetic constants (V_m', K_m') were derived from the concentration-time curves.

Extrapolation to a diameter value of zero in the plots of the kinetic constants V_m' and K_m' against the mean aggregate diameter of the fraction yielded an estimate of the intrinsic kinetic constants V_m and K_m.

Thiophene content in the aggregates was assayed by pouring an accurately-weighed amount of fresh aggregates into liquid nitrogen. The frozen aggregates were crushed in acetone and extracted by about 15 cm^3 of acetone during 24 h in the dark. These crude extracts were filtered through hexane-washed quartz wool, evaporated till about 2 cm^3 under nitrogen gas, and extracted with about 15 cm^3 cyclohexane during 24 h in the dark. (X) After filtration through a 0.22 µm filter, the extract was fully evaporated to dryness under nitrogen gas. The residue was dissolved in 500 mm^3 cyclohexane and HPLC analysed.

Thiophene content in the medium was assayed by extracting an accurately pipetted volume of the medium with the same volume of hexane during 16 h in the dark. In parallel experiments, the efficiency of this extraction was determined and the resulting factor was used to calculate thiophene yields. After separation of the aqueous and hexane phase, the latter was treated as before-mentioned from point (X).

HPLC was done by isocratic elution with hexane:dioxane (95:5 w/v) at a flow of 1.5 cm^3 min^{-1} (Waters model 6000A pump, 10.3 MPa, Serva Silica column 42360, Heidelberg, FRG). The injection volume varied between 50 mm^3 to 250 mm^3. The eluate was scanned (230-400 nm) on-line with a Hewlett-Packard 1040A high-speed spectrophotometer. The signal wavelengths of the detector were routinely set at 270 and 350 nm. Thiophenes were identified by retention time and UV-spectrum (Bohlmann et al, 1973). All peaks with an optimum in the UV-spectra between 320 nm and 380 nm were identified as thiophenes. A standard curve for α-terthienyl (0.3 nM - 3 μM) prepared with our HPLC-UV equipment yielded good linearity at 350 nm. The molar extinction coefficients of natural thiophenes are of comparable magnitude (Norton, 1985) and close to that of α-terthienyl (Bohlmann et al, 1973). The HPLC-UV extinction values could thus be used to estimate the contents of thiophenes in the different samples.

Experimental procedure.

The experimental procedure was as follows. Aggregates from the stock cell culture of Tagetes patula were divided into 7 fractions with aggregate diameters varying from 0.5 to 13.0 mm. These fractions were used for respiration measurements and determination of the intrinsic constants V_m and K_m, which were used as experimental data to calculate oxygen concentration profiles and critical diameters.

All manipulations with the plant cells were carried out under aseptic conditions. From the stock cell line of T.patula, 4 fractions with an aggregate diameter below 6 mm were obtained by sieving. Three fractions with diameters of more than 6 mm were obtained by manual selection of the aggregates. As a control a mixture of all fractions was taken along in this experiment. About 5 g of fresh cell aggregates of each fraction were transferred in duplicate to 100 cm^3 Erlenmeyer flasks and MS-medium was added until a total volume of 40 cm^3. The flasks were closed with cotton wool and aluminium caps, and exposed to the same conditions as described for T.patula cell cultures. Every 3 days, the PCV of each flask was determined, the cells were sieved to keep the aggregate diameter of each fraction within the original values, and subsequently the medium was replaced by fresh MS-medium. The loss of free cells was taken into account in the experimental results. The harvested media were

analysed for thiophene content.

RESULTS AND DISCUSSION

Fractionation

The 7 fractions which were obtained by sieving and manual selection resulted in the following mean aggregate diameters: 1.0, 1.4, 2.8, 5.4, 11.2, 12.3 and 12.8 mm.

Figure 2 shows photographs of six of these cell aggregate fractions of Tagetes patula. Considering the variability in aggregate shape, especially of the large ones, it is clear that the results of the experiments should not be used for establishment of quantitative correlations but merely for the observation of trends.

