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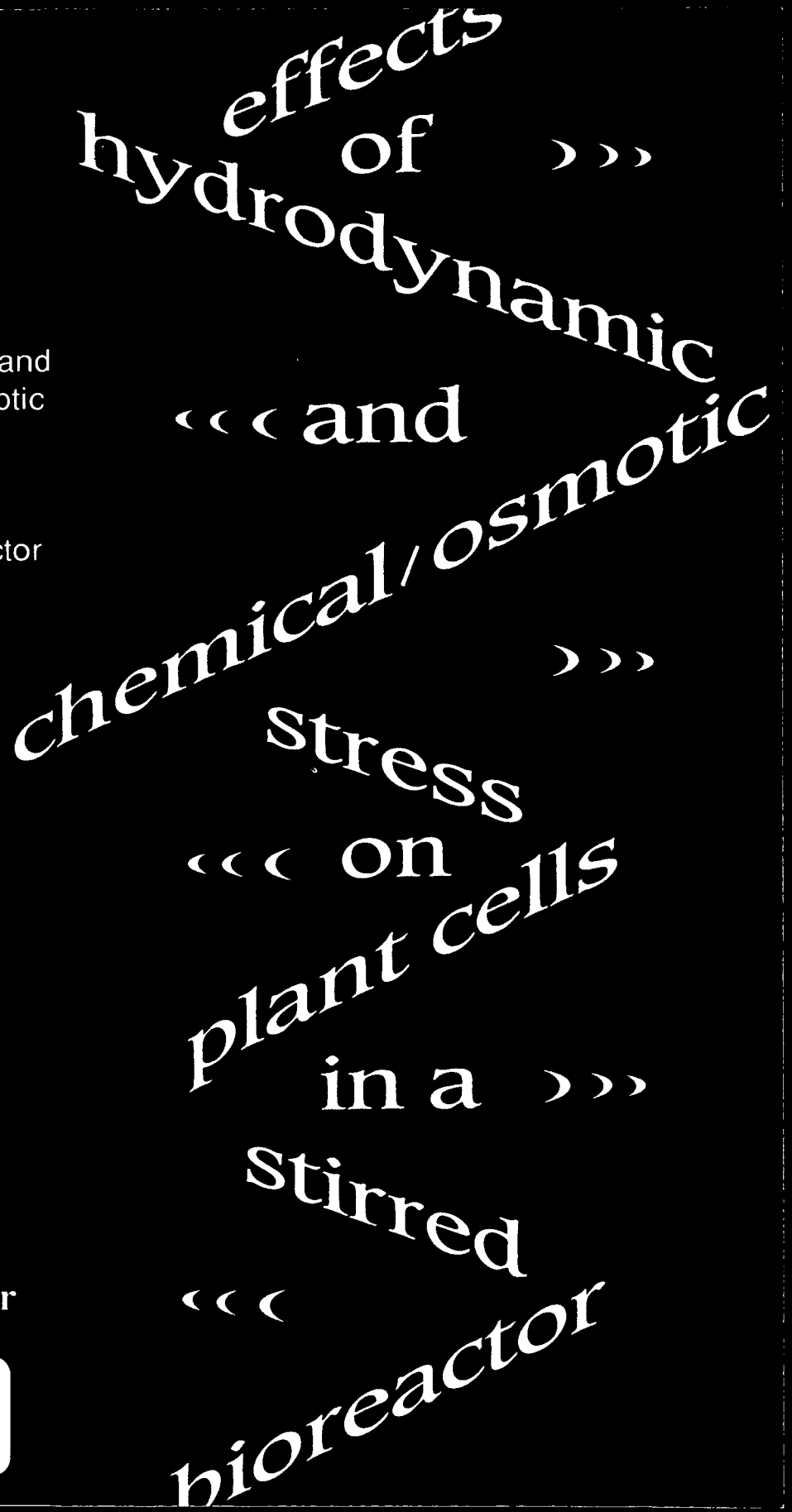

EFFECTS OF HYDRODYNAMIC AND CHEMICAL/OSMOTIC STRESS ON PLANT CELLS IN A STIRRED BIOREACTOR

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus, prof. drs. P.A. Schenck, in het openbaar te verdedigen ten overstaan van een commissie aangewezen door het college van Dekanen op donderdag 7 september te 16.00 uur

door

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geboren op 28 juni 1958 te Den Haag

scheikundig ingenieur

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VOOR MIJN MOEDER, VADER, ERIK

DANKWOORD

Vele mensen hebben bijgedragen aan de totstandkoming van dit proefschrift. Ik wil beginnen met een dankwoord aan de mensen die de basis van ons Kluyver-laboratorium voor Biotechnologie vormen.

Ik bedank de medewerkers van de instrumentmakerij, de glasblazerij, en de electronische dienst voor alle ondersteuning, hulp, en advies die ik van hun kreeg tijdens mijn promotieonderzoek. Voor het doen van innovatief onderzoek zijn goed functionerende ondersteunende diensten onontbeerlijk. Helaas wordt dit door de "hoge heren" die het "beleid?" maken onvoldoende ingezien.

Veel dank gaat uit naar Bart Kerkdijk, Sjaak Lispet, Peter Kroon, en Louise Trompert voor de ontelbare malen dat ze mij geholpen hebben met apparatuur en het oplossen van allerlei technische probleempjes.

Na het uitvoeren van een experiment moeten de analyses worden gedaan. Gelukkig heeft de onderzoeker in dit laboratorium een analysegroep tot zijn beschikking, zodat hij (of zij) de analyses niet zelf hoeft te doen. Uiteraard ben ik de analysegroep daarvoor veel dank verschuldigd en hoop dat hun positie geconsolideerd, zoniet versterkt zal worden.

Hens ten Hoopen, Karel Luyben, Kees Libbenga en Sef Heijnen wil ik bedanken voor de vele nuttige adviezen en het bewaken van de grote lijnen van het onderzoek. Walter van Gulik, Hens ten Hoopen en Ko Vinke hebben door hun fijne samenwerking in grote mate bijgedragen tot het welslagen van deze onderneming.

Wie dit proefschrift leest zal misschien denken dat ik alle experimenten zelf heb uitgevoerd. De experimentele bijdrage van Yvonne van Gameren en Jacqueline Kleiwegt zijn echter van groot belang geweest voor mijn onderzoek. Bovendien wil ik alle andere studenten en stagiaires danken voor hun medewerking en gezelligheid, in het bijzonder: Gerard Marckx, John Metselaar, André van den Broek, Louise Trompert, Laurie Krombeen en Marjan Smit.

Alle andere leden en ex-leden van de plantecelgroep en medewerkers van het Kluyver-laboratorium voor Biotechnologie wil ik bedanken voor de prettige werksfeer gedurende de afgelopen vier jaar.

CONTENTS

GENERAL INTRODUCTION

In principle many valuable products like pharmaceuticals, flavours, fragrances, and dyes can be produced by plant cell culture in liquid media. The production of those secondary metabolites can be performed in a bioreactor. Although the yearly production of most plant derived products is relatively small, they represent by their high prices an enormous economic value. The retail price of medical products containing plant derivatives that are yearly sold in the USA amounts $8 billion.

Developing a process for the production of plant derived chemicals by cell culture is by no means an easy undertaking. Little information is available to design an industrial-scale process properly. Growth and production kinetics of cell lines, process scale-up and down-stream processing of products have been hardly studied until now.

The subject of this thesis mainly concerns the effects of hydrodynamic and chemical/osmotic stress on plant cells in a stirred bioreactor. Hydrodynamic stress ('shear stress'), is exerted on the cells during cultivation in mixed bioreactors. Initially it has been assumed that plant cells are very sensitive to hydrodynamic stress. This would prevent the use of simple impeller stirred tanks for large-scale cultivation of plant cells, and urge the development of (expensive) alternative low-shear bioreactors. Until 1984 data on this subject were scarce, and therefore it was made one of our primary research topics. Chemical/osmotic stress can play a role in the induction of secondary metabolism. Besides, a deliberate application of chemical/osmotic stress could in theory lead to higher attainable biomass concentrations, thereby improving the economic feasibility of the process.

This study was carried out at the Department of Biochemical Engineering of the Delft University of Technology within the project group Plant Cell Biotechnology of the Biotechnology Delft Leiden (BDL) framework during the period of december 1984 until december 1988. There was a close cooperation with the Department of Plant Molecular Biology in Leiden.

The organization of the thesis is as follows. An introduction on some aspects of the large-scale cultivation of plant cells for the production of secondary metabolites is given in **Chapter 1**. Attention is focused on process design, regime analysis, and assessment of hydrodynamic stress

parameters.

In **Chapter 2** a literature review is given on the experimental methods for the assessment of hydrodynamic stress sensitivity of cultured plant cells. Few publications have reported on this subject until now. Relevant papers on the assessment of shear sensitivity and the effects of shear stress on growth and production of other eucaryotic (fungal, mammalian, insect) cells are reviewed to demonstrate methods and techniques employed in this field.

In **Chapter 3** batch culture experiments are described for the determination of the effects of short-term hydrodynamic stress on four different plant cell suspension cultures.

In **Chapter 4** chemostat experiments are described for the determination of the effects of long-term hydrodynamic stress on one cell line in particular: *Catharanthus roseus*.

In **Chapter 5** experimental work on the effects of chemical/osmotic stress on the growth and product formation of *C. roseus* cell suspension cultures is presented.

In **Chapter 6** the outcome of the previous chapters is evaluated in a general discussion.

CHAPTER 1

LARGE-SCALE PROCESS DESIGN FOR THE CULTIVATION OF PLANT CELLS IN STIRRED FERMENTERS FOR THE PRODUCTION OF SECONDARY METABOLITES.

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Introduction

Plants have provided us with food, fuel, and fibres since prehistoric times. They have been also an inexhaustible source of a diverse array of chemicals such as flavours, fragrances, natural pigments, pesticides and pharmaceuticals. This seemingly unrelated collection of chemicals can be grouped together under the broad heading of plant secondary metabolites. Secondary metabolites can be functionally distinguished from primary metabolites such as amino acids, nucleic acids, and carbohydrates in that they do not seem to have any direct physiological function. Generally it is assumed that secondary metabolites act as signals from the plant to its environment. Many secondary metabolites are thought to play a role in the plant's defence against animal, microbial or viral attack. Although much progress has been made in organic chemistry it is still not feasible to synthesize many of these chemicals from simple organic building blocks.

Many efforts were made in the fifties and the sixties by the chemical industry to substitute most of our natural products by synthetic analogs. The advance of modern biotechnology in the seventies has reversed this trend. The growing awareness of the public towards the drawbacks of many synthetic products e.g. artificial fragrances and flavours having unpleasant side-effects like allergies, and the world-wide "back-to-nature" trend of the eighties are reviving the interest in products from natural origin. In pharmacy, plants still have remained the sole source of some 25% of the prescribed medicines. The potential of plants as source of pharmaceuticals is enormous, a systematic survey of this abundance has in fact just begun. Until now only 2500 plant species have been properly screened for pharmaceuticals out of a world population of at least 250,000 (Stafford *et al.*, 1986). Therefore it can be expected that plants will play an increasing role in supplying new and better pharmaceuticals.

In this chapter some aspects of the large-scale cultivation of plant cells for the production of secondary metabolites are studied. After a short introduction on plant cell culture, economic feasibility and large-scale cultivation a process design, regime analysis, and assessment of hydrodynamic stress parameters are treated. Starting from a hypothetical production process the conventional stirred tank bioreactors to be used for this process are calculated using data obtained by experimental work

in our project group, data from literature, and reasonable assumptions.

A regime analysis is performed on the largest fermenter of the process (25 m^3). The same procedure is applied to a geometrically similar down-scaled 5-l fermenter under conditions of constant power input or impeller tip speed with respect to the 25-m^3 reactor. Hydrodynamic stress parameters are assessed for both the industrial-sale and the lab-scale fermenter.

Plant cell culture

Plant cell culture can be described as the culturing of plant cells in an undifferentiated state under axenic conditions. This technique has been established for many years. Already in the forties plant cell culture has been regarded as a potential alternative to the agricultural production of fine chemicals (Gautheret, 1942). The first patent for production of substances by plant tissue culture was obtained in 1956 (Routien and Nickell, 1956). In principle it is possible to grow plant cells in substantial volumes under conditions that are similar to microbial fermentations. The production of plant cell products in an industrial type production system has obvious advantages compared to field grown products. Supply, quality and price are steady and not affected by climactic and political disturbances, and effects of pests and diseases. However, routine plant cell culture production of valuable chemicals is fraught with a number of genetic, biochemical, engineering, and, last but not least, economic difficulties to be overcome.

Economic feasibility

The production of plant cell products by fermentation must be economically feasible. This prerequisite limits the range of products, because of low volumetric production rates and consequently high initial investment in fermentation equipment and downstream processing. The markets for some plant secondary metabolites, their application, and wholesale price are given in *Table 1.1,* (Curtin, 1983).

Best candidates would be products with a production volume of 1 to 100 ton/year, and a price of $1000 - $10,000/kg (Veltkamp, 1985). Presently the only commercial plant cell culture process producing a secondary metabolite is the shikonin production by *Lithospermum erythrorhizon* developed by Mitsui Co. in Japan. It is carried out in 200-l and 750-l

Table 1.1. Markets for some plant secondary metabolites.

Compound	Application	Price	Market
ajmalicine	circulatory problems	$1500/kg	$6M (World)
codeine	sedative	$650/kg	$50M (USA)
digitalis	heart disorders	$3000/kg	$20-55M (USA)
jasmine	fragrance	$5000/kg	$0.5M (World)
pyrethrins	insecticide	$300/kg	$20M (USA)
quinine	malaria, flavour	$100/kg	$5-10M (USA)
shikonin	dye, anti-bacterial	$4500/kg	$0.7M (Japan)
spearmint	flavour, fragrance	$30/kg	$85-90M (World)
vinblastine/ vincristine	leukemia	$5000/g	$18-20M (USA)

stirred-tank reactors (Curtin, 1983). Shikonin is used as dye and anti-bacterial agent. Other pharmaceuticals that might be at the brink of commercial production by plant cells are berberine, rosmarinic acid, digoxine and ginseng.

In many cases products from naturally-grown plants will be far more cheaper than those produced by plant cell culture. This is well illustrated by a comparison of the production of ajmalicine by either *Catharanthus roseus* roots or biomass cultivated on a large scale. Drapeau *et al.* (1987) studied the economic assessment of plant cell culture for the production of ajmalicine by *C. roseus*. Cost estimates have been prepared for ajmalicine obtained from naturally-grown *C. roseus* roots and by large-scale plant cell culture. The cost of naturally produced ajmalicine (≈$600) appeared to be only 20% of the cost of ajmalicine produced by cell culture (≈$3000). The principle reason for the high costs of the cell culture route is the slow specific production rate. Although cell culture gives a production of 0.20 $mg*g^{-1}*day^{-1}$ while the plant gives only 0.0082 $mg*g^{-1}*day^{-1}$ (a ratio of 24), this is not sufficient to give an economically feasible production by cell culture. The advantage of a far more higher specific production rate is more than offset by the investment costs of process equipment.

Large-scale cultivation

Large-scale cultivation of plant cells began nearly 30 years ago when Tulecke and Nickel (1960) described the growth a variety of species (*Holly*, gingko, *Lolium*, rose) on 134-l scale in a stainless steel tank. In the seventies Japanese workers cultivated tobacco cells in an impeller-stirred tank reactor of 20 m^3 (Noguchi *et al.*, 1977). In this large-scale experiment 1.5 m^3 cell suspension was used to inoculate 14 m^3 of MS-medium (Murashige and Skoog, 1962) with 3% glucose as carbon and energy source, three times the normal concentration phosphate, and 0.2 ppm of the growth hormone 2,4-dichlorophenoxyacetic acid. Operating conditions were: temperature: 28°C, aeration: 0.3 vvm, back-pressure: $5*10^4$ $N*m^{-2}$, agitation speed: 30 rpm.

Schiel and Berlin (1987) scaled-up cell cultures of *C. roseus* to volumes of 5 m^3 using conventional reactors with flat-blade impellers.

The German company DIVERSA constructed in 1986 a fermentation cascade consisting of five stirred tank fermenters of 0.075, 0.75, 7.5, 15, and 75 m^3 (gross volume). This plant has been erected to study all aspects of the economic production of secondary metabolites by large-scale cultivation of plant cells. DIVERSA claims that it has designed a low-shear impeller system capable of providing adequate homogeneity and oxygen transfer. Several cell lines, among those *Echinacea purpurea*, were cultivated on a scale of 60 m^3 without contamination problems (BINE, 1988).

It is obvious that large bioreactors are needed for industrial plant cell production processes. One of the major problems associated with mass cultivation of plant cells in bioreactors is to provide adequate mass-transfer, especially of oxygen. An important additional prerequisite for a successful process is the ability to provide the cells with favourable conditions to grow and produce secondary metabolites without exerting too much hydrodynamic stress generated by mixing and/or aeration. For economic reasons it would be advantageous to perform plant cell fermentations in existing conventional impeller stirred tank reactors. However, mixing with conventional impellers creates high levels of hydrodynamic stress to which plant cells have been regarded very sensitive. Therefore, experiments to determine the robustness of a plant cell line should be performed in order to decide whether it can be grown in an impeller-stirred bioreactor.

Assessment of hydrodynamic stress sensitivity

It is infeasible to test the sensitivity to hydrodynamic stress of a cell line on an industrial scale, so a small-scale experiment has to be designed in which the behaviour of the cell line can be studied. This can be done by down-scaling of the large-scale process to lab-scale proportions. A regime analysis has to be performed to determine which mechanism is rate-limiting in the large-scale process. In principle small-scale experiments should be performed under the same rate limiting regime. To test the sensitivity of the plant cells hydrodynamic stress should be exerted equivalent to that experienced by the plant cells on production-scale. In literature it is suggested to keep the tip speed of the impeller or power input constant to create similar hydrodynamic stress conditions on both scales (Märkl *et al.*, 1987). This approach is also adopted in this study but attention is focused on the quantification of the various hydrodynamic stress components.

An estimate of hydrodynamic stress intensity and a proper down-scaling of the large-scale process can be performed when the operating conditions and dimensions of the involved reactor(s) are known. In this example the largest fermenter of a plant cell production process is studied together with a geometrically down-scaled (lab-scale) 5-l fermenter operating at constant tip speed or power input with respect to the large-scale fermenter.

Process design

In the following a **hypothetical** process for the production of a secondary plant product is studied. The main process parameters are briefly summarized in *Table 1.2.* The process comprises two stages:
(1) In the growth phase biomass is grown fed-batch wise in a fermenter cascade.
(2) In the production phase (stationary phase) production medium with a high glucose concentration is fed to the fermenter to induce product formation. After 21 days a product concentration of 2.5% of dry-weight is reached. Data are partly based on growth kinetics research on *C. roseus* carried out in our project group (van Gulik *et al.*, 1989b), adopted from literature, and reasonable assumptions.

Table 1.2. Process parameters.

Design basis		
Production	[kg*year^{-1}]	500
Product loss during down-stream processing	[%]	20
Operation period	[day*year^{-1}]	300
Growth parameters		
Specific growth rate	[h^{-1}]	0.018
Doubling time	[h]	38.5
Initial biomass dry-weight concentration	[kg*m^{-3}]	2.5
Inoculation ratio	[%]	12.5
Biomass yield coefficient on glucose	[Ceq*Ceq^{-1}]	0.65
Maintenance coefficient on substrate	[Ceq*Ceq^{-1}*h^{-1}]	0.0074
Biomass yield coefficient on oxygen	[Ceq*mol^{-1}]	2.1
Maintenance coefficient on oxygen	[mol*Ceq^{-1}*h^{-1}]	0.0073
Maximum oxygen uptake rate	[mol*m^{-3}*h^{-1}]	10.6
Maximum substrate uptake rate	[mol*m^{-3}*h^{-1}]	23.4
Final biomass dry-weight concentration	[kg*m^{-3}]	20
Production parameters		
Final product concentration	[kg*kg^{-1}]	0.025
Production period	[day]	21

From these data the total volume for the production phase can be calculated to be 150 m^3. To diminish the risk of loss of production due to contamination or equipment failure and to increase process flexibility it is adequate to perform the production phase in for example six bioreactors of 25 m^3 each. Therefore the growth phase has to be performed in six parallel three-stage fermenter cascades of 0.063, 1.25, and 25 m^3.

A schematic view of a typical industrial-scale impeller stirred bioreactor is given in *Figure 1.1*, the proportions are given in *Table 1.3* (Oosterhuis, 1984). Some general process data are given in *Table 1.4*.

Using the data of *Table 1.2*, *1.3*, and *1.4* the dimensions and operating conditions of a 25-m^3 reactor can be calculated using a methodology for analysing agitator performance and mass transfer in large multi-turbine

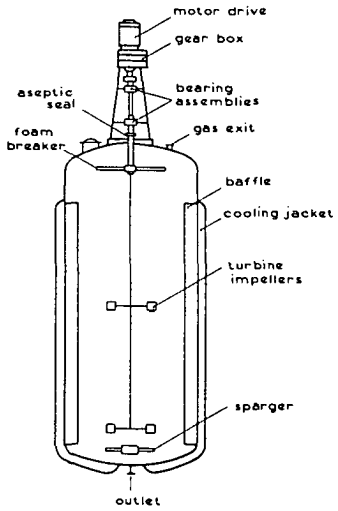

Figure 1.1. Schematic view of an industrial-scale bioreactor. (Oosterhuis, 1984)

Table 1.3. Proportions of industrial-scale reactor.

impeller diameter/vessel diameter	(D_i/D_V)	[-]	0.32
impeller blade width/impeller diameter	(W/D_i)	[-]	0.2
baffle diameter/vessel diameter	(D_b/D_V)	[-]	0.09
height/vessel diameter	(H/D_V)	[-]	2.25
liquid height/vessel diameter	(H_L/D_V)	[-]	1.8

Table 1.4. General process data.

broth density	(ρ_b)	$[kg*m^{-3}]$	1030
kinematic medium viscosity	(η)	$[m^{-2}*s^{-1}]$	$1*10^{-6}$
process temperature	(T_p)	$[^oC]$	25
back-pressure	(p_B)	$[N*m^{-2}]$	$5*10^4$
gas flow/reactor volume x time	(Q/V)	$[m^3*m^{-3}*s^{-1}]$	0.005

production fermenters developed by Bader (1987). The application of this approach provides a method for the determining axial dissolved oxygen profiles under conditions of known mass transfer rates as a function of

agitation-aeration characteristics. A stagewise approach is used which divides the fermenter into a series of mixing cells.

This modelling approach was applied using a computer program written in Turbo-Pascal (Borland International Inc., Scotts Valley, California, USA). The results of the calculations are presented in *Table 1.5* and *1.6* and are compared with the reactor dimensions and operating conditions of a geometrically down-scaled lab-scale fermenter. In the design of the large-scale fermenter it was assumed that the volumetric oxygen transfer coefficient k_La must be sufficiently high to provide non-limiting oxygen transfer under worst case conditions (at the end of the exponential growth phase).

Table 1.5. Dimensions of an industrial-scale fermenter and a geometrically down-scaled lab-scale fermenter.

			scale	
			industrial	lab
gross volume		[m^3]	25	0.005
net volume	(V)	[m^3]	20	0.004
height	(H)	[m]	5.44	0.316
diameter	(D_V)	[m]	2.42	0.141
liquid height (ungassed)	(H_L)	[m]	4.35	0.253
number of impellers	(n)	[-]	3	3
impeller diameter	(D_i)	[m]	0.77	0.045
impeller blade width	(W)	[m]	0.16	0.009
baffle diameter	(D_b)	[m]	0.22	0.013

Table 1.6. Operating conditions for the industrial- and lab-scale fermenter for constant impeller tip speed and power input.

			V [m^3]		
			25	0.005 (v_i=C)	0.005 (P/V=C)
gas flow	(Q)	[m^3*s^{-1}]	0.1	$3.3*10^{-5}$	$3.3*10^{-5}$
superficial gas velocity	(v_s)	[$m*s^{-1}$]	0.02	0.0014	0.0014
hold-up	(1-ε)	[-]	0.043	0.039	0.016
O_2-transfer coefficient	(k_La)	[s^{-1}]	0.0072	0.03	0.004
impeller speed	(N)	[s^{-1}]	1.28	21.9	8.6
impeller tip speed	(v_i)	[$m*s^{-1}$]	3.1	3.1	1.2
power input	(P/V)	[$W*m^{-3}$]	400	7000	400

Regime analysis

In order to establish which mechanisms are rate-limiting in a large-scale process regime analysis can be applied. This technique is performed by comparison of characteristic parameters of the mechanisms involved in the process. In most cases the characteristic time concept is used in which characteristic time (time constant) is a measure of the rate with which a mechanism takes place. Characteristic time is defined as the ratio of a capacity and a flow. Mechanisms with a characteristic time many magnitudes smaller than the process time are in a pseudo steady-state during the process, e.g. metabolic reactions that proceed in times less than one second. On the other hand, mechanisms with characteristic times many magnitudes larger than the process time will hardly proceed during the process, e.g. evolutionary processes.