Figure 2. Photograph of six fractions of Tagetes patula cell aggregates in increasing mean aggregate diameter order, resp. 1.0, 1.4, 2.8, 11.2, 12.3 and 12.8 mm.

Oxygen concentration profiles and critical aggregate diameters

In the Figures 3 and 4, the plots are given of the apparent Michaelis Menten constants V_m' and K_m' against the aggregate diameters. Stirring problems in the biological oxygen monitor in case of the bigger aggregates make the pertinent values less reliable than those of the smaller aggregates. Extrapolation of the curves in Figures 3 and 4, resulted in rough estimates of the values of the intrinsic Michaelis Menten constants V_m ($4*10^{-4}$ mol kg^{-1} s^{-1}) and K_m ($3.5*10^{-2}$ mol m^{-3}).

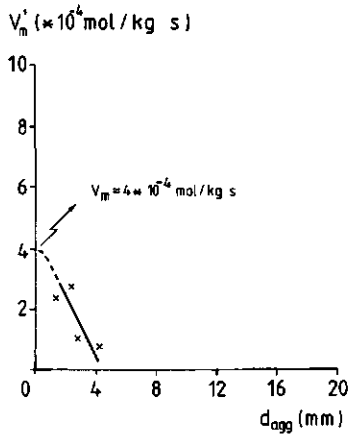

Figure 3.
The apparent maximum consumption rate V_m' as a function of the aggregate diameter.

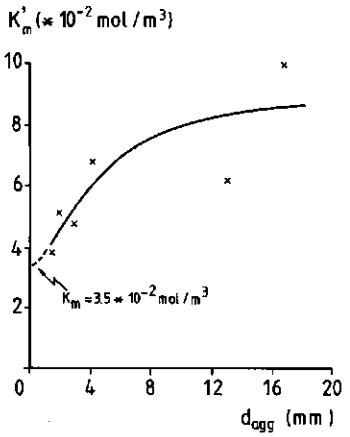

Figure 4.
The apparent Michealis-Menten constant K_m' as a function of the aggregate diameter.

With these values and with the estimated value of $1.9*10^{-9}$ m^2 s^{-1} for the effective diffusion coefficient ID_e of oxygen in the aggregates, the oxygen concentration profiles in the aggregates were calculated. The dry weight of the cells in the aggregates was determined as 10 kg m^{-3}, which also was an input parameter needed for the calculations (see Appendix). The oxygen concentration profiles are given in Figure 5 for aggregate diameters varying from 0.5 to 15.0 mm. As can be seen in Figure 5 the oxygen concentration in the aggregates is approaching zero in the centre when the diameter approximates 3 mm, which can be considered as the critical diameter if Michaelis Menten kinetics are applicable. Assuming zero-order kinetics for the plant cells, the critical aggregate diameter, calculated with equation (1), under otherwise the same conditions, is 1.7 mm, which is somewhat lower, but approximates the value with MM-kinteics quite well. Probably, the ratio of the oxygen saturation concentration in the bulk solution (0.25 mol m^{-3}) and the K_m value (0.035 mol m^{-3}) does not meet the required high value for the assumed zero-order kinetics. In theory, zero-order kinetics can only be reached if this ratio is infinitely high (oxygen concentration = ∞).

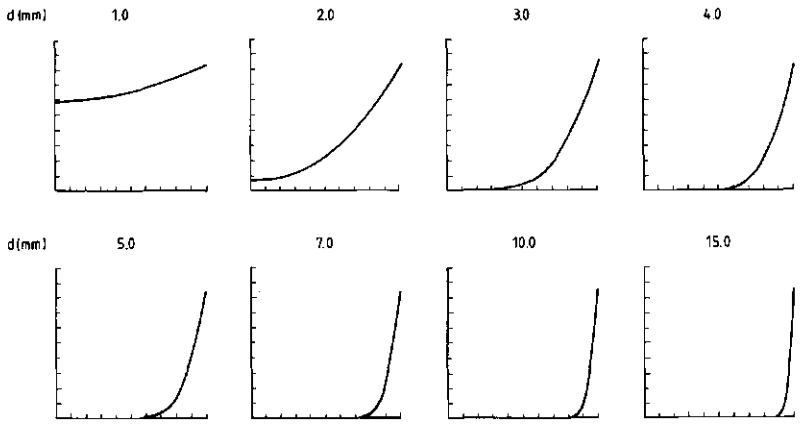

Figure 5. Calculated oxygen concentration profiles in the aggregate as a function of aggregate diameter. V_m = $4*10^{-4}$ mol kg^{-1} s^{-1}, K_m = $3.5*10^{-2}$ mol m^{-3}, cell load 10 kg m^{-3}, surface oxygen concentration 0.25 mol m^{-3}, effective diffusion coefficient of oxygen in aggregate $1.9*10^{-9}$ m^2 s^{-1}.
Range x-axis: from centre (left) to surface (right) of the aggregate.
Range y-axis: from zero (under) to 0.30 mol m^{-3} (above).

Aggregate growth and thiophene production

Preliminary experiments with Tagetes patula aggregates in shake flasks (Ketel, 1987) and in a 1.5 dm^3 bioreactor showed that thiophene production was highest in the lag phase of the growth cycle (Figure 6). With this knowledge the experiments were started as early as possible in the growth cycle of the cell aggregates.

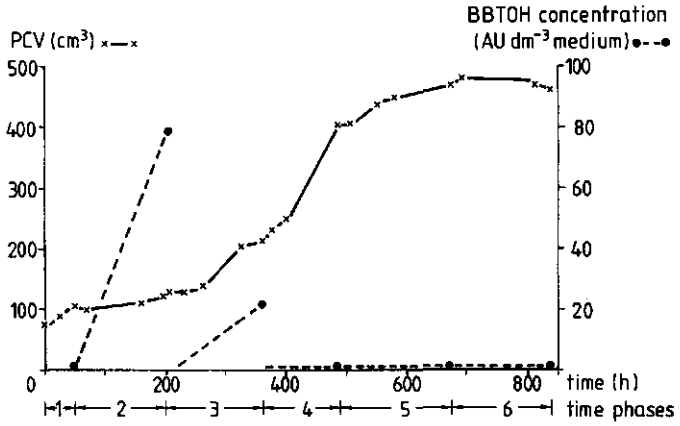

Figure 6. Cell growth and production of BBT-OH in a 1.5 dm^3 bioreactor (CSTR) by Tagetes patula cells. Medium refreshment was performed between each of the time phases 1 to 6. Airflow 0.3 vvm, stirrer speed 100 rpm, temperature 24°C, illumination 2.5 klux. AU = Absorption Unit.

In Figure 7 the aggregate growth and the cumulative thiophene concentration in the media are given for the different cell aggregate fractions. The biomass of all fractions increased during the experiment. The amount of thiophenes excreted into the medium was dependent on the size of the aggregates. In the aggregate fraction with a mean diameter of 1.0 mm, no thiophenes were recovered from the medium. The aggregate fractions with mean diameters of 1.4, 1.9, 2.8 and 5.4 mm showed thiophene excretion in the range of 8 to 40 µmol per cm^3 of cells, but the production was not significantly correlated with the mean aggregate diameter. In these fractions almost all thiophenes were produced during the initial part of the experiment (lag phase). The larger aggregates with a mean diameter of 11.2 and 12.3 mm showed higher productions (50 to 95 µmol per cm^3 of cells). The fraction with the largest mean aggregate diameter (12.8 mm) showed less production in comparison with 11.2 and 12.3 mm (explanation below).