In the following equations are given to calculate the various times constants of a process (Oosterhuis, 1984):

Fluid flow

The time constant for mixing of the gas-liquid dispersion is expressed by the circulation time t_{ci}:

$$t_{ci} = 0.5V_{ic}/\Phi_p \quad (1.1)$$

where V_{ic} is the compartment volume of one impeller in the reactor, and Φ_p is the circulation capacity of a turbine type stirrer expressed as:

$$V_{ic} = 0.08\pi D_v^2 H_L \text{ and} \quad (1.2)$$

$$\Phi_p = 0.75ND_i^3 \quad (1.3)$$

where D_v is the vessel diameter, H_L is the liquid height, N is the stirrer speed, and D_i is the impeller diameter.

An estimate for the mixing time t_m can be calculated from the circulation time t_{ci}:

$$t_m = 4t_{ci} \quad (1.4)$$

Oxygen flow

The time constant for oxygen transfer from gas to liquid t_{Ot} is defined by the reciprocal volumetric oxygen transfer coefficient k_La:

$$t_{Ot} = 1/k_La \quad (1.5)$$

The time constant for oxygen consumption t_{Oc} can be expressed as:

- during growth : $t_{Oc} = C^*_{OL}/r_O^{max}$ (1.6)

- during production : $t_{Oc} = C^*_{OL}/r_O^{main}$ (1.7)

in which C^*_{OL} is the oxygen saturation concentration, r_O^{max} is the maximum oxygen consumption rate during growth, and r_O^{main} is oxygen consumption for maintenance during production:

$$r_O^{max} = (1/Y_{OX})\mu C_X^{max} + m_{OX}C_X^{max} \quad (1.8)$$

$$r_O^{main} = m_{OX}C_X^{max} \quad (1.9)$$

where Y_{OX} is the biomass yield coefficient on oxygen, μ is the specific growth rate, C_X^{max} is the maximum biomass concentration and m_O is the maintenance coefficient on oxygen.

The residence time of the gas bubbles Θ_G is derived from the gas hold-up $(1-\varepsilon)$ in the reactor:

$$\Theta_G = (1-\varepsilon)V_L/Q \tag{1.10}$$

in which V_L is the liquid volume, and Q is the gas flow rate.

Heat flow

The time constant for heat production t_{Hp} can be calculated from:

$$t_{Hp} = \rho_b c_p |T_C - T_p| / (r_{HX} + r_{Hs}) \tag{1.11}$$

in which ρ_b is the density of the culture broth, c_p is specific heat coefficient, T_C is the critical process temperature, T_p is the optimal process temperature, r_{HX} is the heat production by the plant cells:

$$r_{HX} = 460*10^3 r_O \text{ (Cooney } et\ al.\text{, 1969)} \tag{1.12}$$

and r_{Hs} is the heat production by stirring approximated by:

$$r_{Hs} \approx P/V \tag{1.13}$$

in which P is the impeller power consumption.

The time constant t_{Ht} for heat transfer can be expressed as:

$$t_{Ht} = \rho_b V c_p / (aA) \tag{1.14}$$

in which a is the overall heat transfer coefficient, and A is the surface of the heating/cooling device.

Biomass and substrate flow

The time constant for growth t_X can be obtained from the reciprocal

specific growth rate μ:

$$t_X = 1/\mu \tag{1.15}$$

The time constant for substrate consumption t_S can be calculated in the same way as the time constant for oxygen consumption:

$$\text{- during growth} \quad : t_S = C_S/r_S^{max} \tag{1.16}$$

$$\text{- during production} : t_S = C_S/r_S^{main} \tag{1.17}$$

in which C_S is the actual substrate concentration in a fed-batch or a continuous process. For a batch process C_S is equal to C_{S0}, which is the substrate concentration at inoculum time. For all processes r_S^{max} is the maximum substrate consumption rate during growth, and r_S^{main} is the maintenance substrate consumption during the production phase:

$$r_S^{max} = (1/Y_{SX})\mu C_X^{max} + m_S C_X^{max} \tag{1.18}$$

$$r_S^{main} = m_S C_X^{max} \tag{1.19}$$

in which Y_{SX} is the biomass yield coefficient on substrate, and m_S is the maintenance coefficient on substrate.

The time constant for product formation t_P can be expressed as:

$$t_P = C_P^{max}/r_P \tag{1.20}$$

in which C_P^{max} is the maximum attained product concentration, and r_P is the product formation rate.

Using equations 1.1 to 1.20 and the data summed up in the preceding tables the time constants of a 25-m^3 stirred tank fermenter with plant cells were calculated together with those of a 5-l fermenter for constant tip speed and constant volumetric power input (*Table 1.7*). The process comprises a growth and a production phase. For all mechanisms the worst case was analysed being the end of the growth phase as the biomass

concentration attains 20 kg*m^{-3}.

Table 1.7. Comparison of time constants of a 25-m^3 fermenter and a down-scaled 5-l fermenter for constant tip speed (v_i) and constant volumetric power input (P/V).

	V [m^3]		
time constant [s]	25	0.005 (v_i=C)	0.005 (P/V=C)
t_{ci}	8	0.5	2
t_m	32	2	8
t_{Ot}	139	33	250
t_{Oc} (growth)	139	139	139
(production)	303	303	303
Θ_G	9	5	2
t_{Hp}	4800	1000	4800
t_{Ht}	4800	1000	4800
t_X	$2*10^5$	$2*10^5$	$2*10^5$
t_S (growth)	1500*	1500*	1500*
t_P	$18*10^5$	-	-

* Fed-batch process in which C_S is maintained at 10 mol*m^{-3}

Discussion of regime analysis

The growth and production time are the longest characteristic times in this process, thereby determining the total process time. All other times are at least one order of magnitude shorter, so the mechanisms involved are in a pseudo steady-state during the process.

Comparing the characteristic times of the 25-m^3 fermenter it can be concluded that there may be some problems in the oxygen supply to the plant cells. Oxygen transfer and oxygen consumption time are equal on large scale because design has been based on the critical oxygen transfer coefficient. However, mixing time is short compared to oxygen transfer time, so oxygen depletion is unlikely to occur in badly mixed regions of the fermenter. Heat transfer and heat production time are equal. The calculation is based on the assumption that all heat produced is exchan-

ged. No temperature gradients are expected because the time of heat production is long compared to mixing time.

In the 5-l fermenter for P/V=constant oxygen limitation will occur, because the oxygen transfer time is longer than the oxygen consumption time. When v_i=constant the oxygen transfer time is much shorter than the oxygen consumption time, so growth will not be hampered by oxygen limitation. When the process is operated fed-batch wise problems can occur when the substrate supply hampers. From the characteristic time of substrate consumption it can be concluded that in that case substrate limitation will set in after 25 minutes.

It can be concluded that experiments to simulated the large-scale process can not be performed on 5-l scale for P/V=constant. Under this condition oxygen transfer will be the ruling mechanism for growth instead of maximum specific growth rate. For v_i=constant oxygen transfer is more than sufficient, so small-scale experiments should be performed preferably under this condition.

Numerical assessment of hydrodynamic stress in an agitated reactor

Characteristic times for shear effects on the cell population can not be calculated due to various reasons:
(1) It is not possible to define a rate of the incompletely understood shear effects. Low levels of shear stress will have no apparent effect on the cells, resulting in an infinite characteristic time, whereas (too) high levels of shear can only be observed as a decrease of growth rate which is not predictable in advance.
(2) Various effects, each dependant on the flow regimes in the fermenter, are acting simultaneously on the cells.

An analysis of shear effects should therefore be based on comparison of these variety of mechanisms in dependency of the flow characteristics.

Cherry and Papoutsakis (1986) analysed the hydrodynamic effects on cells in agitated tissue culture reactors. Although their objective has been specifically to describe the mechanisms by which hydrodynamic forces can affect microcarrier systems, the same approach can be used to describe the stress mechanisms acting on suspended cells.

The primary mechanisms of cell damage appear to results from (a) direct

interaction between cells and turbulent eddies, (b) collision between cells in turbulent flow and collisions against the impeller or other stationary surfaces, (c) boundary layer shear forces around the solid subjects in the reactor, especially the impeller.

Interaction between cells and eddies

The level of hydrodynamic stress generated by interaction between cells and eddies is governed by the ratio of their sizes.

I. If the scale of the smallest turbulent eddy is sufficiently larger than the cells they will just follow the local flow pattern and no significant levels of hydrodynamic stress will be experienced.

II. If the smallest eddies of turbulent flow are of the same size as the cells they can interact between eddies in several possible ways:

1. A single eddy that cannot engulf the cell entirely can only act on a part of the surface, causing a cell to rotate. This mechanism will generally be accompanied by low levels of hydrodynamic stress.

2. High shear stresses may be exerted on the cells when several eddies with opposed rotation interact with it simultaneously since the cells cannot rotate to cancel each of the shear forces on it.

III. If the eddy size is the same as the intercell spacing cell-cell collisions may occur between cells generating a stress that is dependent on the collision energy and collision frequency.

From Kolmogorov's theory (Kolmogorov, 1941) the size of the smallest eddies d_e and the eddy velocity v_e can be calculated:

$$d_e \approx \eta^{3/4}/(N_p N^3 D_i^5/V_L)^{1/4} \quad \text{and} \tag{1.21}$$

$$v_e \approx \eta/d_e \tag{1.22}$$

where η is kinematic viscosity of the liquid phase, N_p is power number, N is impeller speed, D_i is impeller diameter, V_L is the agitated liquid volume.

According to Smith and Rielly (1988) a tensile stress τ_{te} acting on a spherical cell during interaction with an eddy can be estimated as:

$$\tau_{te} \approx 18.5\eta\rho_L v_e/d_e \tag{1.23}$$

in which v_e/d_e can be considered as the microscale shear rate.

To estimate the duration of the stress event Θ_{te} the tank-averaged, root-mean-square velocity fluctuations σ have to be estimated:

$$\sigma \approx 0.41 N_p^{1/3} N D_i \tag{1.24}$$

in which N_p is the power number, N is the agitation speed, and D_i is the impeller diameter:

The duration of the microscale stress event Θ_{te} can be estimated as:

$$\Theta_{te} \approx d_e/\sigma \tag{1.25}$$

The time constant for this stress event t_{te} can be estimated from the integral length scale for the macroscale turbulence L_t and the root-mean-square velocity fluctuations σ. L_t can be calculated from the reactor diameter D_v, taking into account the reactor proportions of *Table 1.3*:

$$L_t \approx D_b/3 \approx D_v/15 \tag{1.26}$$

The time constant t_{te} can be estimated as:

$$t_{te} \approx L_t/\sigma \tag{1.27}$$

Collisions

The cell-to-cell collision frequency of an individual cell N_{CC} is dependent on the volume fraction cells α, the eddy size d_e, and the diameter of the cells d_c:

$$N_{cc} \approx \pi\eta\alpha/(6 d_e d_c) \tag{1.28}$$

The energy of cell-to-cell collisions E_{cc} can be estimated as:

$$E_{cc} \approx M_c v_e^2 \quad (M_c = \rho_c V_c;\ V_c = \pi d_c^3/6) \tag{1.29}$$

where M_c is the mass of one cell and ρ_c is cell density.

The cell-to-impeller collision frequency of an individual cell N_{ci} can be estimated as:

$$N_{ci} \approx (3/8)\pi n_b D_i^2 N d_c / V_L \qquad (1.30)$$

where n_b is the number of impeller blades.

The energy of cell to impeller collisions E_{ci} is determined by the tip speed of the impeller v_i:

$$E_{ci} \approx 0.5 M_c v_i^2 \quad (v_i = N\pi D_i) \qquad (1.31)$$

Boundary layer shear stresses

Relatively large areas of high shear are expected in boundary layers around the solid objects submerged in the reactor. Because the moving impeller has the highest velocity relative to the liquid only boundary layer shear forces around the impeller will be considered. As a first approximation impeller blades can be modelled as stationary flat plates with fluid moving over them. Because the Reynolds number for transition from laminar to turbulent flow can be substantially reduced by impeller rotation and the possibility of boundary layer separation from the angled blades both laminar and turbulent boundary layers will be considered.

In the laminar case the boundary layer thickness δ_l can be estimated as:

$$3d_c \leq \delta_l \leq 5(\eta W/v_i)^{1/2} \qquad (1.32)$$

where W is impeller blade width.

The laminar boundary layer shear stress can be estimated as:

$$\tau_{wl} \approx 1.66 \rho_L \eta v_i / \delta_l \qquad (1.33)$$

In the turbulent case the boundary layer thickness δ_t can be estimated as:

$$3d_c \leq \delta_t \leq 0.37 W^{4/5} (v_i/\eta)^{-1/5} \qquad (1.34)$$

The turbulent layer shear stress can be estimated as:

$$\tau_{wt} \approx 0.0229 \rho_L \eta^{1/4} v_i^{7/4} \delta_t^{-1/4} \qquad (1.35)$$

Using equations 1.21 to 1.35 *hydrodynamic stress parameters can be* calculated for the 25-m^3 fermenter and the down-scaled 5-l fermenter for v_i=constant and P/V=constant. The results are presented in *Table 1.8.*

Discussion of assessment of hydrodynamic stress

Most of the hydrodynamic stress parameters are of the same order of magnitude for the 25-m^3 and 5-l fermenter at constant tip speed and power input. Two stress parameters diverge considerably: the time constant for macroscale turbulence t_{te}, and the cell-to-impeller collision frequency NC_{ci}. The time between consecutive passages through the impeller region is much smaller on a small scale than on large scale, which leads to a greater time constant for macroscale turbulence.

Table 1.8. Comparison between hydrodynamic stress parameters of a 25-m^3 industrial-scale fermenter and a down-scaled 5-l fermenter under conditions of constant v_i and P/V. Values between ( ) designate the ratio with respect to industrial scale.

		scale		
		industrial	lab (v_i=C)	lab (P/V=C)
reactor parameters				
V_L	[m^3]	20	0.004	0.004
D_i	[m]	0.774	0.045	0.045
N	[s^{-1}]	1.28	21.9	8.6
v_i	[$m*s^{-1}$]	3.1	3.1	1.2
P/V	[$W*m^{-3}$]	400	7000	400
cell parameters				
α	[-]	0.1	0.1	0.1
d_c	[m]	$1*10^{-4}$	$1*10^{-4}$	$1*10^{-4}$

Table 1.8 (continuation)

hydrodynamic stress parameters				*(ratio)*		*(ratio)*
d_e	[m]	$4*10^{-5}$	$2*10^{-5}$	*(0.5)*	$4*10^{-5}$	*(1)*
v_e	[$m*s^{-1}$]	0.026	0.052	*(2)*	0.026	*(1)*
τ_{te}	[$N*m^{-2}$]	12	50	*(4)*	12	*(1)*
Θ_{te}	[s]	$6*10^{-5}$	$3*10^{-5}$	*(0.5)*	$14*10^{-5}$	*(2)*
t_{te}	[s]	0.23	0.014	*(0.06)*	0.035	*(0.15)*
E_{cc}	[J]	$4*10^{-13}$	$15*10^{-13}$	*(4)*	$4*10^{-13}$	*(1)*
E_{cc}/M_c	[J/kg]	$7*10^{-4}$	$30*10^{-4}$	*(4)*	$7*10^{-4}$	*(1)*
NC_{cc}	[s^{-1}]	13	27	*(2)*	13	*(1)*
E_{ci}	[J]	$26*10^{-10}$	$26*10^{-10}$	*(1)*	$4*10^{-10}$	*(0.15)*
E_{ci}/M_c	[J/kg]	5	5	*(1)*	0.75	*(0.15)*
NC_{ci}	[s^{-1}]	$8*10^{-5}$	$2*10^{-2}$	*(250)*	$9*10^{-3}$	*(100)*
τ_{wl}	[$N*m^{-2}$]	3-17	12-17		3-7	
τ_{wt}	[$N*m^{-2}$]	18-40	31-40		5-8	

The specific impeller area ($\approx D_i W/V_L$) increases when volume decreases. This effect combined with a shorter t_{te} results in a far higher cell-to-impeller collision frequency at small scale.

By comparison of the stress parameters it can be concluded that it should be justified to use a small-scale fermenter at constant power input to simulate the stress conditions experienced by the cells on industrial scale. However, there are two objections. Firstly, oxygen limited growth will occur under the condition of P/V=C (regime analysis, *Table 1.7*). Secondly, on large scale the majority of the power is dissipated in a relatively small part of the fermenter, i.e. in the impeller region. So the forces acting on the plant cells will be considerably higher than the averages presented in *Table 1.8*. Therefore, to simulate those conditions it would be necessary to perform small-scale experiments at constant tip speed.

For practical reasons the condition of a constant tip-speed on small scale with respect to industrial-scale has to be weakened a bit. As can be seen in *Table 1.8* the power input for P/V=constant is ≈ 7 $kW*m^{-3}$ at an agitation speed of 21.9 s^{-1} ($\approx$1300 rpm). These conditions are hardly

attainable by the greater part of the commercially available equipment. Besides, performing experiments under those conditions is rather troublesome. Therefore, the experiments described in this thesis were performed at a maximum power input of ≈2-3 $kW*m^{-3}$ and an agitation speed of 17 s^{-1} (≈1000 rpm).

Conclusions

A hypothetical industrial-scale process for the production of a secondary plant product can be designed when enough data are available. In this process assessment of hydrodynamic stress by mixing will be one of the major problems. By proper down-scaling and regime analysis small-scale experiments can be devised for the studying of the behaviour of plant cells under hydrodynamic stress.

To study the shear-tolerance of plant cells a survey is made on methods for the assessment of hydrodynamic stress sensitivity of cultured plant cells (**Chapter 2**). Small-scale fermenter experiments in batch culture to study the effects of short-term hydrodynamic stress are described in **Chapter 3**. Chemostat experiments to study the long-term effects are described in **Chapter 4**.

List of symbols

a	overall heat transfer coefficient	$W*m^{-2}*K^{-1}$
A	surface	m^2
c_p	specific heat coefficient	$J*kg^{-1}*K^{-1}$
C_P	product concentration	$kg*m^{-3}$
C_{S0}	substrate concentration at inoculation	$kg*m^{-3}$
C_X	biomass concentration	$kg*m^{-3}$
C^*_{OL}	equilibrium concentration oxygen	$mol*m^{-3}$
d_c	cell diameter	m
d_e	eddy size	m
D_b	baffle diameter	m
D_i	impeller diameter	m
D_v	vessel diameter	m
E_{cc}	energy of cell-to-cell collisions	J
E_{ci}	energy of cell-to-impeller collisions	J

H	vessel height	m
H_l	liquid height	m
k_La	volumetric oxygen transfer coefficient	s^{-1}
L_t	integral length scale for macroscale turbulence	m
m_O	maintenance coefficient on oxygen	$Ceq*mol^{-1}*h^{-1}$
m_S	maintenance coefficient on substrate	$Ceq*Ceq^{-1}*h^{-1}$
M_c	cell mass	kg
n	number of impellers	-
N	impeller speed	s^{-1}
NC_{cc}	cell-cell collision frequency	s^{-1}
NC_{ci}	cell-impeller collision frequency	s^{-1}
p_B	back pressure	$N*m^{-2}$
P	power	W
Q	gas flow rate	m^3*s^{-1}
r_{HX}	rate of heat production by biomass	$J*m^{-3}*s^{-1}$
r_{Hs}	rate of heat production by stirring	$J*m^{-3}*s^{-1}$
r_O	oxygen consumption rate	$mol*m^{-3}*s^{-1}$
r_P	product formation rate	$kg*m^{-3}*s^{-1}$
r_S	substrate consumption rate	$kg*m^{-3}*s^{-1}$
t_{ci}	circulation time	s
t_{Hp}	time constant for heat production	s
t_{Ht}	time constant for heat transfer	s
t_m	mixing time	s
t_{Oc}	time constant for oxygen consumption	s
t_{Ot}	time constant for oxygen transfer	s
t_P	time constant for product formation	s
t_S	time constant for substrate consumption	s
t_X	time constant for growth	s
t_{te}	time constant for tensile stress	s
T_C	critical process temperature	K
T_p	process temperature	K
v_e	eddy velocity	$m*s^{-1}$
v_i	impeller tip speed	$m*s^{-1}$
v_s	superficial gas velocity	$m*s^{-1}$
V_c	cell volume	m^3
V_L	liquid volume	m^3

V_{ic}	impeller compartment volume	m^3
W	impeller blade width	m
Y_{OX}	biomass yield coefficient on oxygen	$Ceq*mol^{-1}$
Y_{SX}	biomass yield coefficient on substrate	$Ceq*Ceq^{-1}$
α	volume fraction of cells	-
ε	volume fraction of continuous phase	-
η	kinematic viscosity	$m^{-2}*s^{-1}$
μ	specific growth rate	s^{-1}
ρ_b	broth density	$kg*m^{-3}$
ρ_c	cell density	$kg*m^{-3}$
ρ_L	liquid density	$kg*m^{-3}$
τ_{te}	tensile stress by eddy on cell	$N*m^{-2}$
τ_{wl}	wall shear stress in laminar boundary layer	$N*m^{-2}$
τ_{wt}	wall shear stress in turbulent boundary layer	$N*m^{-2}$
θ_G	residence time of gas bubbles	s
θ_{te}	interaction time between eddy and cell	s
Φ_p	pumping capacity	m^3*s^{-1}

Superscripts

main	maintenance
max	maximum

Abbreviations

ppm	parts per million
vvm	volumes of air, per reactor volume, per minute
rpm	revolutions per minute

CHAPTER 2

METHODS FOR THE ASSESSMENT OF HYDRODYNAMIC STRESS SENSITIVITY OF CULTURED PLANT CELLS: A LITERATURE SURVEY.

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Introduction

The effects of hydrodynamic stress on various kinds of living cells in suspension culture have been studied for more than two decades (e.g. Midler and Finn, 1966; Tanaka, 1981; Bronnenmeier and Märkl, 1982; Märkl *et al.*, 1987; Smith *et al.*, 1987; and many others). Experimental work of the shear effects on microbial systems has been reviewed by Märkl and Bronnenmeier (1986). However, reports concerning the effects of hydrodynamic stress on plant cells are scarce (Tanaka, 1981). In fact they are so scarce that in this literature survey publications on the sensitivity of other eucaryotic cells to hydrodynamic stress are cited to demonstrate experimental techniques that have been currently used in this field. They might be applied in future plant cell research.

Plant cells have commonly been regarded as sensitive to hydrodynamic stress generated by agitation and aeration because of their relatively large size compared to microorganisms, their rigid cellulosic cell wall and large vacuoles. Therefore, their cultivation on an industrial scale would require specially designed low-shear bioreactors. For large scale cultivation, however, it would be advantageous to make use of already existing and proven technology, i.e. an impeller-stirred tank reactor. Therefore the lack of conclusive data on the hydrodynamic stress sensitivity of plant cells hampers the progress of industrial plant cell biotechnology.

This review will give a literature survey on the methods that have been used for the determination of hydrodynamic stress sensitivity of cultured living cells and plant cells in particular. Hydrodynamic stress, its effects, and techniques to determine hydrodynamic stress sensitivity of cells are discussed.

Experimental procedure

A schematic representation of possible cause/effect relations and ways to determine the hydrodynamic stress sensitivity of cells is depicted in *Figure 2.1.* In an experimental system hydrodynamic stress is generated. It can be represented as to be composed of at least four stress components. The overall effect is damage, which has many manifestations. Each manifestation of damage can be quantified by determination methods. The outcome

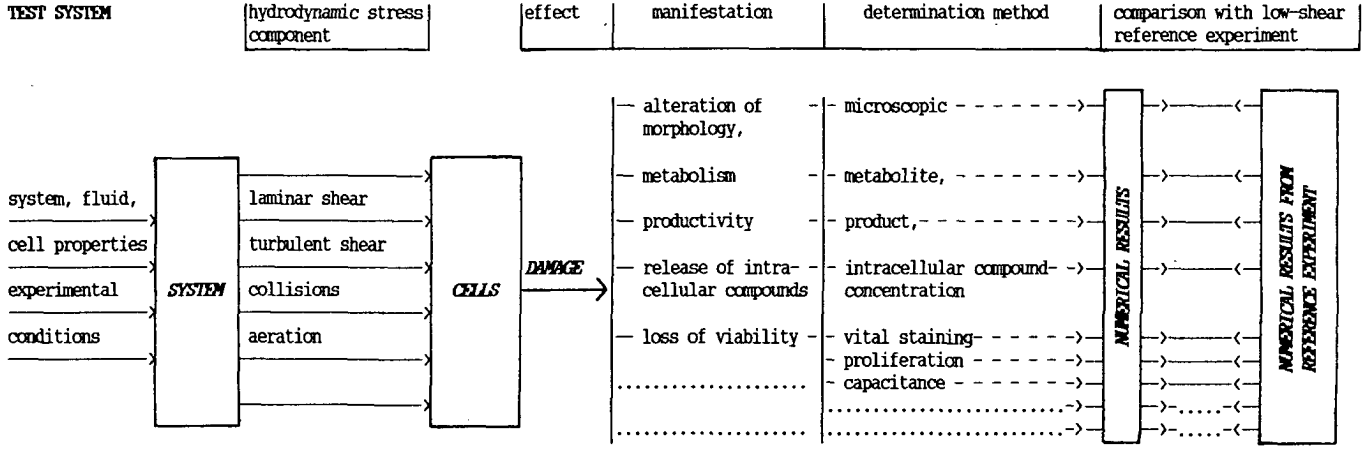

Figure 2.1. Schematic representation of possible cause/effect relations and ways to assess hydrodynamic stress sensitivity.

of the experiment is usually compared with the results of a low-shear reference experiment.

Description of hydrodynamic stress

Cherry and Papoutsakis (1986) analysed the hydrodynamic effects on mammalian cells on microcarriers in agitated reactors. They identified three distinct flow regions in the reactor:
- bulk laminar flow,
- bulk turbulent flow,
- boundary-layer flows.

Their theoretical analysis, which is only valid for diluted suspensions, revealed three major mechanisms of stress exertion in connection with agitation:
- direct interaction between microcarriers and turbulent eddies,
- collision between microcarriers in turbulent flow,
- collision against the impeller or other stationary surfaces.

Eddy size and eddy energy are important factors that determine which mechanism will be predominant. Eddies of the same size of the microcarriers (≈150 µm) may cause high shear-stresses on them if they contain enough energy. Eddies the size of the average mutual distance of the microcarriers may cause bead-bead collisions. Under comparable conditions the energy involved in collisions with the impeller and bead-eddy interactions are the more important. However there is some evidence that collision frequency is also an important factor. In principle the approach of Cherry and Papoutsakis (1986) can also be applied to plant cells, aggregates or other cell systems in an impeller-stirred reactor, provided that their size is has the same order of magnitude as the microcarriers and their volume fraction does not exceed ≈10%.

Stress can also be exerted by the aeration of a bioreactor. Handa-Corrigan *et al.* (1989) studied the effect of gas-liquid interfaces on the growth of suspended mammalian cells and the mechanisms of cell damage by bubbles. Two (sub)mechanisms of cell damage by sparging air have been proposed: damage due to rapid oscillations caused by bursting bubbles, and damage due to shearing in draining liquid films in foams. Hence, hydrody-

namic stress is composed of at least four stress components:
- laminar shear stress,
- turbulent shear stress,
- collisions of any sort,
- aeration

Effect of hydrodynamic stress

A major problem faced by workers in this field is how to measure the effect of hydrodynamic stress on cells. The effect can be both positive and negative. In most cases it will be negative and be designated "damage". Positive effects of shear-stress, e.g. higher production of secondary metabolites should not be excluded. However, it has not yet been shown. Damage can be defined in this context as all effects generated by hydrodynamic stress that affect the growth, metabolism and organisation of a cell, cell aggregate or cell system in a negative sense compared to a low-shear reference situation.

Estimation methods for cell damage

Cell damage can have many manifestations. The prevailing are:

Alteration of morphology

Alteration of morphology of plant cells or aggregates after shear exposure is mostly perceived as mechanical injuries or decrease of aggregate size (Tanaka, 1981; Tanaka, 1987; Scragg *et al.*, 1986; Scragg *et al.*, 1988; Allan *et al.*, 1988).

Midler and Finn (1966) have used light-microscopic observation and cell counting to estimate disruption of protozoa. Smith *et al.* (1987) have used a scanning electron microscope to detect alterations in cell surface morphology of highly sheared hybridomas.

Release of intracellular compounds

Release of intracellular material, a typical indirect method, has often been used as damage indicator. Tanaka *et al.* (1975) reported the results of extensive experiments with mycelial suspensions of *Mucor javanicus* and *Rhizopus javanicus*. Agitation of defined mold suspensions resulted in

leakage of intracellular substances consisting of RNA-related nucleotides, mostly mononucleotides with a maximum absorption at 260 nm. At any constant agitator speed the nucleotides leaked from the mycelia to water directly proportional to agitation time.

Wagner and Vogelmann (1977) reported the release of intracellular material causing drop of pH as a shear-indicator in the case of cultivation of plant cells.

The release of the intracellular enzyme lactate dehydrogenase (LDH) has often been used to quantify cellular lysis resulting from damage to mammalian cells (Smith *et al.*, 1987; Petersen *et al.*, 1988; Chittur *et al.*, 1988).

Alteration of metabolism and productivity

Sittig and Heine (1977) observed drastic alterations of metabolism during fermentation of *Methylomonas clara* when changing the mixing system in the reactor from an air-lift pump (low-shear) to a free jet (high-shear). König *et al.* (1981) reported a decrease in productivity of penicillin by *Penicillium chrysogenum* in a stirred tank reactor at high agitation speed.

The possibility to estimate damage after exertion of hydrodynamic stress by measuring the relative contribution of the alternative respiration to the total respiration in plant cells has been suggested by Hoefnagels (personal communication). However, this hypothesis has not yet been confirmed by experimental results.

It has to be pointed out that one should be cautious in interpreting alterations of metabolism and productivity as manifestations of cell damage. It is very difficult to distinguish between effects of mass-transfer on both metabolism and productivity and damaging effects on the cells. Therefore, alterations of metabolism and productivity should preferably not be used as damage indicator.

Loss of viability

Damage to a cell can result in loss of viability and eventually in cell death. In many cases however, a cell has the ability to recover from the inflicted damage. So a damaged cell can still be viable. This poses another problem of how to define and estimate viability of a cell.

For our purpose the operational definition of viability is: the poten-

tial of cells to grow and divide when placed in favourable conditions. The extent in which cells retain this viability, after subjection to a certain hydrodynamic load, constitutes their tolerance to hydrodynamic stress. This approach has been used in only a few studies (Scragg *et al.*, 1988, Schürch *et al.*, 1988). A major drawback is that the time necessary to discern whether a cell actually has divided disallows a rapid viability test. Therefore other methods have been frequently used in which the integrity of the cytoplasmic membrane is tested. Viability is believed to depend rather strictly upon the possession of a relatively ion-impermeable cytoplasmic membrane (Mackey, 1980).

Methods for estimating viability by testing membrane integrity are mostly staining techniques. Tramper *et al.* (1986) used the exclusion of Trypan Blue as indicator for the integrity of the cell membrane of insect cells. The same method has been used by Smith *et al.* (1987), Petersen *et al.* (1988) and Schürch *et al.* (1988). Fluorescein diacetate (FDA) has been used for both plant cells and mammalian cells as a marker for cellular viability (Widholm, 1972). The percentage viability of a cell suspension has been determined by counting the number of cells with fluorescent cytoplasm using a fluorescence microscope (Chittur *et al.*, 1988; Scragg *et al.*, 1988). The major drawback of these methods is their inherent indirectness, and misinterpretation of the results is common practice.

An alternative method to assess viability by testing membrane integrity could be the measurement of the dielectric permittivity of the cells at radio frequencies (Harris *et al.*, 1987). A cell with intact membrane can be represented as a small capacitor: an internal and an external solution with a certain conductivity are separated by a dielectricum in the form of a relatively ion-impermeable membrane. When the membrane is damaged the ions cross the membrane freely, thereby reducing the capacitance of the cell to nearly zero. Using this principle the fraction cells with an intact membrane can be distinguished from the fraction cells with a damaged membrane by measuring the capacitance of the cell culture. A great advantage of this method would be the possibility to measure 'real-time viability'.

Methods for generation of hydrodynamic stress

It is difficult to apply a defined hydrodynamic stress on cells in

suspension. In literature different types of experimental procedures have been described. Mostly experiments were performed in a turbulent shear device or in a laminar shear device. Methods for studying exclusively the effects of collisions on cells have not yet been described.

Laminar shear devices

Often laminar shear devices (e.g. a Couette viscometer) are used that allow determination of shear-sensitivity under well defined laminar conditions, thus avoiding complex flow patterns. An example of a laminar shear device is given in *Figure 2.2* (Midler and Finn, 1966).

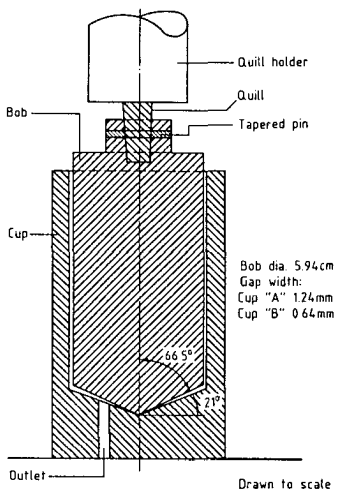

Figure 2.2. Laminar shear device. (Midler and Finn, 1966)

The advantage of this method is the possibility to study the cells under defined shear conditions. The disadvantages are:

(1) A laminar shear device allows studying of only one hydrodynamic stress component: laminar shear.

(2) The experiments are restricted to low-shear rates and shear stresses. For high shear stresses the viscosity of the cell suspension has to be increased by adding polymers which might have unknown effects on the cells.

(3) Large cell aggregates disturb laminar flow. Both cells and aggregates will tend to settle down within the viscometer causing a non-homogeneous suspension.

(4) Experiments are difficult to perform under axenic conditions and

therefore can last only for a short period of time.

From the literature that has been published on methods to determine hydrodynamic stress sensitivity of cells in suspension it appears that in many studies, despite the drawbacks, a laminar shear device was used. It is argued that the main damaging mechanism in stirred bioreactors will be caused by laminar shear when the cells are much smaller than the smallest Kolmogorov eddies (Cherry and Papoutsakis, 1986), (**Chapter 1, *Table 1.8***). This is true for suspended mammalian cells, which have a relatively small diameter (10-20 µm), but not for plant cells, aggregates and mammalian cells on microcarriers (diameters from 50-300 µm). Some interesting studies concerning the effects of laminar shear stress on mammalian-, insect-, and plant cells are discussed below.

A bioreactor with controlled laminar stress has been constructed by Soule *et al.* (1987). It is composed of two coaxial cylinders from which the external cylinder can rotate with speeds between 0 and 80 rpm. Apple cells (*Pirus malus*) were used to test the operation of the reactor. Shear stress in excess of 0.5 $N*m^{-2}$ resulted in cell death after 5 days of cultivation. The results also showed that growth was optimal under conditions of minimal shear stress (0 rpm). Janes *et al.* (1987) constructed a similar device in which the inner cylinder, covered with a gas-permeating membrane, could rotate. Their objective was not to study shear sensitivity, but to develop and test a new low-shear device with bubble-free aeration. This bioreactor, exploiting Taylor-Couette (annular vortex) flow, was successfully used to culture red beet (*Beta vulgaris* L.) cells.

Smith *et al.* (1987) used a Couette viscosimeter to determine the shear sensitivity of mammalian cells in suspension culture. A murine hybridoma was subjected to constant shear rates for periods of 15 hours. Cell damage, estimated by vital staining and release of LDH, was observed at a shear rate of 870 s^{-1}, but not at 420 s^{-1}.

Schürch *et al.* (1988) also studied the effect of laminar shear stress on the behaviour of hybridoma cell cultures. Cells were subjected to shear rates of 500 and 1000 s^{-1} in a sterilizable viscometer. Shear time was varied between 120 and 600 s, viscosity between 3 and 10 mPa*s. After shearing, 250 ml of the cell suspension was transferred for recultivation in order to test viability, growth rate, antibody production and other

metabolic parameters. No differences were found between viable sheared populations and viable control (not sheared) populations. A linear relationship between death rate and applied shear stress was found.

In some studies the determination of shear sensitivity of cells in a laminar shear device was used as a measure of shear sensitivity in an agitated bioreactor.

The shear sensitivity of insect cells (*Spodoptera frugiperda*) in suspension has been studied by Tramper *et al.* (1986). Shear sensitivity experiments were performed both in a viscometer under laminar conditions and in an 1-l stirred vessel with a marine impeller. From this work it has been concluded that insect cells lose their viability in both cases at shear stresses in the order of magnitude of 1 $N*m^{-2}$. Sparging air through the cell suspension resulted in a larger decay rate than growth rate of the cells, probably due to high shear stresses associated with rising and bursting bubbles. Tramper *et al.* (1989) introduced a model describing cell death in a bubble-column and the relation of cell death with oxygen supply in these columns. The model is based on two assumptions. Firstly, the loss of cell viability as a result of aeration is a first-order process. Secondly, a hypothetical volume in which all viable cells are killed ('killing volume') is associated with each air bubble during its lifetime. The model has been validated on laboratory scale.

The shear sensitivity of hybridoma cells was studied by Petersen *et al.* (1988). The cells were subjected to well-defined laminar shear in a Couette viscometer. Increasing levels of shear stress (0-5 $N*m^{-2}$ for 10 minutes) or times of exposure to shear resulted in higher levels of cellular damage and death. Determination of shear sensitivity of cells in a viscometer was found to be a good measure of shear sensitivity in an agitated bioreactor. Cells cultured with low levels of fluid stresses were more sensitive to shear than cells from rapidly agitated cultures. Also, cells from either the lag or stationary phase were more sensitive to mechanical damage than exponentially growing cells.

Turbulent shear devices

As turbulent shear device an impeller-stirred vessel has often been used. It would be obvious to perform shear experiments in a conventional

stirred tank reactor. Different values of stress are generated by different stirring speeds. The behaviour of the cells in the stirred vessel is generally compared with that in low-shear bioreactors (in most cases erlenmeyer flasks or air-lift reactors). Standardization of experimental procedures is almost impossible due to the high variety of stirrers and reactor vessel geometries which makes comparison of literature data difficult. Besides, stress generated by an impeller is badly defined, different stress mechanisms are operating simultaneously. Interpretation of experimental results is therefore rather troublesome.

Wagner and Vogelmann (1977) studied the cultivation of *Morinda citrifolia*, *Catharanthus roseus* and *Beta vulgaris* cell suspensions in various types of bioreactors. Mechanical damage of *Morinda citrifolia* was found in a 75-l draft tube reactor with Kaplan turbine at a stirring speed of 350 rpm. In a stirred vessel of comparable size damage occurred at an agitation speed of 100 rpm. *B. vulgaris* appeared to be even more sensitive to shear, an impeller speed of no more than 28 rpm (propeller diameter 110 mm) caused cell lysis and reduced yield of secondary metabolites. Also the *C. roseus* cell line was shown to be very sensitive to shear generated by a flat blade turbine impeller. At a stirring speed of only 28 rpm (impeller diameter 120 mm) the cells were disrupted completely after a fermentation time of five days.

Scragg *et al.* (1988) have attempted to determine the shear sensitivity of cell suspensions of *Catharanthus roseus* and *Helianthus anuus*. Shear sensitivity was estimated by the cells' viability, defined by the authors as their ability to grow and divide after shear treatment. The cell lines were found to be tolerant to stirrer speeds of 500 rpm and 1000 rpm for up to 5 hours (6-bladed turbine stirrer, diameter 7.3 cm). Subsequently growth in 3-l bioreactors at stirrer speeds of 150-200 rpm was performed. An important finding was that shear tolerance of the *H. annuus* cell line developed as growth rate improved.

The effect of hydrodynamic stress on plant cells in suspension cultures has been studied by Tanaka *et al.* (1988). *Catharanthus roseus* cells were cultivated both in erlenmeyer flasks with two baffle plates and in a 3-l jar fermenter with modified paddle-type impeller that was operated at 175

and 280 rpm. Strong hydrodynamic stress conditions resulted in decrease of growth rate of the cells, the maximum cell mass, and the size of cell aggregates. Cells had a higher wall contents and higher relative amounts of cellulose and hemi-cellulose.

In the aforementioned studies no attempts were made to apply a defined turbulent shear stress to the cells. A different approach for testing hydrodynamic stress sensitivity was followed by Bronnenmeier and Märkl (1982). In a test apparatus (*Figure 2.3*), stable continuous cultures of three types of green algae and two cyanobacteria were exposed to well-defined hydrodynamic loads in a free jet.

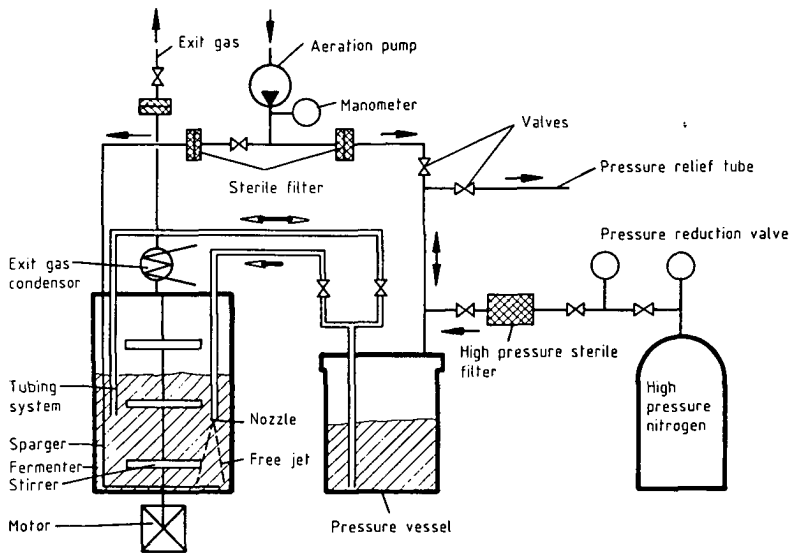

Figure 2.3. Test apparatus for free-jet experiments (Bronnenmeier and Märkl, 1982).

A fraction of the turbidostatically controlled culture was forced out the fermenter vessel by a small increase of pressure into a second vessel. From this vessel the culture was forced back into the fermenter by flow through a nozzle of 0.35 mm diameter. At this nozzle a free jet was formed. From the results of free jet experiments with short stress

exposure and stirring experiments, distinct critical stress values could be determined above which damage of the microorganisms cultures occurred. A correlation between the critical stress values in free-jet and stirring experiments was found. It appeared that the free-jet data, expressed as critical volumetric dissipated energy could be used to calculate the maximum hydrodynamic stress to which microorganisms could be exposed in a bioreactor without suffering damage.

Sometimes it is not the objective to study the hydrodynamic stress sensitivity of cells, but the characterization of the shear properties of a bioreactor, in most cases a stirred fermenter.

Midler and Finn (1966) described a model system for evaluating shear in the design of stirred fermenters. It was developed, using shear sensitive protozoa (*Tetrahymena pyriformis*), in order to characterize the maximum shear in both simple and complex shear fields. Shear experiments were carried out in a constant shear device resembling a Couette viscometer and in two lab-scale turbine-stirred vessels that could be equipped with impellers of different diameter. In the constant shear device only slight damage was observed at shear rates below 1200 s^{-1}. In order to retain laminar conditions in the annular gap at higher shear rates it was necessary to increase the viscosity of the cell suspensions. Shear stress, rather than shear rate, appeared to be the important variable. It came out that in an agitated vessel disruption of cells started at a certain tip speed of the impeller, and there was a clear relationship between the number of survivors and tip speed. The protozoan model system proved to be an effective indicator of disruptive conditions, in both laminar and turbulent shear fields.

Tanaka (1981) developed a method for measuring the intensity of hydrodynamic stress on cells in culture systems. The mass-transfer coefficient (K) for a model solid-liquid system (β-naphthol in water) was proposed as an intensity index of hydrodynamic stress on plant cells in suspension cultures under various conditions in the bioreactor systems. The intensity index was used to select the most optimal bioreactor for the cultivation of *Cudrania tricuspidata* cells at high density. A jar fermenter with a modified paddle-type impeller appeared to be suitable.

Discussion and conclusions

From the reviewed literature it is clear that huge differences in hydrodynamic stress sensitivity exist between various plant cell lines. They range from very sensitive lines such as *Pirus malus* (Soule *et al.*, 1987) and *Cudrania tricuspidata* (Tanaka, 1981) to robust lines such as *Catharanthus roseus* and *Helianthus annuus* (Scragg *et al.*, 1988). Even within one species shear sensitivity diverges. *C. roseus* for example was found to be very shear sensitive by Wagner and Vogelmann (1977). On the contrary, Scragg *et al.* (1988) found this species to be very robust.

Plant cells have commonly been regarded as sensitive to hydrodynamic stress generated by agitation and aeration because of their relatively large size compared to microorganisms, their rigid cellulosic cell wall and large vacuoles. This opinion, first expressed by Mandels (1972) but not substantiated, has been established as a near axiom after subsequent quotations by many authors. It has led to the misunderstanding that all plant cells are intrinsically shear sensitive and by all means difficult to cultivate on a large scale (Aghighi, 1988). Besides, this opinion has led to the development of many low-shear bioreactors for growth of plant cells (Tanaka, 1981; Smart and Fowler, 1984; Tanaka, 1987; Hegglin *et al.*, 1987; Hulst *et al.*, 1987).

Some cell lines are apparently more resistant than was currently assumed. An explanation for these differences is hard to give. Scragg *et al.* (1988) reported that two of their cell lines acquired robustness as culture growth improved, after being initially shear sensitive. This would suggest a relationship between time in culture and shear resistance. However, this is contradicted by their observation of a recently developed suspension culture of *C. roseus* being shear tolerant after only eight subcultures. A possible explanation for shear-tolerance could be the ability of plant cells to repair small lesions in the cell wall by deposition of a polysaccharide, e.g. callose. We suppose that the way in which the stock cultures are maintained can affect shear sensitivity. Their maintenance needs much attention, when stock-cultures are badly attended (oxygen starvation, over-sterilized medium etc.), they might develop shear sensitivity. Experiments to substantiate this assumption has not yet been performed in our research group.

In our opinion one should aim at establishing robust and producing cell

lines instead of spending too much time in developing low-shear reactors for fragile cell lines that eventually might not grow at all in it. This approach has been in fact already followed by Allan *et al.* (1988). They succeeded in transforming an initially shear sensitive, slow growing, non-producing cell line of *Picrasma quassioides* into a suspension culture capable of growing in bioreactors and producing low levels of quassin. This was accomplished by changes in subculture methods and took about two years. If one does not succeed in isolating a robust and producing cell line immobilization can be a good alternative for protecting cells against shear stress (Hulst, 1987).

In literature many different methods have been used to determine the hydrodynamic stress sensitivity of cultured cells. Most of them seem to give satisfactory results, although some general problems are clear:
(1) There is a fundamental lack of knowledge of which circumstances determine the shear sensitivity of a cultured cell.
(2) Determination of damage has often been carried out indirectly which makes the interpretation of the results and the comparison with experimental results from other authors very difficult.
(3) Translation of shear behaviour under experimental conditions to the practical situation.

In our opinion data on hydrodynamic stress sensitivity should be determined preferably in a small-scale system that is a down-scaled version of the production system. Therefore, if the intention is to develop a large-scale process based on an impeller-stirred tank, the best method for determining hydrodynamic stress sensitivity of plant cells is making use of impeller-stirred chemostat (**Chapter 4**). An obvious disadvantage of this method is that hydrodynamic stress is badly defined. In our opinion one should not worry too much about this drawback because results obtained with laminar shear devices or well-defined turbulent shear devices, require in some way or other an awkward translation to the practical situation, i.e. the large-scale fermenter. Also turbidostat culture should be considered, which has been used for studying shear sensitivity of microorganisms (Bronnenmeier and Märkl, 1982). However, performing turbidostat cultures with plant cell has been proven difficult because of problems associated with estimation of cell density by optical means for long periods of time (Wilson *et al.*, 1971).

Both continuous culture and turbidostat offer the advantage of studying cells under constant conditions for long periods of time. Shear stress tolerance is easily estimated by the ability of the cells to maintain themselves in culture under certain stress conditions. Besides, both techniques offer the possibility for selection of shear insensitive cell lines. Disadvantages are the complexity of the experimental set-up and the (especially for plant cells) long duration of the experiments.

A second best choice would be to perform shear experiments in batch culture (**Chapter 3**). This approach offers the advantage of studying the shear sensitivity of plant cells going through consecutive growth phases. Besides, duration of the experiments is relatively short. Disadvantages are:

(1) The cells are subjected to continuously changing hydrodynamic stress because of changing cell and fluid properties.

(2) Viability of the cells is hard to test. Low-shear reference experiments are needed as comparison.