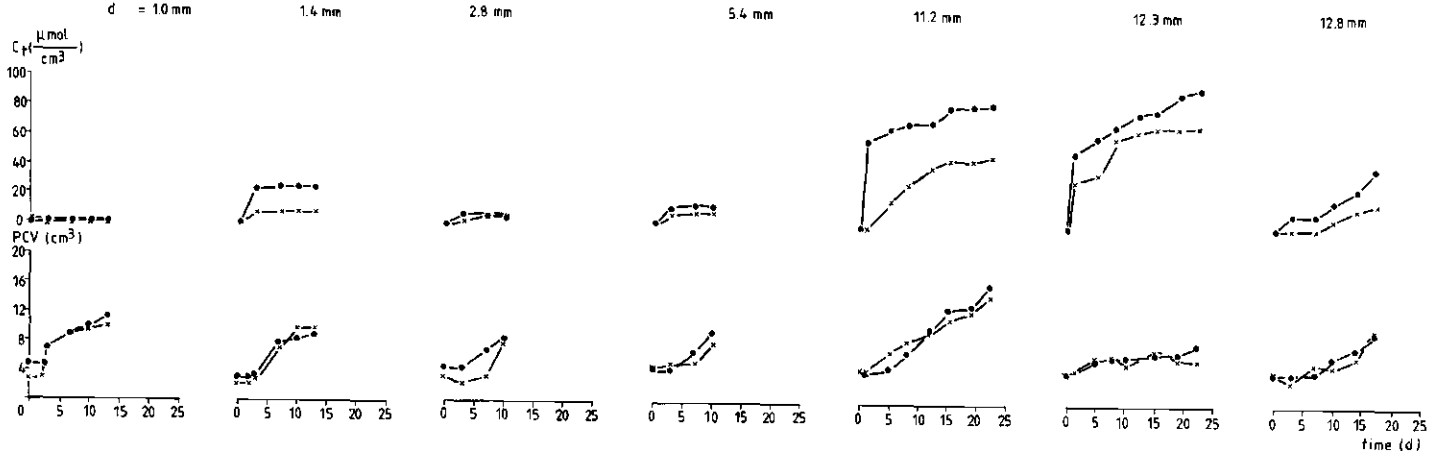

Figure 7. Packed cell volume of aggregates (bottom plot) and accumulated thiophene production in MS-medium (upper plot) during the experiment for aggregate fractions of Tagetes patula plant cells with different aggregate sizes. (o and x are duplicates).

The thiophene content in the aggregates from the different fractions was determined at the beginning and at the end of the experiment. At the beginning of the experiment the thiophene content in the aggregate was positively correlated with the aggregate diameter (Figure 8). At the end of the experiment, the thiophene content in the aggregates of all fractions was negligible. If all thiophenes in the aggregates were assumed to be released into the medium during the experiment, the contribution of thiophenes in the aggregates relative to the actual thiophene content in the medium is less than 1%, indicating that during the experiment production and release of thiophenes occurred.

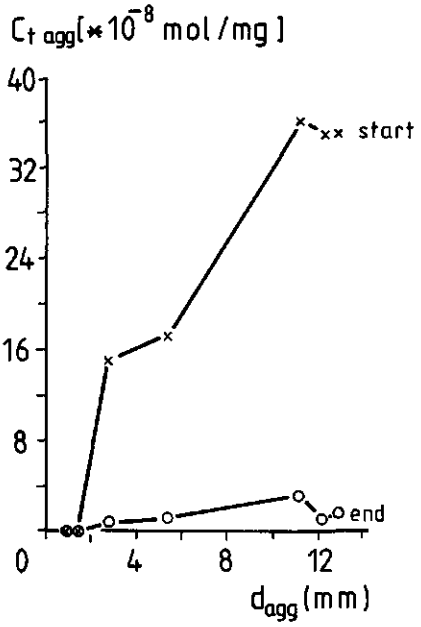

Figure 8.
Thiophene concentration in cell aggregates of Tagetes patula at the start (x) and after the experiment (o) as a function of the aggregate diameter.