The required hydrodynamic stress tolerance for a large scale plant cell production process can easily be estimated from the process parameters (**Chapter 1**). The tip-speed of the impellers and power input for mixing are the two most important parameters that determine the hydrodynamic load on the cells. The magnitude of the hydrodynamic stress components can be assessed as has been described in **Chapter 1**. By a down-scaling procedure small-scale experiments can be devised in which cells can be subjected to hydrodynamic loads that are likely to occur in the large-scale process.

CHAPTER 3.

THE EFFECTS OF HYDRODYNAMIC STRESS ON THE GROWTH OF PLANT CELLS IN BATCH CULTURE

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Introduction

Little information is available on the sensitivity of plant cells for hydrodynamic stress ('shear-stress'). Besides, data on this subject are often contradictory. Until now most experiments to assess this property were carried out by imposing stress during a short period of time, and subsequently determining the effects of stress by one or the other method. As already has been pointed out in detail in **Chapter 2**, these approaches have some disadvantages and difficulties:

(1) Determination of effects of hydrodynamic stress has often been carried out using indirect methods.

(2) The interpretation of the experimental results and the comparison with results from other authors are often difficult.

(3) Translation of hydrodynamic stress sensitivity under experimental conditions to the practical situation is troublesome.

In **Chapter 1** and 2 it has been concluded that performing hydrodynamic stress experiments in a down-scaled version of the bioreactor that one has in view for a large-scale process is the best solution to the aforementioned problems. It has also been concluded that performing experiments with chemostat cultures of plant cells would be the best choice. This technique offers the possibilities to cultivate cells and conduct experiments under strictly defined conditions during long periods of time.

A second best choice would be to perform shear experiments in batch culture. This approach offers the advantage of studying the shear-sensitivity of plant cells going through consecutive growth phases. Disadvantages are that effects of stress are difficult to assess. Low-shear reference experiments are needed as comparison. One is never sure that not only hydrodynamic load, but also other parameters that could affect growth and metabolism of the plant cells were altered. In spite of these drawbacks batch cultures are very useful to get a quick impression of the shear-tolerance of a cell line.

In this study the hydrodynamic stress sensitivity of four cell lines: *Catharanthus roseus, Nicotiana tabacum, Tabernaemontana divaricata,* and *Cinchona robusta* was assessed. Cells were batch cultivated in a lab-scale turbine-stirred fermenter at high agitation speeds and growth characteristics were compared with those at low agitation speeds. An additional

experiment was performed in a turbine-stirred fermenter with *N. tabacum* to assess the hydrodynamic stress sensitivity of nutrient starved cells. Culture growth, respiration, and release of protein and organic carbon by the cells were measured during the course of the experiments to quantify stress effects on the plant cells. To get an impression of the hydrodynamic stress imposed on the cells hydrodynamic parameters related to shear stress were estimated.

Materials and Methods

Cell cultures

The cell suspension cultures of *Catharanthus roseus*, *Nicotiana tabacum*, and *Cinchona robusta* were obtained from the Department of Plant Molecular Biology, Leiden University.

The cell culture of *C. roseus* grown in suspension for more than 5 years, was originally initiated from seeds. It is commonly used within our research group as a model system to study growth kinetics of plant cells in suspension culture.

The cell culture of *N. tabacum*, grown in suspension for more than 10 years, was originally initiated from leaf tissue. It is commonly used within our research group as a model system to study the influence of plant hormones on cell cycle and differentiation, and to study stochastic models for population growth (de Gunst, 1988).

The cell culture of *C. robusta*, grown in suspension for 1 year, was originally initiated from hypocotyl tissue. It is commonly used within our research group to study the production of quinine and quinoline alkaloids, and the induction of anthraquinone production by elicitors.

The cell suspension culture of *Tabernaemontana divaricata* was obtained from the Center for Bio-Pharmaceutical Sciences, Division of Pharmacognosy, Leiden University. This cell culture, grown in suspension for more than 3 years was originally initiated from leaf tissue. It is commonly used within our research group as a model system to study various aspects of secondary metabolism (van der Heijden, 1989).

Maintenance of cell cultures

All four cell lines were cultured in 1000 ml erlenmeyer flasks in the dark at 25^{0}C, and shaken at 100 rpm on an orbital shaker. The flasks were

closed with the cut-off upper halfs of silicone foam stoppers (model T42, Shin Etsu, Tokyo, Japan). Water for medium preparation was demineralized by ion-exchange and subsequently purified by a Milli-Q-UF water purification system (Millipore, Bedford, USA). Growth media were sterilized by autoclaving at 121°C, 1 bar over-pressure, for 20 minutes. Medium pH prior to sterilization was 6.0.

The cell line of *C. roseus* was grown on LS growth medium (Linsmaier and Skoog, 1965) supplemented with 30 $g*l^{-1}$ glucose, 2.0 $mg*l^{-1}$ α-naphthaleneacetic acid (NAA), and 0.2 $mg*l^{-1}$ kinetin. It was subcultured every 7 days by inoculating 35 ml of cell suspension into 165 ml of fresh medium.

The cell line of *N. tabacum* was grown on LS medium containing 30 $g*l^{-1}$ sucrose, and 0.05 $mg*l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D). It was subcultured every 7 days by inoculating 20 ml of cell suspension into 180 ml of fresh medium.

The cell line of *C. robusta* was grown on Gamborg's B5 growth medium (Gamborg *et al.*, 1968), containing 20 $g*l^{-1}$ sucrose, 50 $mg*l^{-1}$ cysteine-HCl, 2.0 $mg*l^{-1}$ 2,4-D, and 0.2 $mg*l^{-1}$ kinetin. It was subcultured every 7 days by inoculating 35 ml of cell suspension into 165 ml of fresh medium.

The cell line of *T. divaricata* was grown on MS growth medium (Murashige and Skoog, 1965) containing 30 $g*l^{-1}$ sucrose, 1.0 $mg*l^{-1}$ 2,4-D, and 1.0 $mg*l^{-1}$ kinetin. Initially it was subcultured in the Center of Bio-Pharmaceutical Sciences every 21 days in a 2000 ml erlenmeyer flasks by inoculating 200 ml of cell suspension into 600 ml fresh medium. In our laboratory the cell line was subcultured in 1000 ml flasks every 14 days by inoculating 35 ml of cell suspension into 165 ml of fresh medium.

Estimation of hydrodynamic stress parameters

Hydrodynamic stress parameters were estimated as has been described in **Chapter 1.**

Batch culture experiments

Batch culturing of the four cell lines was carried out in a 3-l commercially available fermenter (Applikon, Schiedam, The Netherlands) equipped with a six-bladed Rushton impeller with a diameter of 4.5 cm (blade angle=90°), and 3 round baffles with a diameter of 1.4 cm. The fermenter experiments were performed in the dark, at 25°C. The same media and inoculation ratio's were used as for the maintenance of the cell lines.

Aeration rate was controlled by a mass flow controller, and kept on 40 $l*h^{-1}$ (25°C, 1 bar). Effluent air was led through a condensor kept at 4°C to diminish evaporation. The pH of the culture was monitored by a pH electrode in the fermenter, but not controlled. Foaming of the cultures was suppressed by discontinuous addition of a 10% silicone-based antifoam solution (BDH) to the cultures. Addition rate was dependant on the foaming characteristics of the cultures (≈1-5 ml per day, time interval of addition 30 minutes).

Sampling of the culture was performed by aseptic withdrawal of ≈50 ml of suspension through a sampling pipe with an internal diameter of 1.0 cm. Samples were used to determine biomass dry-weight concentration, cell number, and the concentrations of several medium components.

Experiments were carried out at agitation speeds of 4.2 and 16.7 s^{-1}, an additional experiment with *T. divaricata* was carried out at 10.0 s^{-1}. Experiments at 10.0 and 16.7 s^{-1} were started at 4.2 s^{-1}. After 2 days agitation was set at the final speed.

Starvation experiment

This experiment was performed in a 15-l commercially available fermenter (Applikon, Schiedam, The Netherlands) equipped with a six-bladed turbine impeller with a diameter of 7.0 cm (blade angle=90°), and 3 flat baffles with a blade width of 3.0 cm. A volume of 1000 ml of 14 days old *N. tabacum* pre-grown in shake flasks was inoculated into 10 l LS medium without carbon and nitrogen sources. Initial agitation speed was 10.0 s^{-1}, after 11 days it was increased to 16.7 s^{-1}. Other conditions were identical as for the batch culture experiments.

Analytical procedures

Dry biomass concentration was determined in duplicate by filtering 2 x 10.0 ml of cell suspension on a pre-weighted 47 mm glass fibre filter (Type A/E, Gelman Science Inc., Ann Arbor, USA). Filtered cells were washed twice with demi water and subsequently dried at 70°C for 24 hours.

For determination of cell number 2.5 ml suspension culture was macerated with 5 ml 12% CrO_3 solution in a centrifuge tube. After 2 days incubation at room temperature cells were pelleted by centrifugation at 3000 rpm for 2-5 min. The cellular pellet was resuspended in water, final volume was 2.5 ml. Remaining cell aggregates were separated by several

passages through a hypodermal syringe. Samples were diluted with water until a concentration of ≈50-100 cells per 20 µl was attained. From each diluted solution 10 samples of 20 µl were counted using a light microscope. The cell density in the fermenter at a certain time point was estimated by the mean of 10 counts.

Cell free medium was obtained by centrifuging 2 x 10.0 ml of cell suspension in a table centrifuge at 3000 rpm for 5 minutes.

Glucose concentration in cell free medium was determined either with an enzymatic glucose analyser (model 27, Yellow Springs Instruments Co., Yellow Springs, USA) or together with sucrose and fructose using a high performance liquid chromatography (HPLC) system (Waters Associates Inc., Milford, USA). In the latter case an HPX 87P column (Bio-Rad) was used. Its temperature was controlled at 85°C. The eluent was pyrogene-free water (Milli-Q-UF, Millipore); flow rate was 0.6 $ml*min^{-1}$.

Total organic carbon (TOC) concentration in cell-free medium was determined with a TOC analyser (model Tocor 2, Maihak AG, Hamburg, FRG).

Protein concentration in cell free medium was determined colorimetrically by a modified Folin-Ciocalteau method (Herbert *et al.*, 1971). The protein concentration is given with respect to albumine protein.

Cell viability was determined by fluorescein diacetate (FDA) vital staining (Widholm, 1972).

To determine carbon dioxide production rate (rCO_2) and oxygen consumption rate (rO_2) the carbon dioxide and oxygen concentration of effluent air were measured with an infrared CO_2-analyser (model 864, Beckman Instruments Inc, Fullerton, USA) and a double channel paramagnetic oxygen analyser (Taylor Servomex Ltd, Crowborough, UK).

Results

Calculation of hydrodynamic stress parameters

In **Chapter 1** the numerical assessment of hydrodynamic stress parameters has been described. It has also been pointed out that interaction between the smallest eddies and the cells, cell-cell collisions, cell-impeller collisions, and high levels of shear-stress on the surface of the impeller blades are most likely to inflict damage on the cells. The impeller tip speed (v_i), and the power input (P/V) were calculated for various agitation speeds (N) applied in this study together with the eddy size d_e, the

cell-to-cell collision energy and frequency EC_{cc} and NC_{cc}, the cell-to-impeller energy and frequency EC_{ci} and NC_{ci}, and the turbulent and laminar shear stress τ_{wl} and τ_{wt} (*Table 3.1*).

Table 3.1. Calculation of hydrodynamic stress parameters for a 3-l (working volume = 2 l) and a 15-l (working volume = 11 l) turbine-stirred fermenter at various agitation speeds (N). medium kinematic viscosity = $1*10^{-6}$ $m^{-2}*s^{-1}$

bioreactor		3-l fermenter			15-l fermenter	
impeller diameter [m]		0.045			0.07	
volume-fraction biomass [-]		0.1			0.05	
cell (aggregate) diameter [m]		$1*10^{-4}$			$5*10^{-5}$	
N	[s^{-1}]	4.2	10.0	16.7	10	16.7
reactor parameters						
v_i	[$m*s^{-1}$]	0.59	1.41	2.36	2.20	3.67
P/V	[$W*m^{-3}$]	33	460	2100	760	3500
hydrodynamic stress parameters						
d_e	[m]	$7*10^{-5}$	$4*10^{-5}$	$3*10^{-5}$	$3*10^{-5}$	$2*10^{-5}$
E_{cc}	[J]	$1*10^{-13}$	$4*10^{-13}$	$8*10^{-13}$	$6*10^{-14}$	$13*10^{-14}$
E_{cc}/M_c	[J/kg]	$2*10^{-4}$	$7*10^{-4}$	$15*10^{-4}$	$9*10^{-4}$	$19*10^{-4}$
NC_{cc}	[s^{-1}]	7	14	20	16	23
E_{ci}	[J]	$9*10^{-11}$	$54*10^{-11}$	$150*10^{-11}$	$20*10^{-11}$	$50*10^{-11}$
E_{ci}/M_c	[J/kg]	0.2	1	3	2	7
NC_{ci}	[s^{-1}]	$3*10^{-3}$	$7*10^{-3}$	$10*10^{-3}$	$16*10^{-3}$	$26*10^{-3}$
τ_{wl}	[$N*m^{-2}$]	1-3	4-8	8-13	6-24	13-40
τ_{wt}	[$N*m^{-2}$]	1-2	8-10	19-25	15-26	38-64

In the calculations a medium kinematic viscosity of $1*10^{-6}$ $m^{-2}*s^{-1}$ and a mean cell aggregate diameter of $1*10^{-4}$ m were assumed for all experiments. Together with a volume-fraction biomass of 0.1 for the batch culture experiments this represents conditions likely to occur at the

start and during the first four days. However, both medium viscosity and biomass volume-fraction increased considerably during the batch culture experiments. In the starvation experiment the volume-fraction biomass appeared to be 0.05 throughout.

Batch culture experiments

For large scale cultivation impeller tip speeds in excess of 2 $m*s^{-1}$ are needed for efficient dispersion of air into the liquid phase (van 't Riet, 1975). Therefore this speed was chosen as a maximum for our 3-l batch culture experiments. A tip speed of 2 $m*s^{-1}$ corresponds with an agitation speed of about 1000 rpm (16.7 s^{-1}) on a 3-l scale. For low-shear experiments 250 rpm (4.2 s^{-1}) was chosen. At this agitation speed hydrodynamic stress is moderate, and oxygen transfer capacity is sufficient to avoid oxygen limited growth of the cultures.

During the last three years cultivation of *C. roseus* in 3-l and 15-l fermenters at an agitation speed of 4.2 s^{-1} has become a routine within our research group. Both *T. divaricata* and *C. robusta* were, as far as we know, never grown before in a stirred bioreactor.

Catharanthus roseus

The results of batch cultures of *C. roseus* in a 3-l fermenter at agitation speeds of 4.2 and 16.7 s^{-1} are shown in *Figure 3.1A*.

There was no difference in growth characteristics between a culture grown at 4.2 and 16.7 s^{-1}, also the time course of CO_2-production and O_2-consumption rate were nearly identical (results not shown). In order to assess the damaging effect of hydrodynamic stress at 4.2 and 16.7 s^{-1} the release of organic carbon to the medium (TOC) was followed (*Figure 3.1B*). In the experiment at 16.7 s^{-1} the TOC concentration increased linearly from 50 after 7 days to 100 $mC\text{-}mol*l^{-1}$ after 13 days (One C-mole is defined as the amount containing one mole of the element carbon). In the experiment at 4.2 s^{-1} TOC rose from 32 after 8 days to 94 $mC\text{-}mol*l^{-1}$ after 13 days. Since both cultures yielded nearly equal amounts of TOC it can be concluded that the release of organic carbon into the medium can not be attributed to effects of a high hydrodynamic load. Besides, release of TOC originating from cell disruption would be accompanied by a substantial decrease in biomass dry-weight concentration.

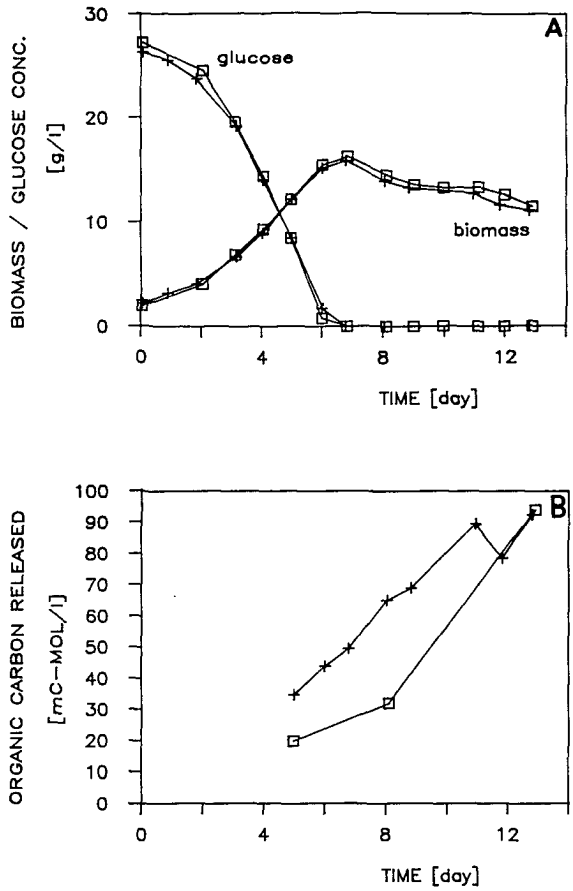

Figure 3.1A,B. Growth by a *C. roseus* suspension culture in a 3-l fermenter stirred with a 4.5 cm turbine impeller at agitation speeds of 4.2 and 16.7 s^{-1}. **A.** biomass dry-weight; glucose [$g*l^{-1}$]. **B.** organic carbon carbon released into medium (TOC) [$mC\text{-}mol*l^{-1}$].
(□) 4.2 s^{-1}; (+) 16.7 s^{-1}

As can be seen in *Figure 3.1A* the course of this parameter is nearly identical in both experiments. The release of organic carbon into the medium is presumably due to excretion of polysaccharides involved in cell wall metabolism (Takeuchi and Komamine, 1980).

Nicotiana tabacum

The results of an experiment at an agitation speed of 16.7 s^{-1} are presented in *Figure 3.2.*

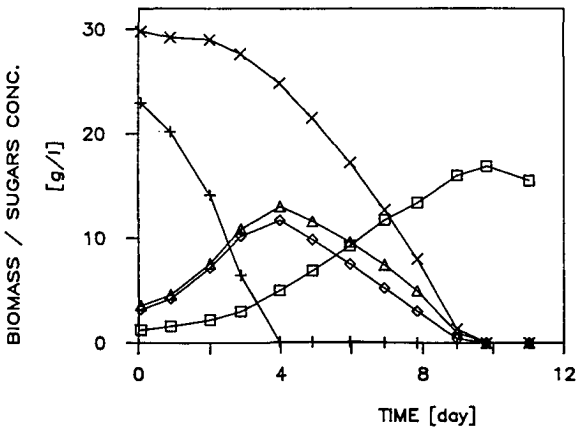

Figure 3.2. Growth by a *N. tabacum* suspension culture in a 3-l fermenter stirred with a 4.5 cm turbine impeller at an agitation speed of 16.7 s^{-1}. (□) biomass dry-weight; (+) sucrose; (◇) glucose; (△) fructose; (×) total sugars [$g*l^{-1}$]

Cell number increased from $2*10^8$ l^{-1} after inoculation to $15*10^8$ l^{-1} after 7 days. From that time point cell number remained constant. Recently *N. tabacum* was grown in a 3-l fermenter at a low agitation speed (2.5 s^{-1}) by de Gunst (1988). This experiment, initially performed to test a model for plant cell population growth, is used in this study as reference experiment. There was no apparent difference in growth characteristics between a culture at 2.5 and 16.7 s^{-1} (*Figure 3.3*).

The supernatants of the samples from day 6 on were increasingly viscous, probably due to polysaccharides. This was observed for both cultures. The concentration of organic carbon in the cell-free medium of both cultures was ≈150 mC-$mol*l^{-1}$ after 8 days. We think this carbon to be attributed to the synthesis and subsequent release of extracellular polysaccharides into the medium, as was also observed with *C. roseus*.

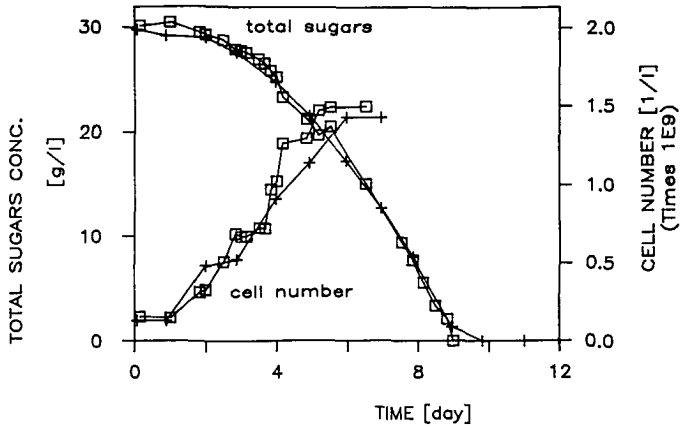

Figure 3.3. Growth by a *N. tabacum* suspension culture in a 3-l fermenter stirred with a 4.5 cm turbine impeller at agitation speeds of 2.5 and 16.7 s^{-1}. cell number [l^{-1}]; total sugars [$g*l^{-1}$]. (□) 2.5 s^{-1}; (+) 16.7 s^{-1}

Cinchona robusta

The growth and sugar uptake by a *C. robusta* culture grown at agitation speeds of 4.2 and 16.7 s^{-1} are shown in *Figure 3.4.* At an agitation speed of 4.2 s^{-1} growth was slow, biomass dry-weight concentration increased from 1 to 5 $g*l^{-1}$ after 7 days (*Figure 3.4A*), cell number increased from $3*10^8$ to $22*10^8$ l^{-1} (results not shown). After 8 days the experiment was stopped because a beginning of infection. The culture grown at 16.7 s^{-1} showed hardly any growth, biomass dry-weight concentration increased from 3 to 4 $g*l^{-1}$ after 11 days, cell number remained $4*10^8$ l^{-1} throughout the experiment. The O_2-consumption rate (rO_2) followed the growth pattern of the cultures. At 4.2 s^{-1} rO_2 increased progressively after inoculation, at 16.7 s^{-1} rO_2 was much lower, and decreased from day 4 on (*Figure 3.4B*).

To assess the damaging of the cells, the amounts of protein released into the medium were determined. The protein in the medium probably originated from damaged cells that released their cell content through ruptures of the cell wall. Because protein concentration will be proportional to the biomass concentration one should compare specific protein concentrations (protein concentration divided by the biomass dry-weight concentration).

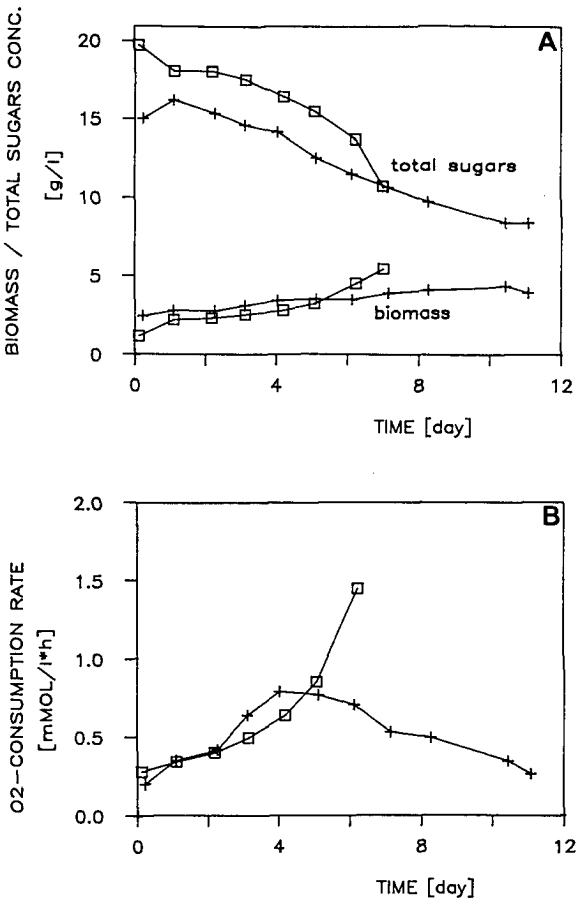

Figure 3.4A,B. Growth by a *C. robusta* suspension culture in a 3-l fermenter stirred with a 4.5 cm turbine impeller at agitation speeds of 4.2 and 16.7 s^{-1}.

A. biomass dry-weight; total sugars [$g*l^{-1}$]. **B.** O_2-consumption rate (rO_2) [$mmol*l^{-1}*h^{-1}$]. (□) 4.2 s^{-1} (+) 16.7 s^{-1}

Results are shown in *Figure 3.5.* It was not possible to determine the concentration of released carbon with sufficient accuracy due to the high background of sugars in the medium. From this figure it is clear that protein release at 16.7 s^{-1} was elevated with respect to 4.2 s^{-1}. After about 4 days this increase correlated with oxygen uptake and growth.