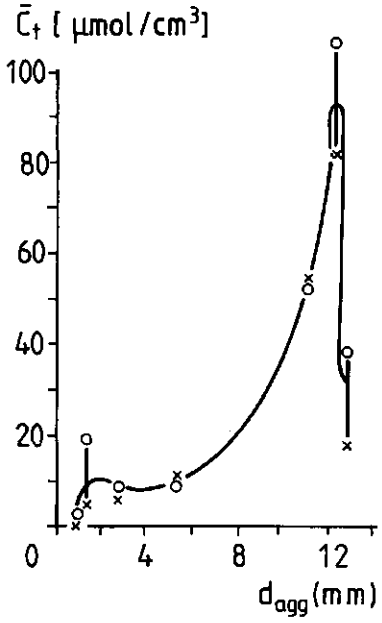

Figure 9.
The time-averaged thiophene production of Tagetes patula plant cell aggregates as a function of the aggregate diameter. The mean thiophene production is the ratio of the cumulative amount of excreted thiophenes and the averaged amount of cell aggregates in the experimental time lapse. (o and x are duplicates).

In Figure 9 the mean thiophene production of the T.patula aggregates is

plotted against the aggregate diameter. The thiophene production clearly appears to be related to the aggregate size of the T.patula plant cells. The mean thiophene production increased especially at aggregate diameters exceeding 3 mm (Figure 9). Also, Figure 9 suggests an optimum value in the mean aggregate diameter in the range of 11 to 13 mm.

An explanation for the higher production of aggregates exceeding 3 mm diameter is oxygen exhaustion, which is supported by the calculated value of the critical diameter (about 3 mm).

The decrease in thiophene production of aggregates with a diameter of 12.8 mm was very likely due to cell lysis in the centre of these aggregates which causes cavitation in the aggregates. This was visualized after the experiment by observation of the cross-sectional areas after cutting the aggregates. Only about 30-40% of the volume of the aggregates in this fraction consisted of intact plant cells at the periphery of the aggregates. To a lesser extent, cavitation was also observed in aggregates with mean diameters of 5.4, 11.2 and 12.3 mm. In these fractions cavitation appeared positively correlated with the aggregate diameter. This observation leads to the hypothesis that lack of oxygen does lyse the cells in the aggregate centre which impel the remaining viable cells to thiophene production. The occasion of hollow aggregates was not related to increased pH in the medium as reported for bean cells by Bertola (1980).

CONCLUSIONS

Thiophene production by Tagetes patula cell aggregates is dependent on the aggregate size. The thiophene production per unit volume of cell aggregates increases with increasing diameter starting at 3 mm, with an optimum diameter in the range of 11 to 13 mm. Above the optimum aggregate size the production is decreasing.

The aggregates with a diameter exceeding 5.4 mm show cavitation in the centre, as a result of cell lysis.

The critical diameter for oxygen uptake by the plant cell aggregates of T.patula was calculated to be about 3 mm, based on the measured respiration of the aggregates and the assumption that the effective diffusion coefficient for oxygen in the aggregate values $1.9*10^{-9}$ m^2 s^{-1}. The results lead to two possible hypotheses for increased thiophene production for aggregates diameters exceeding 3 mm. First, the lack of oxygen in the centre of the aggregate

directly stimulates the inner cells to produce thiophenes and second, the lack of oxygen causes cell lysis in the aggregate centre which impels the remaining viable cells to thiophene production. Experiments to test these hypotheses are in progress.

ACKNOWLEDGEMENT

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NOMENCLATURE

ID_e	= effective diffusioncoefficient	[$m^2\ s^{-1}$]
C_s	= substrate concentration at surface	[mol m^{-3}]
C_{eq}	= substrate concentration at equilibrium	[mol m^{-3}]
r_s	= consumption rate	[mol $m^{-3}\ s^{-1}$]
d_{crit}	= critical aggregate diameter	[m]
d_{agg}	= aggregate diameter	[m]
L	= length of aggregate	[m]
W	= width of aggregate	[m]
V_m	= intrinsic maximum consumption rate	[mol $kg^{-1}\ s^{-1}$]
K_m	= intrinsic Michaelis Menten constant	[mol m^{-3}]
V_m'	= apparent maximum consumption rate	[mol $kg^{-1}\ s^{-1}$]
K_m'	= apparent Michaelis Menten constant	[mol m^{-3}]
*	= multiplication sign	

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CHAPTER 9

GENERAL DISCUSSION

The research work described in this thesis was executed in close cooperation with the Research Institute Ital in Wageningen, within the framework of NOVAPLANT, and was part of a multi-disciplinary project titled 'Biotechnological production of non-persistent bioinsecticides by means of plant cells in vitro'. This project was already started in 1981 at the Research Institute Ital. Their main share in the project was the initiation of callus from suitable plants, development of appropriate growth and production media, fundamental research on plant physiology, and to develop high-producing cell lines in liquid media. At the start of the project the expectation was that after one year, one or more producing cell lines would be available. Our part of the project initially concerned the production of secondary metabolites in a bioreactor with these high-producing plant cell line(s). However, due to unforeseen circumstances, no producing cell lines became available until recently. This situation caused a necessary shift in our research subject which resulted in the work presented in this thesis. The thesis-'history' is outlined below.

In view of upscaling of bioreactors in the future with immobilized plant cells, it was necessary to look at the upscaling of the immobilization techniques in order to provide pilot-plant bioreactors with immobilized plant cells. Until that time most of the techniques routinely used, in particular immobilization in hydrogels, only were of limited production capacity. The resonance nozzle technique, which we applied for immobilization of biocatalysts in large quantities, was suitable for immobilization of plant cells in calcium alginate as well as some other gels (agar, κ-carrageenan and gellan gum). Moreover, the resonance nozzle technique also appeared to give even better results in case of immobilization of bacterial cells and yeasts. Thus, the applicability of the resonance nozzle is much wider than within the scope of this research work on plant cells (Chapter 3+4).

It was known from the literature (Chapter 2) that, once immobilized, plant cells could produce more secondary metabolites in some cases, for unexplained reasons. Substrate diffusion limitation could be a possible explanation and

because in our department already some know-how existed on this subject, this was extended in the area of plant cells.

In the measurements of respiration of plant cells in different gels (Chapter 5) the uncertainty of the value of the effective diffusion coefficient (ID_e) of oxygen showed up to be a great handicap. Likewise, the mathematical model for the calculation of the oxygen concentration profiles in beads was not experimentally tested until then. These uncertainties resulted in the ID_e-measurements (Chapter 6) and the experimental measurements of the oxygen concentration profiles in gel beads containing plant cells (Chapter 7). The ID_e measurements showed values that were close to the assumed values used in the respiration measurements in Chapter 5. The used measurement technique for ID_e was also reported in the literature for glucose as substrate. In case of well-soluble substrates the technique is rather simple. However, in case of gaseous substrates like oxygen, high demands on the experimental equipment in order to keep it leak-free for gases must be met. The experimental determinations of the oxygen concentration profiles in agarose beads containing the plant cells in Chapter 7 was a check afterwards on the validity of the mathematical model for the profile calculations.

Finally, a thiophene producing cell line of Tagetes patula was used to investigate the role of oxygen limitation in rather large cell aggregates on the thiophene production (Chapter 8). Here, the previously described subjects were integrated. Artificial cell immobilization in gels was impossible from a practical point of view because of the heterogeneous nature of the cell suspension and the presence of real large aggregates. In this case it was regarded as a spontaneous cell immobilization.

SUMMARY

The study described in this thesis is part of the integrated project 'Biotechnological production of non-persistent bioinsecticides by means of plant cells *in vitro*' and was done in close cooperation with the research Institute Ital within the framework of NOVAPLANT. The plant cells used in this project were *Tagetes* species which produce thiophenes, naturally occurring biocides, particularly against nematodes.

The objective of the study described in this thesis was to use immobilized plant cells or large plant cell aggregates for secondary metabolite production. In particular the upscaling of immobilization techniques for plant cells, the role of diffusion limitation of oxygen as a substrate on the immobilized plant cells and its effect on secondary metabolite production of the immobilized plant cells were subject of research.

A literature survey on immobilized plant cells is presented in Chapter 2. The advantages of immobilized plant cells, several aspects concerning immobilization techniques, consequences of plant cell immobilization, immobilized plant cell reactors, and future prospects of immobilized plant cells are discussed in this Chapter.