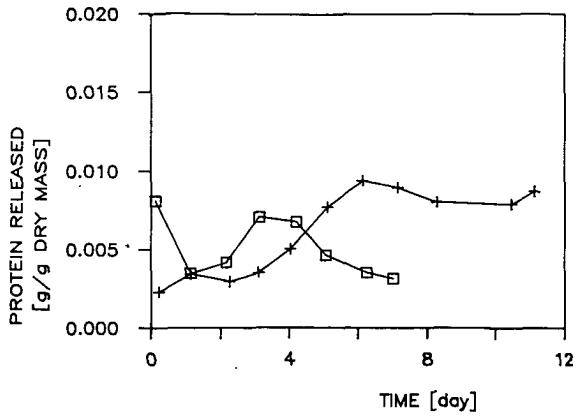

Figure 3.5. Specific protein concentration in cell free medium by a *C. robusta* suspension culture grown in a 3-l fermenter stirred with a turbine impeller at agitation speeds of 4.2 and 16.7 s^{-1}.
(□) 4.2 s^{-1} (+) 16.7 s^{-1}

Tabernaemontana divaricata

The growth patterns of a *T. divaricata* culture grown in a fermenter at agitation speeds of 4.2, 10.0, and 16.7 s^{-1} are presented in *Figure 3.6*.

At low agitation speed growth appeared not impaired by hydrodynamic stress. Biomass dry-weight concentration increased from 3 to 18 $g*l^{-1}$ after 10 days (*Figure 3.6A*). At 10.0 and 16.7 s^{-1} there was hardly any growth and sugar uptake. Determination of O_2-consumption rate reflected this difference in growth behaviour (*Figure 3.6B*). Impaired growth at high agitation speeds resulted in a very low rO_2, notably at 16.7 s^{-1}. The (specific) concentration of released protein was elevated at 10.0 and 16.7 s^{-1} with respect to 4.2 s^{-1} (*Figure 3.7*).

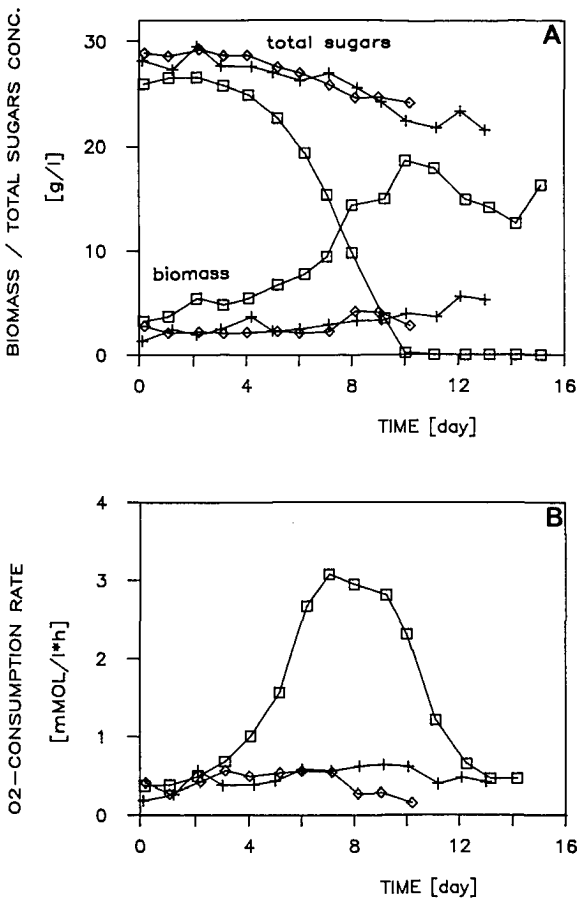

Figure 3.6A,B. Growth by a *T. divaricata* suspension culture in a 3-l fermenter stirred with a 4.5 cm turbine impeller at agitation speeds of 4.2, 10.0, and 16.7 s^{-1}.

A. biomass dry-weight; total sugars [$g*l^{-1}$]. **B.** O_2-consumption rate (rO_2) [$mmol*l^{-1}*h^{-1}$]. (□) 4.2 s^{-1}; (+) 10.0 s^{-1}; (◇) 16.7 s^{-1}

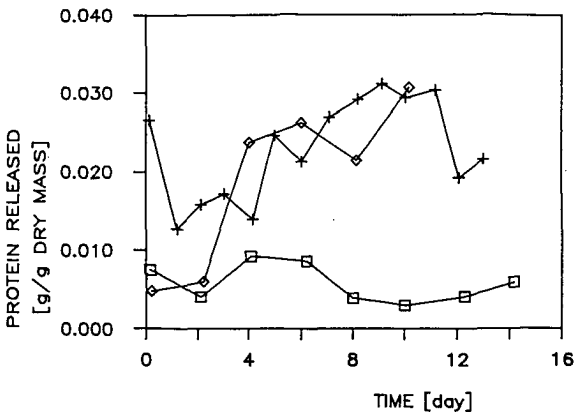

Figure 3.7. Specific protein concentration in cell free medium of a *T. divaricata* suspension culture grown in a 3-l fermenter stirred with a 4.5 cm turbine impeller at agitation speeds of 4.2, 10.0, and 16.7 s^{-1}. (□) 4.2 s^{-1}; (+) 10.0 s^{-1}; (◇) 16.7 s^{-1}

Nutrient starvation experiment

In order to study whether nutrient starved cells are much more susceptible to hydrodynamic stress, an experiment was performed in which *N. tabacum* was kept on LS medium without carbon and nitrogen source. It was to be expected that exposing a cell population weakened by nutrient starvation to hydrodynamic stress would result in rapid cell disruption.

To allow withdrawal of large samples this experiment was conducted in a 15-l fermenter instead of a 3-l fermenter. Agitation speed (10.0 s^{-1}) was in accordance with the maximum tip speed used in batch culture experiments. To attain a comparable power input agitation speed was eventually raised to 16.7 s^{-1}.

The time course of the biomass dry-weight concentration of a starved *N. tabacum* cell suspension kept in a 15-l fermenter stirred with a turbine impeller is shown in *Figure 3.8*.

The dry-weight concentration decreased only slightly with time, after 10 days decrease was more rapidly, indicating the start of massive cell disruption. To confirm this finding the TOC concentration and protein release were determined during this experiment (*Figure 3.9*).

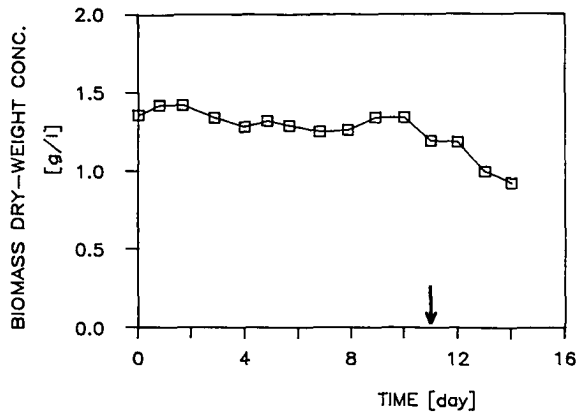

Figure 3.8. Effect of hydrodynamic stress on a *N. tabacum* suspension culture kept under nutrient starvation conditions in a 15-l fermenter stirred with a 7.0 cm turbine impeller. Initial agitation speed was 10.0 s^{-1}, after 11 days (arrow in figure) agitation was increased to 16.7 s^{-1}.
(□) biomass-dry weight [$g*l^{-1}$]

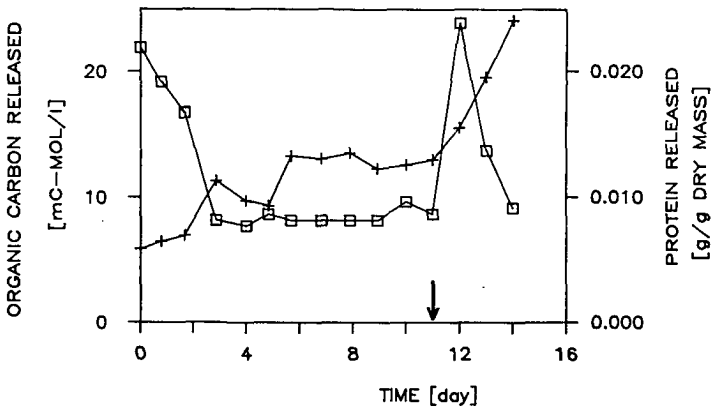

Figure 3.9. Organic carbon concentration and specific protein concentration in cell free medium of a *N. tabacum* suspension culture kept under nutrient starvation conditions in a 15-l fermenter stirred with a 7 cm turbine impeller. Initial agitation speed was 10.0 s^{-1}, after 11 days (arrow in figure) agitation was increased to 16.7 s^{-1}.
(□) organic carbon [$mC\text{-}mol*l^{-1}$]; (+) specific protein [$g*l^{-1}/gDW*l^{-1}$]

After the start of the experiment TOC fell sharply presumably due to the consumption of the last traces of carbohydrates present in the inoculum (*Figure 3.9*). From day 3 on respiration could not be measured (results not shown), indicating a cessation of the metabolism of the culture. From day 3 to day 11 TOC remained constant. After 11 days the agitation speed was increased step-wise from 10.0 to 16.7 s^{-1}. This resulted in a sharp increase of TOC from 8 to 24 mC-mol*l^{-1}, probably due to release of cell content. However, increase of TOC was followed by a sharp decline (day 12 to 14). We could explain this finding by assuming that the carbon released by the cells was consumed by the cells that had survived the unfavourable conditions in the fermenter. Indeed, a viability test (FDA vital staining) performed after 14 days revealed that ≈20% of the cell population was intact.

There was a steady increase of the amount of protein released into the medium (*Figure 3.9*). From this observation it can be concluded that cell disruption occurred. When the results of TOC and protein release are compared it is obvious that only the latter is suitable as damage indicator.

Discussion and conclusions

The experiments show a variation in the shear-sensitivity of the four cell lines. Both *C. roseus* and *N. tabacum* are shear-resistant under the conditions applied in the experiments. *T. divaricata* and *C. robusta* show impaired growth at agitation speeds in excess of 4.2 s^{-1}, and can therefore be considered shear-sensitive. A small fraction of starved *N. tabacum* cells were able to endure high hydrodynamic loads during 14 days. These cells were probably able to survive by metabolizing the remnants of the fragmented cells.

Five cultural parameters were measured to assess the effects of hydrodynamic stress on the cell lines, namely growth rate, respiration rate, release of organic carbon and protein, and viability (*Table 3.2*). From the experiments it is clear that release of organic carbon can not serve as a suitable indicator of damage by hydrodynamic stress. Both *C. roseus* and *N. tabacum* released substantial amounts of organic carbon, although they appeared to be shear-tolerant.

Table 3.2. Summary of batch culture experiments with four cell lines in a 3-l fermenter stirred with a 4.5 cm turbine impeller at various agitation speeds. The specific growth rate μ (based on biomass dry-weight), the maximum oxygen consumption rate rO_2, the release of organic carbon and protein, and the remaining viability (FDA vital staining) at comparable times are given.

cell line	N [s^{-1}]	μ [h^{-1}]	rO_2 (max) [mmol*l^{-1}*h^{-1}]	organic C [mC-mol*l^{-1}]	protein [g*g^{-1}]	viability [%]
C. roseus	4.2	0.014	3.5	94	ND	80
	16.7	0.014	3.5	100	ND	80
N. tabacum	2.1	0.023^{+}	2.8	150	ND	80
	16.7	0.017^{+}	3.6	150	ND	70
C. robusta	4.2*	0.012*	0.9*	ND*	0.007*	ND*
	16.7	≈0	0.8	ND	0.008	30
T. divaricata	4.2	0.010	3.2	36	0.006	60
	10.0	≈0	0.6	ND	0.022	50
	16.7	≈0	0.6	ND	0.031	50

* Experiment stopped prematurely (beginning of infection)

$^{+}$ Based on cell number

ND = not determined/determinable

Further analysis of medium samples of the batch cultures of *C. roseus* revealed that they contained a polysaccharide consisting of arabinose and galactose. Presumably this polysaccharide (by)product plays a role in cell wall turnover (Takeuchi and Komamine, 1980). *N. tabacum* possibly synthesized polysaccharides as a (by)product in the stationary phase due to excess carbon. With *T. divaricata* the levels of three cultural parameters were in accordance with each other, bad growth was accompanied by low and/or declining respiration rate, and release of protein. Viability assessed by FDA vital staining proved to be an unreliable indicator in the experiments with *T. divaricata* (*Table 3.2*).

The difference in shear-tolerance between the cell lines cannot be explained from the experimental data. However, it is possible that this

finding has a trivial explanation. It has been hypothesized that the shear-tolerance of a cell line may be correlated to the number of subcultivations after initiation, in the sense that prolonged subcultivation would result in cells with better growth performance and shear-resistance (Scragg *et al.*, 1988). Both the *C. roseus* and *N. tabacum* cell lines were maintained during several years as relatively fast growing and stable lines under a constant subcultivation regime. On the contrary, the *C. robusta* cell line was initiated recently. Its growth behaviour during subcultivation appeared to be unstable. Although the *T. divaricata* cell line was not initiated recently, it has been kept for three years under a subcultivation regime optimized for secondary metabolite production, but unfavourable for growth (van der Heijden, personal communication). In our lab we attempted to improve the subcultivation regime, but we did not accomplish in obtaining a cell line with stable growth characteristics. Therefore we are of the opinion that the difference in shear-sensitivity can very well be explained by the difference in stability of the four cell lines. Besides we think that eventually every cell line of which the growth can be optimized by subcultivation will appear to be shear-tolerant.

Interestingly, the order of shear-tolerance of the four cell lines is identical to the order of ease with which they can be cryopreserved (van Iren, personal communication). Therefore, it is likely that both findings have a common explanation. To endure a high hydrodynamic load and the burden of cryopreservation a plant cell should have a tough and flexible cell wall and cell membrane. Besides, the cell should have a high capacity to repair lesions and ruptures in wall and membrane. Both properties are presumably present in the cell lines of *C. roseus* and *N. tabacum* we are keeping in our project group. To understand the circumstances that govern the shear- and cryogenic tolerance of a plant cell much more research should be focused on the build-up and properties of cell wall and cell membrane.

CHAPTER 4

GROWTH OF A *CATHARANTHUS ROSEUS* SUSPENSION CULTURE IN A CHEMOSTAT UNDER CONDITIONS OF LONG-TERM HYDRODYNAMIC STRESS.

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Introduction

For large scale cultivation of plant cells the use of classical mechanically stirred bioreactors should be, from an economic point of view, the most attractive option. Today, this type of reactor, ranging from several litres up to volumes of 500,000 l, is commonly used in microbial fermentation processes for its relatively high reliability and simplicity. However, in an impeller-stirred reactor high levels of hydrodynamic stress ('shear-stress') are generated which can lead to cell damage and eventually cell death.

In some papers the negative effects of hydrodynamic stress on cell growth and production of secondary metabolites have been reported (Wagner and Vogelmann, 1977; Tanaka, 1987), which has led to a common opinion among workers in this field that plant cells are intrinsically shear-sensitive. This property is often explained by stating that they possess a rigid cellulosic cell wall and have a relative large cell volume compared to microorganism (Mandels, 1972). Moreover, this opinion has given rise to the development of a range of alternative low-shear bioreactors for the cultivation of plant cells (Hegglin *et al.*, 1987; Hulst *et al.*, 1987; Soule *et al.*, 1987; Tanaka, 1987). In general low-shear bioreactors perform well at small sizes. For industrial use, however, scale-up is needed. In most cases little knowledge is available on scale-up of this kind of bioreactors, so expensive research is needed to gain this knowledge. Therefore one should carefully investigate whether a cell line is indeed shear-sensitive in order to make a justified choice of reactor.

Until now most experiments to assess the shear-sensitivity of plant cells have been performed in shear devices in which cells were exposed for relatively short periods of time (**Chapter 2**). In this chapter experiments are described in which cells are exposed to high hydrodynamic loads for long periods of time in a turbine-stirred chemostat. A continuous culture, and a chemostat in particular, are very versatile tools to determine the influence of hydrodynamic stress on plant cells:

(1) Loss of cell viability by cell damage or cell death is unambiguously reflected by decline of growth rate or wash-out of the cell population.

(2) A cell population can be exposed to hydrodynamic stress for long periods of time under steady-state conditions.

(3) Selection of shear-tolerant cells can be performed by imposing slowly increasing hydrodynamic stress on the cell population.

This chapter describes the cultivation of a *Catharanthus roseus* cell suspension in a 3-l chemostat. Hydrodynamic stress was generated by mixing with a six-bladed turbine impeller. This type of impeller has been chosen because it generates by its shape high levels of hydrodynamic stress. Therefore it represents a worst case situation. In a preliminary experiment a *C. roseus* cell suspension was cultivated for 80 days in a chemostat in which the agitation speed and dilution rate were increased step-wise.

In two consecutive experiments steady-state growth was obtained at an agitation speed of 16.7 s^{-1} and dilution rates of 0.0056 and 0.0115 h^{-1} for several reactor residence times. Results are compared with results from analogous experiments performed at the same dilution rates, but at much lower agitation speeds.

Materials and methods

Cell culture

The cell suspension culture of *Catharanthus roseus* was obtained from the Department of Plant Molecular Biology, Leiden University. This culture, grown in suspension for more than 4 years, was originally initiated from seeds. The stock cell line was used to inoculate chemostats.

Culture medium

The stock cell line of *C. roseus* was grown on LS growth medium (Linsmaier and Skoog, 1965) containing 30 $g*l^{-1}$ glucose, 2.0 $mg*l^{-1}$ α-naphthaleneacetic acid (NAA), and 0.2 $mg*l^{-1}$ kinetin. For medium preparation pyrogene free water produced by a water purification system (Milli-Q-UF, Millipore, Bedford, USA) was used. The initial medium pH was 6.0. Sterilization was carried out by autoclaving at 121°C, 1 bar overpressure, for 20 minutes. For chemostat experiments the glucose concentration in the feed medium was 8 $g*l^{-1}$ while the pH was adjusted to 5.0. The medium was filter-sterilized by a 0.2 µm hydrophilic filter (Millidisk System, Millipore) and collected in a sterile 10-l glass vessel containing 1 ml silicone antifoaming agent (BDH Chemicals Ltd., Poole, UK) dissolved in 100 ml water.

Culture conditions

The cells were cultured in 1000 ml Erlenmeyer flasks in the dark at 25°C, and shaken at 100 rpm on an orbital shaker. The cultures were subcultured every 14 days by inoculating 35 ml of culture into 165 ml of fresh medium. The flasks were closed with the cut-off upper halfs of silicone foam stoppers (model T42, Shin Etsu, Tokyo, Japan).

The chemostat culture was inoculated by transferring 300 ml of a 7 days old culture together with 1500 ml of LS medium containing 8 $g*l^{-1}$ glucose into the fermenter. Dilution with feed medium was started after 3 days of batch cultivation.

Continuous culture system

The continuous culture system used is shown in *Figure 4.1.* It is based on a commercially available 3-l fermenter (Applikon, Schiedam, The Netherlands) equipped with a six-bladed turbine impeller (blade angle=90°) with a diameter of 4.5 cm, and 3 round baffles with a diameter of 1.4 cm. The fermenter has been specially modified for the continuous culturing of plant cells.

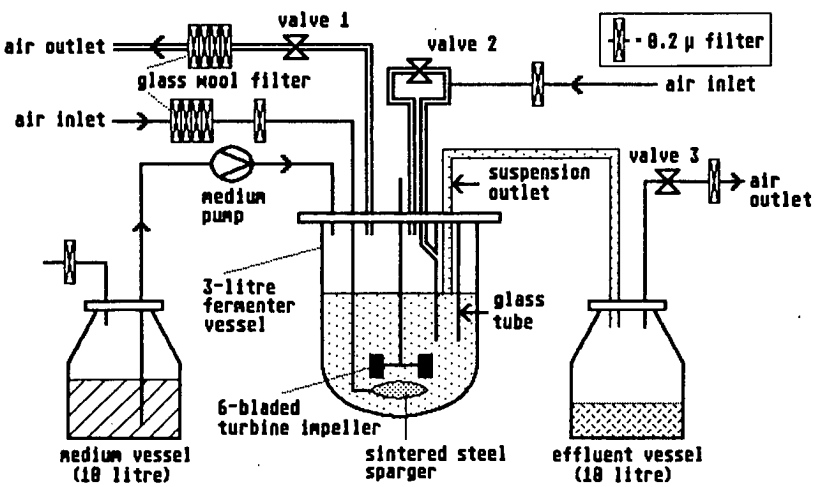

Figure 4.1. Schematic representation of the continuous culture system.

The culture in the fermenter was aerated by means of a sintered steel sparger at the bottom of the vessel, aeration rate was 40 $l*h^{-1}$. Pre-dried influent air passed through a cotton wool filter and a 0.2 µm hydrophobe membrane filter (Millex FG50, Millipore). Aeration flow was kept constant by means of a mass flow controller system (Brooks Instrument BV, Veenendaal, The Netherlands). Effluent air left the fermenter through a glass condenser kept at 4°C by a cryostat (Lauda Meßgeräte, Lauda, FRG). The amount of water which was lost by evaporation in spite of the use of a condenser was determined by leading the effluent air through a preweighted silica column. Both dilution rate and influent glucose concentration were corrected for evaporation loss.

The temperature in the fermenter was maintained at 25°C by circulating water from a thermostated heating bath (Lauda) through the reactor's jacket.

The pH of the culture was monitored continuously by a sterilizable pH electrode (Ingold Meßtechnik AG, Urdorf, Switzerland) and during continuous culture maintained at 5.0 with 1 N NaOH by an automatic pH control system (Metrohm AG, Herisau, Switzerland).

Fresh medium was fed continuously to the culture by means of a peristaltic pump (Varioperpex-Pharmacia LKB Biotechnology, Uppsala, Sweden).

Effluent was taken discontinuously from beneath the fluid surface with a special device which was developed and built in our laboratory's workshop. This device consisted of a glass tube (internal diameter=18 mm) mounted vertically inside the reactor at the under side of the lid of the reactor. The glass tube reached into the suspension about half-way between the culture surface and the bottom of the vessel. This tube contained a smaller (internal diameter=8 mm) stainless steel tube which passed through the lid of the reactor and was connected to the effluent vessel by a flexible silicone tube (internal diameter=8 mm). The lower end of the stainless steel tube reached exactly to the culture surface. The glass tube was continuously emptied by a small flow of sterile air through a pipe which passed through the lid into the reactor and was connected to the glass tube inside (*Figure 4.2A*).

After a fixed time interval the air outlet of the reactor was shut off by valve 1 while at the same time the pressure inside the glass tube was

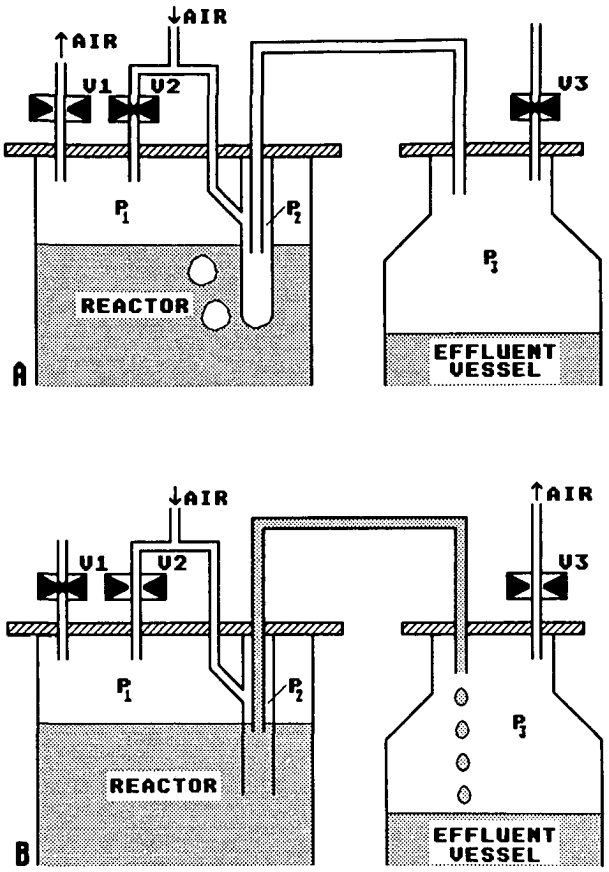

Figure 4.2A,B. Principle of the effluent removal system. P_1=pressure inside the headspace of the reactor. P_2=pressure inside the effluent removal tube. P_3=pressure inside the headspace of the effluent vessel.
A. If valve **V1** is open, and valves **V2** and **V3** are closed a small air stream keeps the effluent removal tube empty. In this case P_2 ($=P_3$) exceeds P_1.
B. When **V1** is closed while at the same time **V2** and **V3** are opened, this will cause equilibration of P_1 with P_2, resulting in a rise of the culture in the effluent removal tube. Because of the aeration of the reactor, P_1 ($=P_2$) will exceed P_3, resulting in withdrawal of all the culture fluid above the inlet of the effluent removal tube.

equilibrated with the pressure in the reactor by releasing valves 2 and 3. This resulted in a rapid rise of the suspension inside the glass tube and suspension was driven out of the reactor as long as the culture level was above the inlet of the stainless steel tube (*Figure 4.2B*). When the liquid level was lowered until just below the stainless steel tube, only air was driven out. So the position of the stainless steel tube determined the minimum culture volume during operation. The effluent removal took about 5 seconds and time intervals between effluent removal were chosen in such a way that changes in culture volume never exceeded ± 1%.