Chapter 3 deals with the application of the resonance nozzle as an immobilization technique with a high production capacity for plant cells as well as yeast cells in calcium alginate gel beads. It is found that this technique has a production capacity of two orders of magnitude larger than the conventional dripping technique with a needle. The viability of the cells after immobilization with the resonance nozzle was preserved. An extension of the applicability of the nozzle technique for thermogelling gel supports (κ-carrageenan, agar and gellan gum) is described in Chapter 4. Plant cells, yeast cells, bacterial cells and insect cells were used as model systems in the experiments.

In Chapter 5, the occurrence of oxygen diffusion limitation of *Daucus carota* cells in agarose, calcium alginate and κ-carrageenan is determined by respiration measurements of the immobilized cells in order to explain the enhanced pronounced secondary metabolite production with alginate immobilized plant cells from in the literature reported experiments. However, in our experiments no differences between the support materials could be observed.

The effective diffusion coefficient for oxygen (ID_e) in the gel material is an important factor in mathematical model calculations in order to quantify the occurrence of oxygen diffusion limitation. Chapter 6 deals with the experiments in which ID_e was determined in different gel materials (calcium alginate, κ-carrageenan, gellan gum, agar and agarose) by measuring the oxygen diffusion from a well-stirred solution into gel beads, which were initially free of oxygen. A mathematical model was fitted on the experimental data resulting in the value of ID_e, which was used in the following experiments.

In several parts of this thesis a mathematical model was used for calculation of oxygen concentration profiles in gel beads containing plant cells or cell aggregates in order to visualize the occurrence of oxygen diffusion limitation. In Chapter 7 this model is tested on validity by experimental measurement of the oxygen concentration profiles in agarose beads containing respiring plant cells of Tagetes minuta. This was done with the aid of an oxygen microelectrode with a tip of 15 μm. The experimental and calculated oxygen concentration profiles correspond quite well.

Chapter 8 deals with the effects of aggregate size and oxygen diffusion limitation on thiophene production and cell growth by cell aggregates of Tagetes patula. It is concluded that aggregate size is related to thiophene production by the observation of an 'optimum' aggregate size where the production is highest. Calculations of the oxygen concentration profiles showed that this could be due to the absence of oxygen in the centre of the aggregates.

SAMENVATTING

Het onderzoek, beschreven in dit proefschrift, is een gedeelte van het geïntegreerde projekt getiteld: 'Biotechnologische produktie van milieuvriendelijke bio-insecticiden door middel van plantecellen in vitro'. Plantecellen van de Tagetes soorten (afrikaantjes) werden in dit projekt onderzocht voor de produktie van thiofenen. Deze verbindingen werken als natuurlijk voorkomende biociden, speciaal tegen nematoden (aaltjes). Het onderzoek, in het kader van NOVAPLANT, is in nauwe samenwerking uitgevoerd met het researchinstituut Ital in Wageningen.

Het doel van het beschreven onderzoek was het produceren van secondaire metabolieten met behulp van geïmmobiliseerde plantecellen. In het bijzonder is in dit onderzoek aandacht besteed aan het opschalen van immobilisatietechnieken voor plantecellen, de rol van de diffusielimitatie van het substraat zuurstof op de geïmmobiliseerde plantecellen en de invloed hiervan op de produktie van secondaire metabolieten.

Een literatuuroverzicht op het gebied van geïmmobiliseerde plantecellen is in Hoofdstuk 2 weergegeven. De voordelen van geïmmobiliseerde plantecellen, verscheidene aspekten van immobilisatietechnieken, de gevolgen van immobilisatie van plantecellen, de reactoren voor geïmmobiliseerde plantecellen en de toekomstverwachtingen worden in dit hoofdstuk besproken.