To let the above described continuous culture system work under aseptic conditions, sterilizable valves were needed. For this purpose an apparatus was constructed which could pinch two silicone tubes and release another or vice versa. The device that was equipped with a bi-directional pneumatic cylinder was driven by compressed air of 3 bar. Pneumatics were controlled by a set of electric valves and a programmable sequential timer (Omron, Tokyo, Japan).

Sampling was performed by aseptic withdrawal of 100 ml of suspension from the culture through a sample pipe connected by flexible silicone tubing to a bottle holder containing a sterile serum bottle of 100 ml. After sampling the full bottle was replaced aseptically by a sterile empty bottle. At regular time intervals samples were taken for determination of biomass, glucose, ammonium, nitrate and (by)product concentration. Besides carbon dioxide production rate and oxygen consumption rate were measured.

Estimation of hydrodynamic stress parameters

Hydrodynamic stress parameters were estimated as has been described in **Chapter 1.**

Analytical procedures

Dry biomass concentration was determined in duplicate by filtering 2 x 10.0 ml of cell suspension on a pre-weighed 47 mm glass fiber filters (Type A/E, Gelman Sciences Inc., Ann Arbor, USA). After washing twice with demi water cells were subsequently dried at 70°C for 24 hours. Ash content was determined by heating dried biomass to 600°C until constant weight.

Cell free medium was prepared by centrifuging of 2 x 10.0 ml of cell suspension in a table centrifuge for 5 minutes at a speed of 50 s^{-1}. Cell free medium was frozen and stored at -20°C prior to analysation.

Glucose concentration in the cell free medium was determined with an enzymatic glucose auto-analyser (model 27, Yellow Springs Instruments Co., Yellow Springs, USA).

Total organic carbon (TOC) concentration was determined with a TOC analyser (model Tocor 2, Maihak AG, Hamburg, FRG). (By)product concentration was determined by subtracting the carbon from glucose in the spent medium from the overall TOC concentration.

Ammonium concentration was determined colorometrically by an assay based on the phenol-hypochlorite-ammonium reaction. Nitrate concentration was determined by means of ion high performance liquid chromatography (ion-HPLC).

To determine carbon dioxide production rate (rCO_2) and oxygen consumption rate (rO_2) the carbon dioxide and oxygen concentration of effluent air were measured with an infrared CO_2-analyser (model 864, Beckman Instruments Inc, Fullerton, USA) and a double channel paramagnetic oxygen analyser (Taylor Servomex Ltd, Crowborough, UK). Both the specific oxygen uptake rate (qO_2) and specific carbon dioxide production rate (qCO_2) were calculated by dividing rO_2 and rCO_2 by the biomass dry-weight concentration.

Results

Calculation of hydrodynamic stress parameters

The eddy size d_e, the cell-to-cell collision energy and frequency EC_{cc} and NC_{cc}, the cell-to-impeller energy and frequency EC_{ci} and NC_{ci}, and the turbulent and laminar shear stress τ_{wt} and τ_{wl} were calculated.

As can be seen in *Table 4.1* the size of the smallest eddies d_e ranges from $14*10^{-5}$ at 1.7 s^{-1} to $3*10^{-5}$ m at 16.7 s^{-1}. So the eddy size is of the same scale as the size of the cells, which can result in rotation of the cell accompanied by low levels of hydrodynamic stress, or high shear stresses when several eddies with opposed rotation interact with it simultaneously. The latter mechanism will dominate when agitation speed increases. A ten-fold increase of agitation speed results in an increase of 3 orders of magnitude of power-input, and the severity of cell-impeller collision ($E_{ci}*NC_{ci}$). The severity of cell-cell collision ($E_{cc}*NC_{cc}$) increases 2 orders of magnitude. At an agitation speed of 16.7 s^{-1} the maximum shear stress is 8-25 $N*m^{-2}$.

Table 4.1. Calculation of hydrodynamic stress parameters for a 3-l (working volume=1.7 l) turbine-stirred fermenter. Volume-fraction biomass=0.05, cell aggregate diameter=$1*10^{-4}$ m, impeller diameter=4.5 cm, medium kinematic viscosity=$1*10^{-6}$ $m^{-2}*s^{-1}$.

reactor parameters						
N	[s^{-1}]	1.7	3.4	5.0	6.7	16.7
v_i	[$m*s^{-1}$]	0.24	0.47	0.71	0.94	2.36
P/V	[$W*m^{-3}$]	2	20	68	160	2500
hydrodynamic stress parameters						
d_e	[m]	$14*10^{-5}$	$8*10^{-5}$	$6*10^{-5}$	$5*10^{-5}$	$3*10^{-5}$
E_{cc}	[J]	$3*10^{-14}$	$8*10^{-14}$	$14*10^{-14}$	$22*10^{-14}$	$85*10^{-14}$
E_{cc}/M_c	[J/kg]	$5*10^{-5}$	$14*10^{-5}$	$26*10^{-5}$	$40*10^{-5}$	$159*10^{-5}$
NC_{cc}	[s^{-1}]	2	3	4	5	10
E_{ci}	[J]	$2*10^{-11}$	$6*10^{-11}$	$14*10^{-11}$	$24*10^{-11}$	$150*10^{-11}$
E_{ci}/M_c	[J/kg]	0.03	0.1	0.25	0.4	3
NC_{ci}	[s^{-1}]	$1*10^{-3}$	$3*10^{-3}$	$4*10^{-3}$	$6*10^{-3}$	$14*10^{-3}$
τ_{wl}	[$N*m^{-2}$]	0.3-1.3	0.7-3	1-4	2-5	8-13
τ_{wt}	[$N*m^{-2}$]	0.3-0.5	1-2	2-3	4-5	19-25

Outflow efficiency

In various experiments (results not shown) no difference could be detected between effluent biomass concentration and biomass concentration in the fermenter. This meant that the previously described effluent removal system provided for an outflow efficiency of 100%.

Preliminary experiment

In order to get an impression of the shear sensitivity of the *C. roseus* cell line a chemostat was started at a median dilution rate of 0.0093 h^{-1} and a moderate agitation speed of 1.7 s^{-1}. After 24 days agitation speed was increased to 3.3 s^{-1}, after 38 days to 5.0 s^{-1}. After 48 days dilution rate was increased to 0.0139 h^{-1} and agitation speed to 6.7 s^{-1} and finally after 63 days dilution rate was increased to 0.0168 h^{-1}. Biomass concentration slightly increased during the period from day 24 until day 48 indicating no inhibition of growth by increasing hydrodynamic stress

(*Figure 4.3A*).

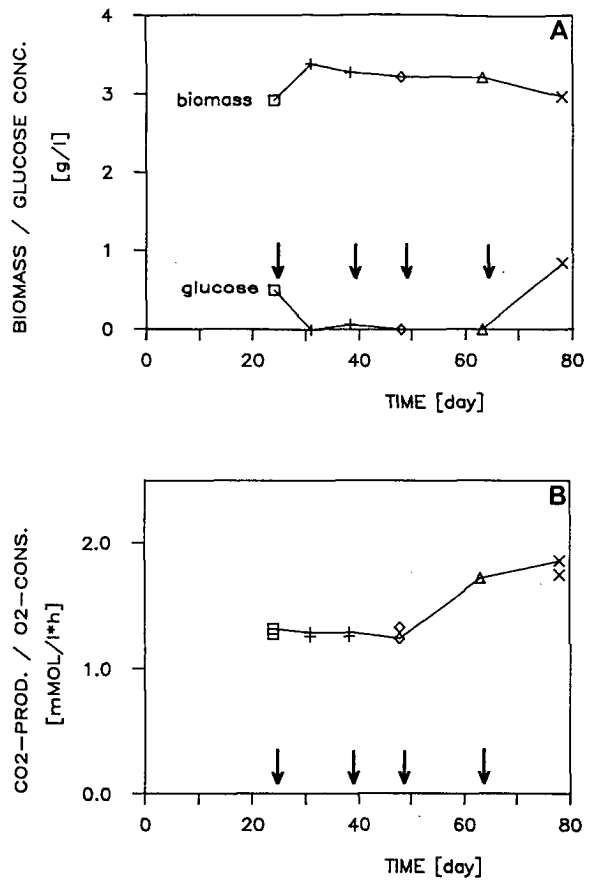

Figure 4.3A,B. Growth by a *C. roseus* suspension culture in a glucose-limited chemostat stirred with a 4.5 cm turbine impeller. Dilution rate and agitation speed were gradually increased from 0.0093 h^{-1} and 1.7 s^{-1} to 0.0168 h^{-1} and 4.7 s^{-1} (marked by arrows in figure).
A. biomass dry-weight; residual glucose [$g*l^{-1}$]. **B.** CO_2-production (symbols connected by line) ; O_2-consumption (symbols) [$mmol*l^{-1}*h^{-1}$]
(□) 0.0093 h^{-1}, 1.7 s^{-1} (+) 0.0093 h^{-1}, 3.3 s^{-1}
(◇) 0.0093 h^{-1}, 5.0 s^{-1} (△) 0.0139 h^{-1}, 6.7 s^{-1}
(×) 0.0168 h^{-1}, 6.7 s^{-1}

Glucose was converted quantitatively as it was not detectable in the

effluent (less than 0.1 $g*l^{-1}$). At a dilution rate of 0.0168 h^{-1} glucose was not converted quantitatively. Presumably the dilution rate had reached a value in the vicinity of the maximum specific growth rate (μ_{max}) of the cell line. Van Gulik *et al.* (1989b) found a maximum specific growth rate of 0.018 h^{-1} for this cell line grown in an identical chemostat set-up at moderate agitation speeds. Increase of dilution rate resulted in a rise of the carbon dioxide production and oxygen consumption, indicating adaptation of respiration to the increasing growth rate (*Figure 4.3B*).

Steady state experiments

The results of the afore-described experiment indicated that the *C. roseus* cell line was able to tolerate an agitation speed of 6.7 s^{-1} without loss of viability. Batch culture experiments with *C. roseus* in a comparable set-up at an agitation speed of 16.7 s^{-1} (**Chapter 3**) showed the *short-term* shear-tolerance of this cell line. So it was decided to perform also chemostat experiments at the same agitation speed to determine the *long-term* effects of high levels of hydrodynamic stress on *C. roseus*.

Two separate chemostats at a dilution rate of respectively 0.0056 h^{-1} and 0.0115 h^{-1} and an agitation speed of 16.7 s^{-1} were started. The evolution of biomass, glucose, and (by)product concentration during seven reactor residence times for D=0.0056 h^{-1} is shown in *Figure 4.4* and for D=0.0115 h^{-1} in *Figure 4.5* together with results from analogous chemostat experiments performed at nearly identical dilution rates, but at moderate agitation speed (van Gulik *et al.*, 1989a,b). Results are briefly summarized in *Table 4.2*.

At a dilution rate of 0.0056 h^{-1} glucose-limited growth was attained after 3 residence times (*Figure 4.4A*), compared to 6 residence times at a dilution rate of 0.0115 h^{-1} (*Figure 4.5A*). Ammonium was preferred over nitrate as nitrogen source (*Table 4.2*). During both steady states nearly 50% of the ammonium in the feed medium was consumed compared to nearly 10% of the nitrate. This is in accordance with results obtained from batch cultures of *C. roseus* on LS medium in which ammonium was always the preferred nitrogen source (ten Hoopen *et al.*, 1988).

In the spent medium a small quantity of organic carbon was detected by total organic carbon (TOC) analysis when no residual glucose was present anymore (*Figure 4.4B, 4.5B*). It amounted to 4% (D=0.0115 h^{-1}) and 8%

(D=0.0056 h^{-1}) of the in-going carbon flow (glucose). Chemostat experiments carried out at nearly the same dilution rates, but significantly lower agitation speed showed comparable levels of (by)product (*Table 4.2*). Therefore, release of organic carbon can not be attributed to the effect of a high hydrodynamic load.

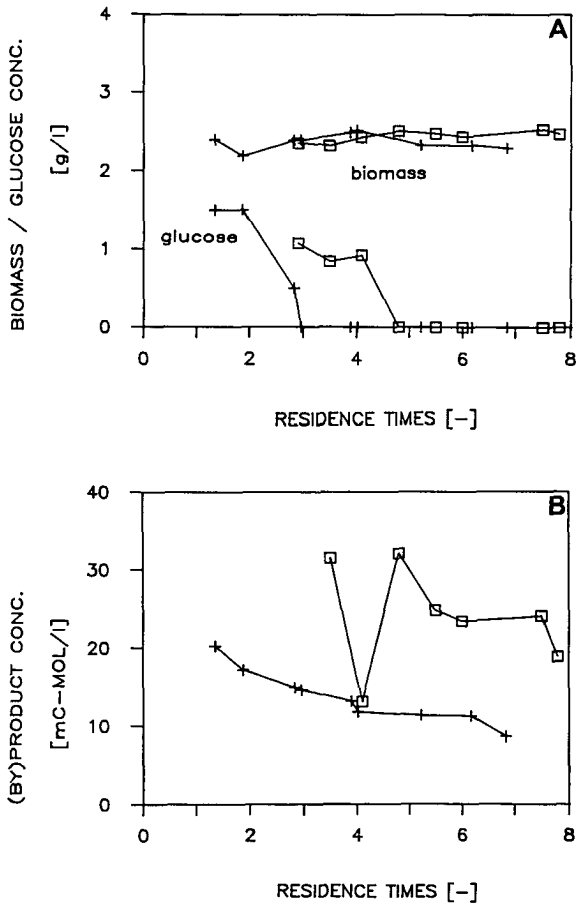

Figure 4.4A,B. Growth by a *C. roseus* suspension culture in a glucose-limited chemostat stirred with a 4.5 cm turbine impeller at agitation speeds of 2.1 and 16.7 s^{-1}, dilution rate=0.006 h^{-1}. **A.** biomass dry-weight; residual glucose [$g*l^{-1}$]. **B.** (by)product [$mC\text{-}mol*l^{-1}$].
(□) 2.1 s^{-1}, D=0.0060 h^{-1}; (+) 16.7 s^{-1}, D=0.0056 h^{-1}

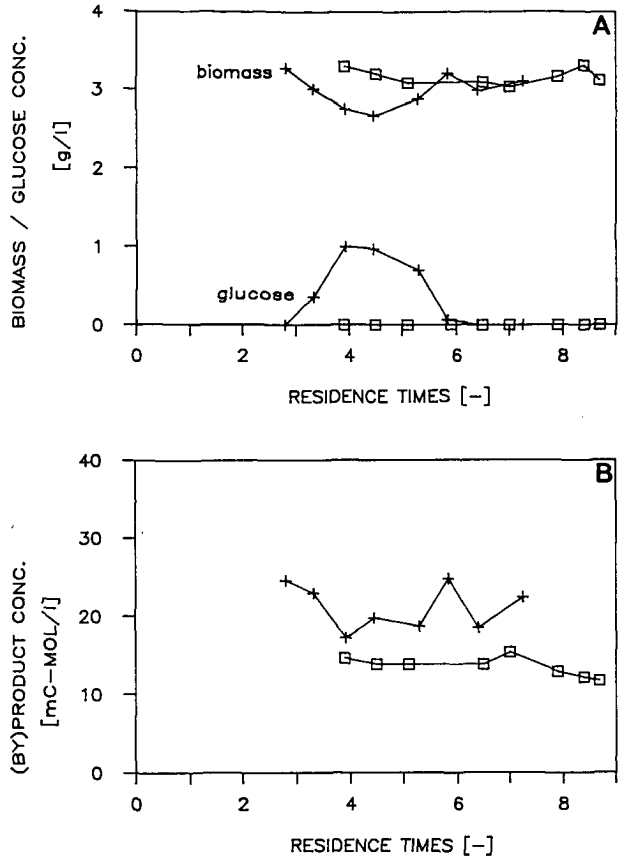

Figure 4.5A,B. Growth by a *C. roseus* suspension culture in a glucose-limited chemostat stirred with a 4.5 cm turbine impeller at agitation speeds of 4.2 and 16.7 s^{-1}, dilution rate=0.012 h^{-1}. **A.** biomass dry-weight; residual glucose [$g*l^{-1}$]. **B.** (by)product [$mC\text{-}mol*l^{-1}$].
(□) 4.2 s^{-1}, D=0.0117 h^{-1}; (+) 16.7 s^{-1}, D=0.0115 h^{-1}

Analysis of medium samples of batch cultures of *C. roseus* revealed that they contained a polysaccharide consisting of arabinose and galactose. As has been discussed in Chapter 3 this polysaccharide (by)product presumably plays a role in turnover of the cell wall (Takeuchi and Komamine, 1980). Whereas the (by)product concentration seemed to increase with increasing dilution rate in the case of the high-agitation speed experiments, this trend seemed reversed in the low-agitation speed experiments. However, from 11 glucose-limited chemostat experiments performed with *C. roseus* at low agitation speeds (van Gulik, 1989b) it has been concluded that there is no clear relation between specific rate of (by)product formation and growth rate.

In *Table 4.2* and *4.3* a comparison is made between low agitation speed and high agitation speed chemostat experiments.

Table 4.2. Cultural parameters of four steady states of a *C. roseus* suspension culture grown in a glucose-limited chemostat stirred with a 4.5 cm turbine impeller at low (**bold**) and high agitation speeds.

N	[s^{-1}]	**2.1**	16.7	**4.2**	16.7
D	[h^{-1}]	**0.0060**	0.0056	**0.0117**	0.0115
biomass	[$g*l^{-1}$]	**2.48**	2.37	**3.13**	3.05
ammonium	[$mmol*l^{-1}$]	**15.8**	10.3	**10.1**	11.2
nitrate	[$mmol*l^{-1}$]	**31.6**	36.8	**32.0**	35.1
rCO_2	[$mmol*l^{-1}*h^{-1}$]	**1.05**	1.00	**1.70**	1.89
qCO_2	[$mmol*g^{-1}*h^{-1}$]	**0.42**	0.42	**0.54**	0.62
rO_2	[$mmol*l^{-1}*h^{-1}$]	**0.90**	1.00	**1.68**	1.98
qO_2	[$mmol*g^{-1}*h^{-1}$]	**0.36**	0.42	**0.54**	0.65
(by)product	[$mC\text{-}mol*l^{-1}$]	**24.7**	10.8	**13.3**	21.1

Most cultural parameters of a steady-state at a high agitation speed do not deviate significantly from those at a low agitation speed (*Table 4.2*). Biomass grown at a high agitation speed contained a little bit more hydrogen and oxygen (*Table 4.3*). From *Table 4.2* it can be concluded that the oxygen consumption rate was slightly elevated for a steady-state at

0.0115 h^{-1} and 16.7 s^{-1} with respect to that at 0.0117 h^{-1} and 4.2 s^{-1}. This might be explained by a higher availability of oxygen at a high agitation speed. However, care must be taken when drawing conclusions. The degree of reproducibility of the chemostat experiments has not been studied in detail yet.

Table 4.3. Biomass elemental composition and ash content of four steady states of a *C. roseus* suspension culture grown in a glucose-limited chemostat stirred with a 4.5 cm turbine impeller at low (**bold**) and high agitation speeds.

D [h^{-1}]	N [s^{-1}]	elemental composition	ash content [%]
0.0060	**2.1**	**$CH_{1.71}O_{0.66}N_{0.17}$**	**14.9**
0.0056	16.7	$CH_{1.74}O_{0.85}N_{0.16}$	8.4
0.0117	**4.2**	**$CH_{1.63}O_{0.64}N_{0.16}$**	**9.9**
0.0115	16.7	$CH_{1.76}O_{0.76}N_{0.16}$	9.8

Discussion and conclusions

Until 1985 only one research group had published reports concerning the growth of plant cell suspensions in an impeller-stirred chemostat. Bertola and Klis, 1979) described a system for continuous culturing of bean cells (*Phaseolus vulgaris* L.) in a 2-l fermenter stirred at ≈4 s^{-1}. A major drawback of this system was that it had a poor outflow efficiency, due to improper design of the effluent removal system. We managed to solve this problem by designing an effluent removal system providing for an outflow efficiency of 100%.

The general opinion holds that plant cells are intrinsically sensitive to hydrodynamic stress. With this assumption we performed the preliminary experiment which showed the feasibility of growing this cell line in a turbine-stirred chemostat under moderate stress.

From the chemostat experiments performed at an agitation speed of 16.7 s^{-1} it can be concluded that it is possible to grow *C. roseus* cell suspension cultures in a turbine-stirred vessel under high hydrodynamic

stress conditions during several generations. Experimental results did show no significant deviation from results obtained with chemostat experiments under moderate stress conditions (van Gulik *et al.*, 1989a,b). The robustness of this cell line was beyond expectation.

It is hard to give an explanation for this finding. From literature it is known that the shear sensitivity of *C. roseus* can diverge to a large extent. Wagner and Vogelmann (1977) found their *C. roseus* cell line to be very shear-sensitive. On the contrary, Scragg *et al.* (1988) exposed a cell line for several hours to stress generated by a 7.2 cm turbine stirrer at 16.7 s^{-1} without significant loss of viability. Therefore, sensitivity to hydrodynamic stress seems to diverge strongly between cell lines and is presumably dependent on the way in which stock cultures were initiated and have been maintained.

Tanaka *et al.* (1988) reported that *C.* roseus cells cultured under strong hydrodynamic stress conditions were rich in hemi-cellulose and cellulose. The cell wall in cells cultured under these conditions could be considered to be physically strong in comparison with that in cells cultured under mild hydrodynamic stress conditions. From literature it is known that cells of whole plants can repair inflicted damage to some extent by depositing callose. This 1,3-β-glucan polysaccharide can be rapidly deposited at localized positions onto plant cell walls in order to reinforce and seal cells and tissues after mechanical injury, and during defence reactions against pathogens (Kauss, 1987). It is not known whether *C. roseus* is able to synthesize callose at a high rate during exposure to high hydrodynamic loads and whether there is a relation with shear-tolerance. So this should be subject of further research.

It is unlikely that selection for shear-tolerant cells occurred in the chemostat. A shake flask culture of the same cell line could be cultivated in the same vessel at an agitation speed of 16.7 s^{-1} as a batch culture (**Chapter 3**). Selection for shear-tolerant cells through subculturing of the stock line is not likely when only shear is considered since cells in a (unbaffled) shake flask are only exposed to very low levels of hydrodynamic stress. A second alternative for selection can be the subculturing procedure itself. It is generally accepted that an - in all respects - optimal procedure for maintenance of stock cultures leads to selection of subpopulations with increasing growth performance. It is

likely that this also leads to cells that are increasingly tolerant to hydrodynamic stress. The *C. roseus* cell suspension culture used in this investigation has been in culture for 4 years under a constant subculture regime.

This study shows that the general opinion of plant cells being shear sensitive should be reconsidered. The *C. roseus* cell line which was capable of growth under short term hydrodynamic stress could also tolerate long-term exposure in a chemostat. Future research should be focused on circumstances determining the shear-sensitivity of a cell line. With this knowledge it should be possible to transform delicate cell lines into robust lines capable of growth in impeller stirred vessels.

CHAPTER 5.

THE EFFECTS OF CHEMICAL/OSMOTIC STRESS BY POLYETHYLENE GLYCOL ON GROWTH AND PRODUCT FORMATION OF A *CATHARANTHUS ROSEUS* CELL SUSPENSION CULTURE.

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Introduction

Plant cells cultivated in a bioreactor for the purpose of producing pharmaceutically active compounds are exposed to conditions that may induce various stresses which can affect their growth and secondary metabolism considerably. Commonly only negative effects of stress have been studied. Stress generated by mixing in a bioreactor ('shear stress') is considered as a major problem in large-scale cultivation of plant cells, and therefore has often been studied (Tanaka, 1987; Scragg *et al.*, 1988).