Hoofdstuk 3 behandelt de toepassing van de resonantienozzle als immobilisatietechniek met een hoge produktiecapaciteit, voor zowel plantecellen als gistcellen in calciumalginaat bollen. Deze techniek heeft, vergeleken met de conventionele druppeltechniek door een naald, een produktiecapaciteit die twee ordegroottes hoger is. Na immobilisatie met behulp van de resonantienozzle bleven de cellen in leven. Een uitbreiding van de toepassing van de nozzle voor andere gelen (κ-carrageen, agar en gellaan) wordt in Hoofdstuk 4 beschreven. In deze experimenten werden plantecellen, gistcellen, bakteriële cellen en insektecellen als modelsystemen gebruikt.

In Hoofdstuk 5 wordt de mate van zuurstof diffusielimitatie bij Daucus carota cellen geïmmobiliseerd in agarose, calciumalginaat en κ-carrageen bepaald met behulp van respiratiemetingen aan deze geïmmobiliseerde cellen. Deze metingen zijn uitgevoerd om de in de literatuur vermelde verhoogde secondaire metabolietvorming, speciaal bij plantecellen geïmmobiliseerd in

calciumalginaat, te verklaren. Echter in onze experimenten werden geen verschillen tussen de onderzochte dragermaterialen gevonden.

De effektieve diffusiecoëfficient voor zuurstof (ID_e) in een gel vormt een belangrijke faktor in berekeningen om het optreden van zuurstof diffusielimitaite te kwantificeren. Hoofdstuk 6 behandelt de experimenten waarmee ID_e in verschillende gelen (calciumalginaat, κ-carrageen, gellaan, agar en agarose) is bepaald. In deze proeven werd de zuurstofdiffusie vanuit een goed geroerde oplossing in gelbollen, die aanvankelijk zuurstofloos waren, gemeten. Een model, dat een dergelijk systeem beschrijft, werd 'gefit' op de experimentele gegevens. De ID_e's, die op deze manier werden bepaald zijn in de vervolgexperimenten gebruikt.

Op verschillende plaatsen is in dit proefschrift gebruik gemaakt van een wiskundig model om zuurstof concentratieprofielen binnen gelbollen met plantecellen of celaggregaten te berekenen om het optreden van zuurstof diffusielimitatie aan te tonen. In Hoofdstuk 7 is dit model getoetst door middel van het meten van zuurstofprofielen in agarose bollen met Tagetes minuta plantecellen. Dit gebeurde met behulp van een zuurstof microëlektrode met een tip van 15 μm. De gemeten en berekende zuurstofprofielen komen goed met elkaar overeen.

Hoofdstuk 8 behandelt de invloed van aggregaatgrootte en zuurstof diffusielimitatie op de thiofeenproduktie en celgroei van Tagetes patula celaggregaten. Geconcludeerd kan worden dat de aggregaatgroote verband houdt met de mate van thiofeenproduktie. Een 'optimale' aggregaatgrootte is gevonden voor de produktie van thiofenen. Berekeningen van zuurstof concentratieprofielen tonen aan dat dit het gevolg kan zijn van de afwezigheid van zuurstof in het midden van de aggregaten.

CURRICULUM VITAE

De auteur van dit proefschrift werd te Elburg geboren op 3 november 1955. Na de middelbare school opleiding aan het Carolus Clusius College te Zwolle, welke werd afgesloten met het behalen van het diploma Atheneum B, begon hij in 1974 met de studie Levensmiddelentechnologie aan de Landbouwhogeschool te Wageningen. Deze studie, met als hoofdvakken levensmiddelenchemie, proceskunde en informatica, werd in 1982 afgerond. In de periode september 1982 tot maart 1987 vervulde hij de functie van onderzoeks-assistent bij de sectie Proceskunde van de vakgroep Levensmiddelentechnologie aan de Landbouwhogeschool te Wageningen. Het promotieonderzoek op het gebied van de plantecelbiotechnologie werd gesubsidieerd door de Programma Commissie Biotechnologie. Tijdens deze periode is hij ook enige maanden als tijdelijk universitair docent werkzaam geweest. Sinds 1 maart 1987 is hij als research medewerker in dienst bij Cacao de Zaan in Koog a/d Zaan.