However, stress may also have positive effects, in particular on the stimulation of the production of secondary metabolites (DiCosmo and Towers, 1984). The ecological significance of many secondary metabolites is ascribed to the improvement of the adaptation of plants to unfavourable environmental conditions. It is therefore plausible that environmental stress has a modulating effect on secondary metabolism. Analogously, stress conditions experienced by plant cells in a bioreactor could affect product biosynthesis rate.

Plant cells in suspension seldom synthesize substantial amounts of secondary metabolites spontaneously. In general cultural conditions are optimized for growth, therefore conditions that may impose stress of any kind are avoided. Furthermore, proliferating cells are undifferentiated. Both these facts are usually put forward to explain the lack of secondary metabolite production capacity of plant cells during growth. Therefore it is hypothesized that creation of artificial stress situations could stimulate the production of particular classes of secondary metabolites. Broadly speaking, secondary metabolites can be divided into two classes: those that can be induced by stress factors of all kind (elicitors), and those that are a consequence of cell morphological differentiation.

The first class of secondary metabolites, the stress metabolites designated as phytoalexines, are usually absent in a non-stressed culture. Examples of phytoalexines are berberine, rosmarinic acid, shikonin and anthraquinones. Their production can be enhanced by stress from virtually nil to several grams per litre cell culture. In contrast to phytoalexines, secondary metabolites coupled to morphological differentiation are usually already present in low concentrations in a non-stressed culture. Their production is not stimulated as strongly by stress as in the case of

phytoalexines. Examples of differentiation coupled secondary metabolites are ajmalicine, quinine, codeine and morphine.

Some examples of stresses used to stimulate secondary metabolism

Nutrient stress by low concentrations of nitrogen and phosphate sources combined with high sucrose concentrations has already been reported as an effector for secondary product formation in *Catharanthus roseus* cell suspensions (Knobloch and Berlin, 1980). Mérillon *et al.* (1984) investigated the influence of sucrose concentration in suspension cultures of *C. roseus*. Cells grown in medium supplemented with 6% sucrose yielded 9 times more ajmalicine and 4 times more serpentine than cells grown in 2% sucrose. Frischknecht and Baumann (1985) found a stimulation of the production of the purine alkaloid caffeine in *Coffea arabica* cell suspensions by stressors such as high light intensity and - depending on culture type - high NaCl concentration. Stimulation by temperature and light rhythms of indole alkaloid formation in cell cultures of *C. roseus* has been found by Giger *et al.* (1985). Rudge and Morris (1986) and Frischknecht *et al.* (1986) found a stimulation of alkaloid accumulation in *C. roseus* cell suspensions by osmotic stress generated by mannitol supplemented growth media. Stimulation of indole alkaloid formation by *C. roseus* in response to chemical stress generated by vanadyl sulphate has been found by Smith *et al.* (1987). Vanadium has been shown to inhibit the plasmamembrane ATPase, thus changing the membrane potential. It has been hypothesised that in this way vanadium acts analogously to a biotic elicitor (Hagendoorn, personal communication). Arfmann *et al.* (1985) studied the effect of 5-azacytidine, a powerful inducer of cell differentiation, on the formation of secondary metabolites in *C. roseus* cell suspension cultures. They found the formation of a lignan type compound, lirioresinol B mono-β-D-glucoside, not found before in *C. roseus* plants or cell cultures.

Osmotic stress

For this study osmotic stress was chosen as subject. Osmotic stress can be induced by lowering the water potential of the medium, which can be accomplished by dissolving a certain amount of a solute. When a plant cell is exposed to osmotic stress, three different phases of adaptation can be distinguished (Kleiwegt, 1988). Firstly, the plant cell controls its

volume by the regulation of the plasticity of the cell wall as well as the regulation of the hydraulic conductivity of the cell membrane. Secondly, the plant cells controls its own osmotic potential by the active uptake of ions or low molecular weight organic solutes. The ions are preferably K^+ ions, but when not available K^+ can be replaced by Na^+, Ca^{2+}, and Mg^{2+}. However, besides the active uptake of ions, which is energized by the hydrolysis of ATP, a co-transport is probably available in the cell membrane as well. In this case, ATP is hydrolysed to generate a proton gradient across the cell membrane. Passive diffusion of these protons back into the cell is linked to the active uptake of another substance like K^+ ions or sugars. In the third place, plant cells are able to synthesize and accumulate organic solutes in order to control their osmotic potential. This is probably a secondary mechanism, which is induced either by a high concentration of K^+ ions in the cytoplasm or by the effect of the intracellular ionic strength. The major function of these accumulated compatible solutes is to protect the structure and functions of the proteins in the cytoplasm. Compatible solutes are e.g. proline, betaine, organic acids and polyalcohols.

Plant cells exposed to osmotic stress show a reduced fresh weight gain, a reduced cell expansion and an increase in turgor pressure. Therefore adapted cells remain small, probably due to a change of the cell wall composition which leads to a higher compressive modulus of the cell wall. Thus, osmotic stress is apparently an effective tool in controlling the biomass fresh-weight to dry-weight ratio. In theory this could be applied for attaining higher biomass dry-weight concentrations in large-scale fermenter cultures, which has been shown to increase the commercial feasibility of a production process.

Chemical/osmotic stress

It is not clear whether the stimulation of secondary metabolism of plant cells by osmotic stress exerted by solutes such as sugars, polyalcohols, or salts must be attributed to pure osmotic effects (e.g. lowering of water potential of cytoplasm or vacuole) that are exerted on the cells, or to chemical effects (e.g. membrane permeabilization), or to a combination of both. Therefore we are of the opinion that one should designate stress exerted by solutes as chemical/osmotic stress rather than osmotic stress.

To study the effects of chemical/osmotic stress on plant cells non-metabolizable, non-penetrating, non-toxic osmotica should be used. A low molecular weight is necessary when the effects of high osmotic stress are to be studied. These prerequisites are only fulfilled by a small number of osmotica such as melibiose (Dracup *et al.*, 1986), and low molecular weight polyethylene glycol (PEG) (Handa *et al.*, 1982). Sorbitol or mannitol are in most cases not suitable for long-term experiments. Although seldom serving as carbon and energy source, they are taken up by some cell suspension cultures (Dracup *et al.*, 1986; van den Broek, 1987).

In this study PEG was chosen because of its availability in a wide range of molecular weights. Chemical/osmotic stress by PEG with a molecular weight of 600 and 6000 was tested as stimulator of secondary metabolism in *C. roseus*. Besides, effects on growth and biomass fresh-weight to dry-weight ratio were studied. It was attempted to differentiate between effects of chemical and osmotical stress. We assumed that osmotic stress would be dominant with PEG 600 as it has a low molecular weight. On the contrary, we expected that PEG 6000 would exert mainly chemical stress. Experiments were performed both in a shake flask and in a fermenter.

Materials and Methods

Cell culture and medium

The cell culture of *Catharanthus roseus* was obtained from the Department of Plant Molecular Biology in Leiden. This cell line was grown on LS medium (Linsmaier and Skoog, 1965) supplemented with 30 $g*l^{-1}$ glucose, 2.0 $mg*l^{-1}$ α-naphtaleneacetic acid (NAA), and 0.2 $mg*l^{-1}$ kinetin and was subcultured every 7 days with an inoculation ratio of 1 to 5. The culture was grown in the dark on an orbital shaker at 100 rpm and at 25°C.

Chemical/osmotic stress media

Polyethylene glycol (PEG) 600 stress media were prepared by dissolving various quantities of synthesis grade PEG 600 (Merck, Hohenbrunn, FRG) in LS growth medium. Five stress media with 250, 300, 350, 400, and 450 $g*l^{-1}$ PEG 600 were prepared for shake flask experiments. For a fermenter experiment medium with 200 $g*l^{-1}$ PEG 600 was prepared.

PEG 6000 stress media were prepared by dissolving various quantities of

synthesis grade PEG 6000 (J.T. Baker Chemical Co., Phillipsburg, USA) in LS growth medium. Four stress media with 100, 200, 300, and 350 $g*l^{-1}$ PEG 6000 were prepared for shake flask experiments. For a fermenter experiment medium with 250 $g*l^{-1}$ PEG 6000 was prepared. All media were sterilized by autoclaving at 121°C, 1 bar over-pressure, for 20 minutes, pH was adjusted to 6.0 before sterilization.

Calculation of water activity and water potential

Norrish (1966) proposed a correlating equation for predicting the water activity a_w in binary non-electrolyte solutions:

$$a_w = X_1 \exp(-KX_2^2) \qquad (5.1)$$

where X_1 and X_2 are molar fractions of water and solute, respectively, and K is the correlating constant. PEGs are typical non-ideal solutes and therefore exhibit a very strong negative deviation from Raoult's law as compared to other polyols (Chirife and Ferro Fontan, 1980; Chirife *et al.*, 1980). K values appear to increase substantially with increasing molecular weight of the PEG. K can be calculated from the molecular weight MW of the PEG (Chirife and Ferro Fontan, 1980):

$$K = 1.6(MW/100)^2 \quad (200 < MW < 8000) \qquad (5.2)$$

The water potential P can be calculated from the water activity:

$$P = 55.5(RT\ln a_w) \qquad (5.3)$$

where R is the gas constant (8.31 $J*K^{-1}*mol^{-1}$), T is the (absolute) temperature (K).

Shake flask experiments

Fifty erlenmeyer flasks of 250 ml containing 40 ml LS growth medium were inoculated with 10 ml of a 7 day old *C. roseus* cell suspension and were incubated in the dark at 25°C on an orbital shaker at 100 rpm. The cut-off lower halfs of silicone foam stoppers (model T42, Shin-Etsu, Tokyo, Japan) were used as shake flask closures. After 7 days the 50 flasks were divided in 10 series of 5. To 9 series 40 ml chemical/osmotic

stress medium was added, each series of flasks received 1 of the 9 chemical/osmotic stress media. To the 10th series, that was intended as control experiment, 40 ml of standard LS medium was added. After that the flasks were put back on the shaker. Final concentrations of PEG 600 were 111, 133, 156, 178, and 200 $g*l^{-1}$ respectively. Final concentrations of PEG 6000 were 44, 89, 133, and 156 $g*l^{-1}$ respectively. During 12 days with a time interval of 2 or 3 days 10 flasks (one of each series) were harvested for the measurement of culture growth, glucose, and production of ajmalicine and serpentine.

Fermenter experiments

Fermenter experiments were carried out in a 3-l fermenter (Applikon, Schiedam, The Netherlands) equipped with a 4.5 cm diameter six-bladed turbine impeller, and three round baffles with a diameter of 1.4 cm. Experiments were performed at 25°C in the dark. Aeration rate was 40 $l*h^{-1}$ agitation speed was 250 rpm. The fermenter containing 1900 ml of fresh LS growth medium was inoculated with 400 ml of a *C. roseus* suspension culture pre-grown in shake flasks. After 7 days batch cultivation aeration was stopped and cells were allowed to settle for one hour. A volume of 1500 ml spent medium was withdrawn aseptically by a pipe mounted through the fermenter's lid and collected in a sterile flask. A volume of 1500 ml chemical/osmotic stress medium containing either 200 $g*l^{-1}$ PEG 600 or 250 $g*l^{-1}$ PEG 6000 was poured into the fermenter by means of a sterile flask connected to an inlet port of the fermenter. Final concentration of PEG 600 and PEG 6000 were 171 and 155 $g*l^{-1}$ respectively. During 26 days with time intervals of 3 or 4 days a sample of 100 ml was taken from the culture for the measurement of culture growth, glucose, and production of ajmalicine and serpentine for 26 days.

Analytical procedures

Biomass was determined as fresh and dry-weight by filtering 10.0 ml of cell suspension on a pre-weighted 47 mm glass fibre filter (type A/E, Gelman Sciences Inc. Ann Arbor, USA). Filtered cells were washed twice with demi water. For dry-weight determination cells were subsequently dried at 70°C for 24 hours.

Glucose concentration in cell free medium was determined with an enzymatic glucose analyser (model 27, Yellow Springs Instruments Co.,

Yellow Springs, USA).

Viability of the shake flask culture cells was estimated by fluorescein diacetate (FDA) vital staining according to Widholm (1972).

Viability of the fermenter cultures was estimated by measuring respiration of the total cell population. Oxygen concentration and carbon dioxide concentration of effluent air was measured using a double channel paramagnetic oxygen analyser (Taylor Servomex Ltd., Crowborough, UK) and an infrared CO_2-analyser (model 864, Beckman Instruments Inc., Fullerton, USA).

For alkaloid analysis biomass was collected by filtering cell suspension on a 10 µ nylon mesh. Filtrate was collected for extracellular ajmalicine and serpentine analysis. To 20.0 ml filtrate 0.5 ml of a 200 $mg*l^{-1}$ dihydrokinine internal standard in HPLC grade methanol was added. Biomass and filtrate were frozen and stored at -20°C. Intracellular ajmalicine and serpentine were extracted as follows: a mixture of 200 mg freeze-dried cells in 5.0 ml of HPLC grade methanol was sonicated at 60 W for 5 minutes using a sonifier with microtip (model B12, Branson Power Company, Danbury, USA). After sonication 0.5 ml of a 200 $mg*l^{-1}$ dihydroquinine internal standard in was added to the mixture. Extracellular ajmalicine and serpentine were extracted from the remainder of 20.5 ml freeze-dried filtrate with 10.0 ml methanol. Samples were filtered through a 0.45 µm membrane filter for HPLC injection. For analysis of ajmalicine and serpentine content a high performance liquid chromatography (HPLC) system (Waters Associates Inc., Milford, USA) was used. A Waters µ-Bondapack phenyl column P/N 27198 was used together with a Waters Bondapack phenyl/corasil (37-50 µm) precolumn. The mobile phase was 0.05M sodiumdihydrogen phosphate in water with 15% acetonitrile and 5% 2-methoxyethanol adjusted to pH 3.9. The flow rate was 2 $ml*min^{-1}$. Ajmalicine peaks were detected by an UV detector (model Lambda-Max 481, Waters) set at 254 nm. Serpentine peaks were detected by a fluorimetric detector (model RF 530, Shimadzu Corporation, Kyoto, Japan), excitation was set at 346 nm, emission measured at 455 nm.

Results

Calculation of water activity and water potential of stress media

Using equations 5.1 to 5.3 the remaining water activity and water

potential in the shake flask and fermenter cultures after depletion of nutrients were calculated (*Table 5.1*). For comparison: the water potential of LS medium with 3% glucose is -6.6 bar.

Table 5.1. Remaining water activity and water potential in the shake flask and fermenter cultures after depletion of glucose and salts.

PEG 600 conc. [g*l^{-1}]	a_w [-]	P [bar]	PEG 6000 conc. [g*l^{-1}]	a_w [-]	P [bar]
shake flask cultures					
111	0.9955	-6.2	44	0.9997	-0.5
133	0.9942	-8.0	89	0.9988	-1.6
156	0.9927	-10.1	133	0.9973	-3.6
178	0.9912	-12.2	156	0.9963	-5.1
200	0.9894	-14.7			
fermenter cultures					
171	0.9917	-11.5	155	0.9963	-5.1

Shake flask experiments

I. Effects of PEG 600 on growth

The cell cultures survived concentrations of PEG 600 up to 200 g*l^{-1}. At all concentrations biomass increased (*Figure 5.1A*) until a maximum between 14 and 20 g*l^{-1} was attained after 7 days.

Glucose was consumed completely within 5 days (data not shown). The initial fresh to dry-weight ratio (FW/DW ratio) of the cells fell sharply from 11 to 3-5 after addition of the stress media and stayed on this value until the end of the experiment (*Figure 5.1B*). This observation illustrates that stress imposed by all PEG concentrations kept the cells small. The control experiment performed with LS medium without PEG 600 showed a rapid increase of FW/DW ratio from 8 after 2 days to 19 after 12 days. Viability tests by FDA vital staining performed after 12 days revealed clearly a detrimental effect of PEG 600 at concentrations of 178 and 200 g*l^{-1}. Loss of viability was 70-90%.

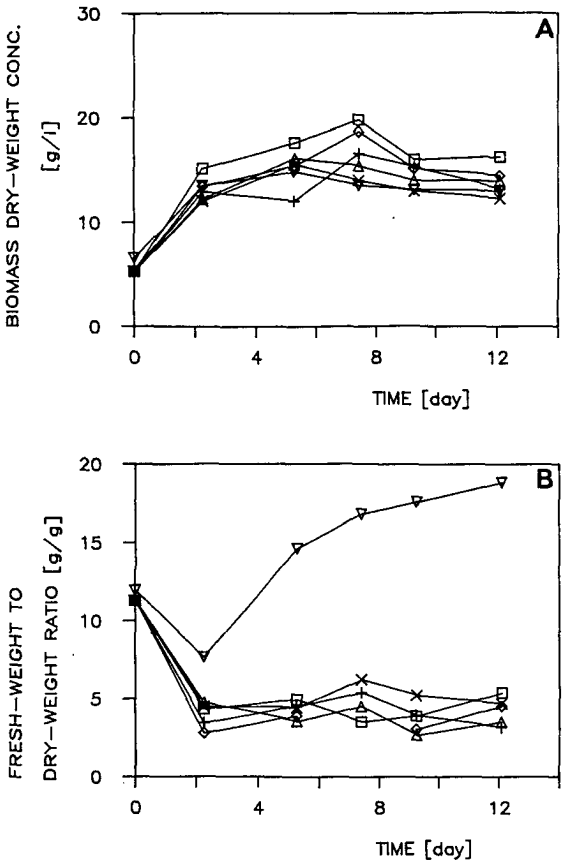

Figure 5.1A,B. Growth by a *C. roseus* suspension culture in shake flasks on LS medium supplemented with various PEG 600 concentrations.
A. Biomass dry-weight [$g*l^{-1}$]. **B.** Biomass fresh-weight to dry-weight ratio [-]. (▽) control; (□) 111; (+) 133; (◇) 156; (△) 178; (×) 200 $g*l^{-1}$

II. Effects of PEG 600 on secondary metabolism

After 12 days the cells had accumulated 0.05-0.4 $mg*gDM^{-1}$ ajmalicine and 0.003-0.05 $mg*gDM^{-1}$ serpentine (*Figure 5.2A* and *5.2B*). In the cell free medium of the stressed cultures ajmalicine concentrations of 11-42 $mg*l^{-1}$, and serpentine concentrations of 0.3-1.4 $mg*l^{-1}$ were found (*Figure 5.2A* and *5.2B*, see also *Table 5.2* at the end of this paragraph).

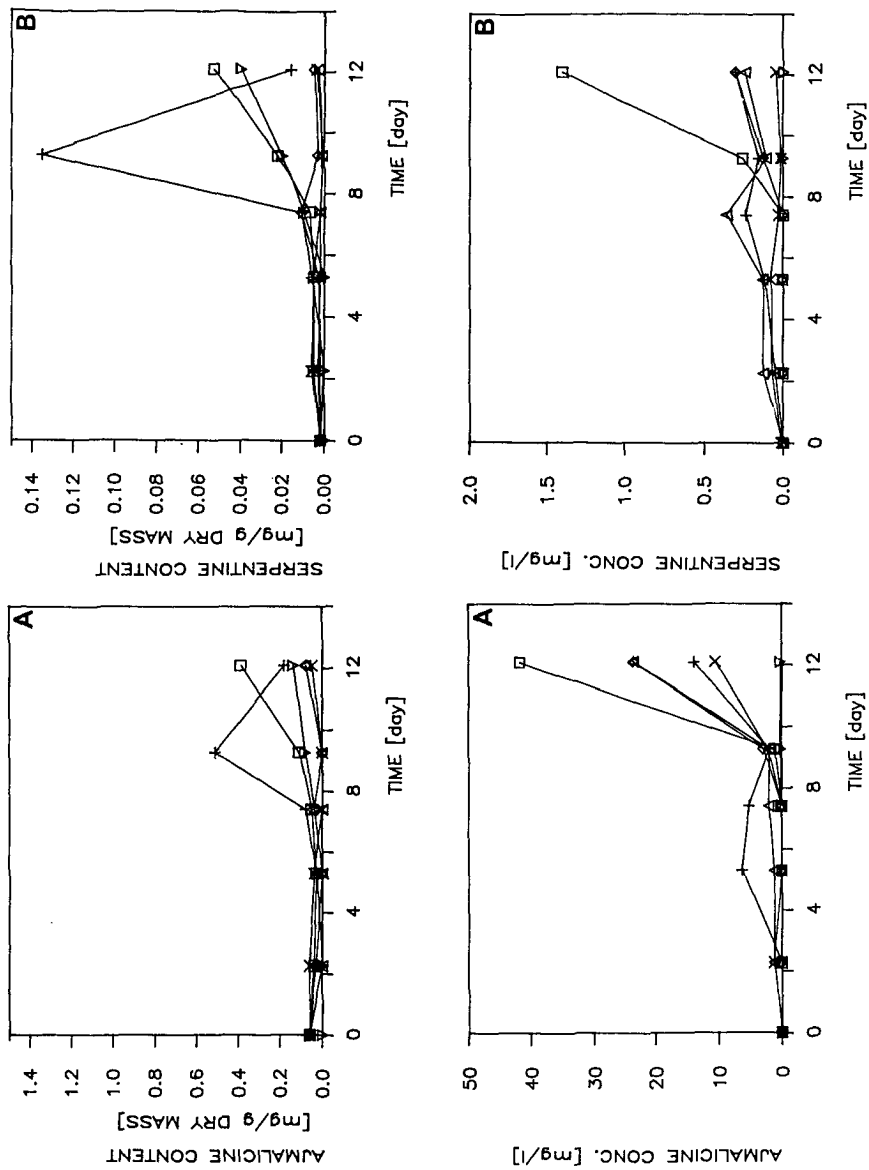

Figure 5.2A,B. Alkaloids accumulated in biomass and released into medium by a *C. roseus* suspension culture grown in shake flasks on LS medium supplemented with various PEG 600 concentrations. **A.** ajmalicine in biomass [mg*gDM^{-1}], and in medium [mg*l^{-1}]. **B.** serpentine in biomass [mg*gDM^{-1}], and in medium [mg*l^{-1}].
(▽) control; (□) 111; (+) 133; (◇) 156; (△) 178; (×) 200 g*l^{-1}

These results show that ajmalicine was the predominant alkaloid formed under these conditions, the major part of it was released into the medium. Cells grown on medium without PEG accumulated 0.14 $mg*gDM^{-1}$ ajmalicine and 0.04 $mg*gDM^{-1}$ serpentine, and both alkaloids were not found in cell free medium.

It is hard to decide whether there was an 'optimal' PEG 600 concentration. The results suggest an optimal concentration of 111 $g*l^{-1}$. However, these results were not verified by a duplicate experiment. So the finding of an 'optimal' concentration of could be a mere coincidence. One should be aware of the fact that each data point in the course of the experiments, owing to the experimental procedure, was the result of sampling an independently growing culture. For this reason results can diverge considerably. The total alkaloid concentration (ajmalicine and serpentine in biomass and medium added together) varied between 12-47 $mg*l^{-1}$ (culture) after 12 days at an average biomass dry-weight concentration of 14 $g*l^{-1}$. From these data an average alkaloid production rate of about 0.2 $mg*g^{-1}*day^{-1}$ can be calculated.

III. Effects of PEG 6000 on growth

The effects of various concentrations of PEG 6000 ranging from 44 to 156 $g*l^{-1}$ on growth of *C. roseus* cell suspensions are shown in *Figure 5.3.* After addition of the media biomass rapidly increased until a dry-weight of 15-17 $g*l^{-1}$ was reached after 5 days (*Figure 5.3A),* glucose was consumed entirely within 5 days (results not shown). The initial FW/DW ratio of 11 fell after addition of the stress media and attained 6-8 after 5 days (*Figure 5.3B).* Comparison with the control experiment reveals a distinct effect of PEG 6000 on the FW/DW ratio. PEG 6000 apparently kept the cells small, although the effect was not as definite as with PEG 600.

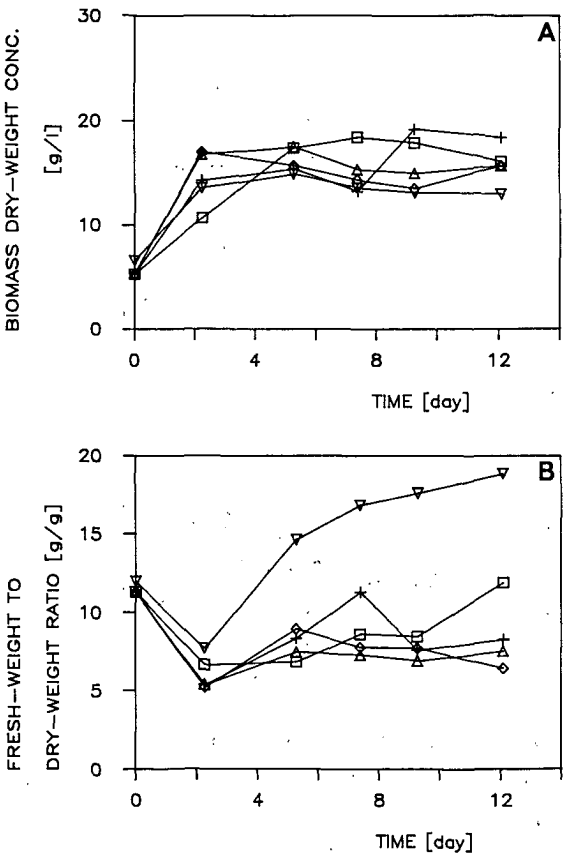

Figure 5.3A,B. Growth by a *C. roseus* suspension culture in shake flasks on LS medium supplemented with various PEG 6000 concentrations.
A. Biomass dry-weight [$g*l^{-1}$]. **B.** Biomass fresh-weight to dry-weight ratio [-]. (▽) control; (□) 44; (+) 89; (◇) 133; (△) 156 $g*l^{-1}$ PEG 6000

IV. Effects of PEG 6000 on secondary metabolism

After 12 days the stressed cells had accumulated 0.5-1.1 mg*gDM^{-1} ajmalicine and 0.02-0.06 mg*gDM^{-1} serpentine (*Figure 5.4A* and *5.4B*, see also *Table 5.2* at the end of this paragraph).

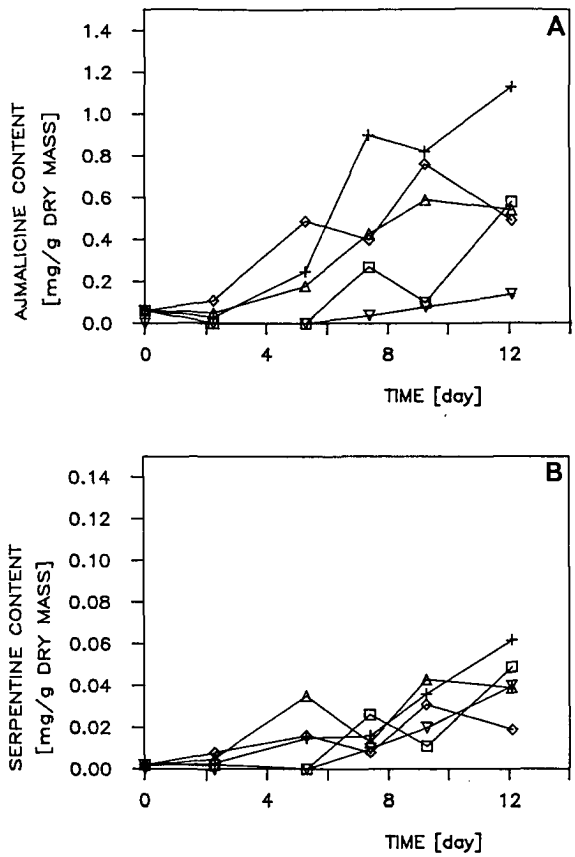

Figure 5.4A,B. Alkaloids accumulated in biomass by a *C. roseus* suspension culture grown in shake flasks on LS medium supplemented with various PEG 6000 concentrations. A. ajmalicine [mg*gDM^{-1}]. B. serpentine [mg*gDM^{-1}]. (▽) control; (□) 44; (+) 89; (◇) 133; (△) 156 g*l^{-1} PEG 6000

Low concentrations of ajmalicine were found in the medium after 7 days (≈4 mg*l^{-1}), after 12 days, however, no ajmalicine could be detected (data not shown) . Extracellular serpentine was hardly detectable (≤ 0.1 mg*l^{-1},

results not shown). Ajmalicine was the predominant alkaloid formed by *C. roseus* under these conditions. However, in contrast to the experiments performed with PEG 600 as stressor, the cells had accumulated the alkaloids instead of released them into the medium. The average alkaloid production rate of the PEG 6000 cultures was 0.07 $mg*g^{-1}*day^{-1}$.

Summary of shake flask experiments

The experimental results are briefly summarized in *Table 5.2.*

Table 5.2. Alkaloids in biomass and medium, and biomass fresh-weight to dry-weight ratio after 12 days of shake flask cultures of *C. roseus* grown on LS medium supplemented with various concentrations PEG 6000 or PEG 600.

		BIOMASS		MEDIUM		
PEG conc. $[g*l^{-1}]$	P [bar]	ajmalicine $[mg*gDM^{-1}]$	serpentine $[mg*gDM^{-1}]$	ajmalicine $[mg*l^{-1}]$	serpentine $[mg*l^{-1}]$	FW/DW [-]
0	0	0.14	0.04	0.3	0	18.9
PEG 6000						
44	-0.5	0.58	0.05	0	0	11.9
89	-1.6	1.13	0.06	0	0	8.3
133	-3.6	0.49	0.02	0	0	6.4
156	-5.1	0.54	0.04	0	0	7.5
PEG 600						
111	-6.2	0.39	0.05	41.8	1.4	5.4
133	-8.0	0.18	0.02	14.0	0.3	3.2
156	-10.1	0.08	0.005	23.5	0.3	4.6
178	-12.2	0.08	0.003	23.4	0.3	3.5
200	-14.7	0.05	0.004	10.6	0.3	4.7

Fermenter experiments

In order to duplicate results obtained from the previously described experiments in shake flasks, experiments on 2-l scale in a fermenter were performed. Fermenter experiments not only facilitate culturing on a far larger scale than attainable with shake flasks, but also allow frequent sampling of one and the same culture during a considerable time period. Serious drawbacks of shake flask experiments, as discussed previously, can thereby be circumvented.

I. Effects of PEG 600 on growth

Unintentionally too much stress medium with PEG 600 was added to the culture. Instead of ≈100-120 $g*l^{-1}$ a final concentration of 171 $g*l^{-1}$ was attained. In the shake flask experiments this concentration resulted in a high loss of viability. After addition of the stress medium growth nearly ceased, although glucose uptake was considerable (*Figure 5.5A*).

Microscopic examination revealed plasmolysis of nearly all the cells and damage to a major part, there were no signs of cell division. O_2-consumption and CO_2-production rate, which are reliable indicators of culture viability, also decreased (data not shown) However, after 9 days, the culture showed recovery as respiration was increasing. After 18 days a biomass dry-weight concentration of 10 $g*l^{-1}$ was attained. The FW/DW ratio of the cells dropped sharply from 18 just before addition of the stress medium to 8 just after addition. After 5 days a FW/DW ratio of 5 was attained, from that point it gradually increased to 9 after 26 days (*Figure 5.5B*).

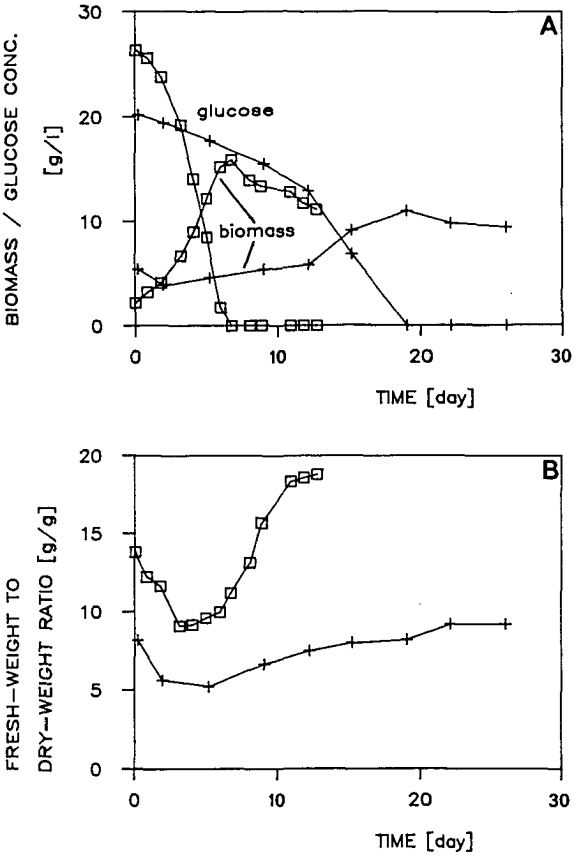

Figure 5.5A,B. Growth by a *C. roseus* suspension culture in a 3-l fermenter on LS medium supplemented with PEG 600 (final concentration 171 $g*l^{-1}$) and LS medium without PEG (control). **A.** biomass dry-weight; glucose [$g*l^{-1}$]. **B.** biomass fresh-weight to dry-weight ratio [-].
(□) control; (+) 171 $g*l^{-1}$ PEG 600

II. Effects of PEG 600 on secondary metabolism

The determination of alkaloid content of the biomass showed that only trifling amounts of both ajmalicine and serpentine had accumulated (≤ 0.03 $mg*gDM^{-1}$, results not shown). Small amounts of ajmalicine (3.5 $mg*l^{-1}$ after 15 days) and trace amounts of serpentine (≤ 0.2 $mg*l^{-1}$) were released into the medium (*Figure 5.6*).

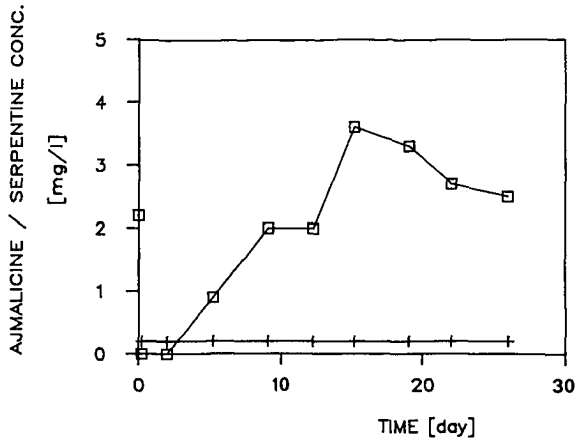

Figure 5.6. Alkaloids released into medium by a *C. roseus* suspension culture grown in a 3-l fermenter on LS medium supplemented with PEG 600 (final concentration 171 $g*l^{-1}$). (□) ajmalicine; (+) serpentine [$mg*l^{-1}$]

In contrast to the shake flask experiments the presence of PEG 600 in the medium did not result in an enhancement of secondary metabolite formation. The results show a detrimental effect of PEG 600 on the growth potential and secondary product formation of *C. roseus* grown in a fermenter.

III. Effects of PEG 6000 on growth

After addition of the stress medium with PEG 6000 (final concentration 155 $g*l^{-1}$) rapid growth was accompanied by rapid uptake of glucose (*Figure 5.7A*). After 5 days a maximum biomass dry-weight concentration of 23 $g*l^{-1}$ was attained that slowly decreased to 17 $g*l^{-1}$ after 21 days. After 28 days dry-weight attained 21 $g*l^{-1}$. This sudden rise in dry-weight could be attributed to PEG 6000 penetrating the cells due to permeabilization of the cell membrane. By microscopic examination it was observed that the refraction of the major part of the cells was identical to that of the surrounding medium. This indicated equal concentration of PEG 6000 in medium and cells. The FW/DW ratio of the cells fell slightly from 11 to 8 after 4 days when glucose was depleted. From that point it rose linearly to 18 after 18 days (*Figure 5.7B*).

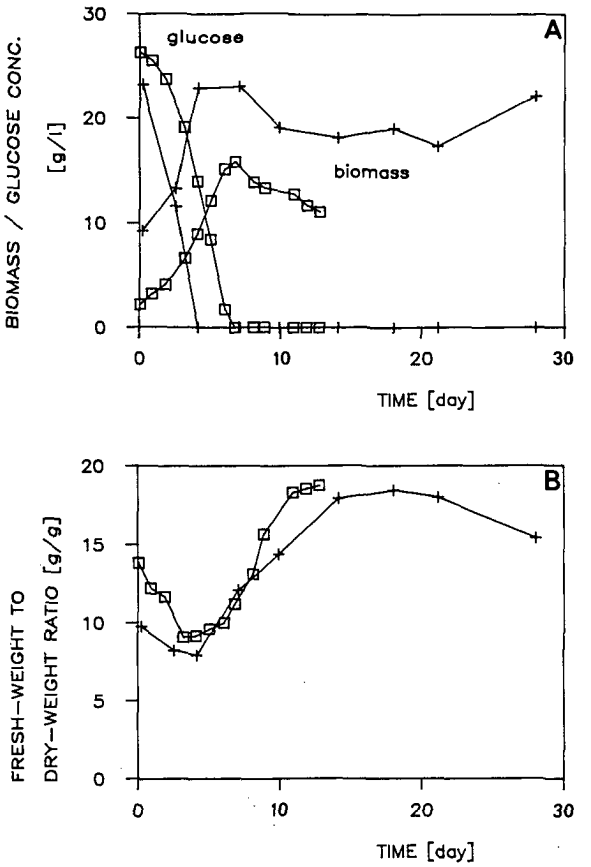

Figure 5.7A,B. Growth by a *C. roseus* suspension culture in a 3-l fermenter on LS medium supplemented with PEG 6000 (final concentration 155 $g*l^{-1}$), and LS medium without PEG (control).
A. biomass dry-weight; glucose $[g*l^{-1}]$. **B.** biomass fresh-weight to dry-weight ratio [-]. (□) control; (+) 155 $g*l^{-1}$ PEG 6000

IV. Effects of PEG 6000 on secondary metabolism

Determination of ajmalicine and serpentine content in biomass and medium showed accumulation of only trace amounts of serpentine in biomass ($\leq$ 1 $\mu g*gDM^{-1}$, results not shown) and no release at all of alkaloids into the medium as they were not detectable ($<$ 0.1 $mg*l^{-1}$, results not shown). In the control fermenter experiment on LS 3% glucose no ajmalicine or serpentine could be detected in both biomass and medium.

In contrast to the shake flask experiments the presence of PEG 6000 in the medium did not result in enhancement of accumulation of ajmalicine in the biomass.

Discussion

Both PEG 600 and PEG 6000 had a stimulating effect on secondary metabolism of *C. roseus* grown in shake flasks. Alkaloid yields were similar in magnitude. It is not clear from these experiments to which effect the stimulation of product formation should be attributed. When there would be a relationship between exerted osmotic pressure and product formation rate the PEG 6000 cultures would have yielded far less product. It is therefore likely that stimulation of secondary metabolism by the PEGs used in this study was an effect of chemical stress.

The presence of PEG 600 in the medium resulted in nearly complete release of ajmalicine to the medium, whereas PEG 6000 had no releasing effects. PEGs have been shown to have a weak surfactant activity (Hopwood, 1981), dehydrate membrane bilayers (Arnold *et al.*, 1985), and make membranes abnormally permeable to ions and molecules (Aldwinckle *et al.*, 1982). It has also been shown that permeabilization of the plasmalemma and the tonoplast resulted in release of alkaloids from *C. roseus* cells (Brodelius and Nillson, 1983). Besides, surfactants like Triton X-100 have been shown to facilitate release of indole alkaloids by *C. roseus* with preserved viability (Brodelius, 1988). The difference in releasing effect between PEG 600 and PEG 6000 could be explained by the difference in penetrating potential of both PEGs. It is likely that PEG 600 molecules, due to their small size, can penetrate the plant cells and serve as extractant for the alkaloids from the vacuole.

The results from the shake flask experiments could not be duplicated by fermenter experiments. Growth was retarded considerably by PEG 600, alkaloid production was not stimulated by both PEGs. We are of the opinion that they can be explained by considering the differences between the cultivation systems used.

Firstly, with regard to growth, it is conceivable that the combination of osmotic stress by PEG 600 and hydrodynamic stress generated by the impeller damaged the major part of the cells in the fermenter. In

contrast, hydrodynamic stress in a the shake flasks was nearly absent. It has been shown that this *C. roseus* cell line can withstand short-term and long-term stress in a comparable set-up (**Chapter 3, 4**). Nevertheless, it is likely that plant cells become susceptible to hydrodynamic stress during rapid plasmolysis. As has been shown by the fermenter experiment PEG 6000 does not impede the proliferation of the cells. PEG 6000 has, due to it's high molecular weight, no significant osmotic effect on the cells and thus there will be no synergistical effect with hydrodynamic stress. However, in spite of the absence of any adverse effect on the cells, no alkaloids were formed.

Secondly, the gas exchange in the shake flask was quite different from that in the fermenter. In a shake flask gas exchange is poor, due to the diffusion barrier formed by the commonly used shake flask closure like aluminium foil and silicone foam stoppers (ten Hoopen, 1989). This will result in accumulation of gaseous metabolites as carbon dioxide and ethylene to concentrations that might have physiological effects, especially stimulation of secondary metabolism. In plants it has been found that a variety of environmental stresses rapidly induce ethylene production in plants. Ethylene triggers response mechanisms, among others things secondary metabolism (Liebermann, 1979; Ecker and Davis, 1987). Enhancement of secondary metabolites synthesis by ethylene has already been found with plant cell cultures of *Coffea arabica* and *Thalictrum rugosum* (Cho *et al.*, 1988). A fermenter is usually aerated by a fresh air stream, resulting in very low concentrations of gaseous metabolites, consequently secondary metabolism may be repressed. Scragg *et al.* (1987) studied the effect of scale-up on serpentine formation by *C. roseus* suspension cultures. They found very low serpentine formation when their cell lines were grown in air-lift bioreactors when compared with shake flasks.

The difference in productivity between shake flask and fermenter might therefore be attributed to a difference in gas exchange of both culture systems. Ethylene produced as a response on the chemical/osmotic stress generated by PEG 600 and PEG 6000 could have accumulated to physiological concentrations in the shake flask cultures. This may have triggered secondary metabolism. Because this conclusion is rather speculative, it should be substantiated by further experimental work. When substantiated it would complicate the scale-up of some plant cell production processes considerably. Until now most research has been carried out in shake

flasks, whereas large-scale processes will be carried out in fermenters. Duplicating promising shake flask experiments as quick as possible in a small-scale fermenter is therefore advisable.

List of symbols

a_w	water activity	-
K	correlating constant	-
P	water potential	$N*m^{-2}$ or bar
R	gas constant	$J*K^{-1}*mol^{-1}$
T	temperature	K
X	molar fraction	-

CHAPTER 6.

GENERAL DISCUSSION

When this research work was started by late 1984 to study the effects of hydrodynamic stress ('shear stress') on plant cells in suspension culture little information was available on this subject. Initially we shared the same opinion about the shear-sensitivity of plant cells as most other workers within this field: plant cells are (inherently) shear-sensitive, and therefore cultivation in impeller-stirred bioreactor will be infeasible, or at least problematic.

However from the work of Fowler (1982) and Scragg *et al.* (1986) we learned that cell lines of *Catharanthus roseus* appeared to be less shear-sensitive than was initially assumed. In the meanwhile we managed to cultivate *C. roseus* suspension cultures in turbine-stirred lab-scale bioreactors at moderate (1-3 s^{-1}) agitation speeds in batch culture as well as in chemostat culture. In the middle of 1986 a small (student) research project was performed in which the influence of the agitation speed on the growth of *C. roseus* was studied. It appeared that our cell line could be batch cultivated in a 2-l turbine stirred fermenter at 16.7 s^{-1} without any problem. In the meanwhile chemostat cultures of *C. roseus* were performed in which the agitation speed was 16.7 s^{-1}. *C. roseus* appeared to be capable of sustaining short-term (in batch culture) as well as long-term (in a chemostat) hydrodynamic stress.

These results urged to reconsider our view on the shear-sensitivity of plant cells. In order to test whether the shear-tolerance of *C. roseus* was an unique feature of this cell line, three other cell lines used in our project group were tested on their shear-tolerance: *Nicotiana tabacum, Tabernaemontana divaricata,* and *Cinchona robusta.* The cell line of *N. tabacum* appeared to be shear-tolerant under short-term hydrodynamic stress. The two other cell lines appeared to be less shear-tolerant, although they could be cultivated in a 3-l turbine-stirred fermenter at a moderate agitation speed of 4.2 s^{-1}.

From the experimental results it is not possible to give an explanation for the divergence in shear-tolerance of the cell lines. However, there might be some trivial explanations. Firstly, it can be hypothesized that the stability of a cell line affects its shear-tolerance, in a sense that stable lines that are being kept for some years or more are inherently shear-tolerant. *C. roseus*, *N. tabacum*, and *T. divaricata* are stable cell lines that were initiated more than 3 years ago, *C. robusta* was initiated recently. Scragg *et al.* (1988) found that the shear-tolerance of *Picrasma quassioides* cultures increased as culture growth improved. However, a recently developed culture of *C. roseus* appeared to be shear-tolerant to.

Secondly, the methods for maintenance and subcultivation of the stock cell lines could affect shear-sensitivity, in a sense that circumstances preventing optimal growth would induce weakness of the cells. In our laboratory *C. roseus* had been maintained as a stable cell line for more than a year when we started the shear experiments. *N. tabacum*, *T. divaricata*, and *C. robusta* were imported from Leiden University just prior to the experiments. *N. tabacum* was easily adapted to our maintenance and propagation methods. Both *T. divaricata* and *C. robusta* gave some complications. We did not succeed in obtaining lines with a stable subcultivation regime.

The fact that most workers within this field found their cell lines shear-sensitive would lead to the conclusion that most cell lines were kept under sub-optimal conditions. After careful examination of the materials and methods and experimental results from a number of papers on this subject one concludes that there are reasons to assume that most methods of maintenance and propagation have been inadequate. Often shake flask closures with a very low gas permeability - like aluminium foil - were applied, resulting in oxygen limited growth and build-up of high concentrations of potentially toxic gaseous metabolites. In some cases growth curves of cultures appeared to be linear instead of exponential, indicating oxygen limitation, or growth ceased when excess carbon was present indicating limitation of a vital nutrient.

This study shows that the general opinion that plant cells are inherently shear-sensitive should be reconsidered. Future research should be focused on circumstances determining the shear-sensitivity of a cell

line. To understand the conditions that govern the shear-tolerance of a plant cell much more research should be focused on the build-up and properties of cell wall and cell membrane. With this knowledge it should be possible to transform delicate cell lines into robust lines capable of growth in impeller-stirred vessels.

Chemical/osmotic stress can play a role in stimulating secondary product formation by cultured plant cells as has been shown in Chapter 5. However, the mechanism by which chemical/osmotic stress affects secondary metabolism is not clear. It can be speculated that stress triggers the formation of a stress hormone like ethylene, which in its turn effects the onset of secondary metabolism.

In the shake flask experiments production of considerable amounts of ajmalicine was found. With PEG 600 as stressor the majority of it was released in the medium. In the fermenter experiments the production was very low. It is likely that this finding can be attributed to the difference in gas exchange of the culture vessels employed in the experiments. In the shake flasks the volatile stress hormone accumulated due to poor gas exchange, in the fermenters it was driven out by aeration. The most important conclusion from the study of the effects of chemical/osmotic stress on plant cells is: Promising results obtained by shake flask experiments should always be verified in the down-scaled version of the large-scale production bioreactor (in this case an aerated impeller-stirred tank).

SUMMARY

The objective of the research described in this thesis was to study the effects of hydrodynamic and chemical/osmotic stress on plant cells in a stirred bioreactor. The study was carried out within the framework of the cooperation between Delft University of Technology and Leiden University in the field of biotechnology (BDL) in the project group Plant Cell Biotechnology. The aim of this project is to acquire knowledge and expertise for the development of large-scale processes for the production of valuable compounds by plant tissue cultures.

In **Chapter 1** some aspects of the large-scale cultivation of plant cells for the production of secondary metabolites are treated: process design, regime analysis, and assessment of hydrodynamic stress parameters. Starting from a hypothetical production process the conventional stirred tank bioreactors to be used for this process are calculated using data obtained by experimental work in our project group, data from literature, and reasonable assumptions. A regime analysis is performed on the largest fermenter of the process (25 m^3). The same procedure is applied to a geometrically similar down-scaled 5-l fermenter under conditions of constant power input or impeller tip speed with respect to the 25 m^3 reactor. Hydrodynamic stress parameters are assessed for both the industrial-sale and the lab-scale fermenter.

In **Chapter 2** a review is given on the methods for the assessment of hydrodynamic stress sensitivity of plant cells. It appears that this subject has seldom been studied, this in contrast to shear-sensitivity of mammalian cells and hybridomas. The main conclusion of this chapter is that experiments to assess hydrodynamic stress sensitivity should preferably be studied in a down-scaled version of the bioreactor which has been chosen for a large-scale process.

In **Chapter 3** results are presented of experiments that were aimed at studying the effects of short-term hydrodynamic stress on plant cells in suspension culture. *Catharanthus roseus*, *Nicotiana tabacum*, *Tabernaemontana divaricata*, and *Cinchona robusta* were batch-cultivated in a 3-l fermenter at various agitation speeds. *C. roseus* and *N. tabacum* appeared to be unaffected by hydrodynamic stress generated by a 4.5 cm turbine impeller at a rotational speed of 16.7 s^{-1}. *T. divaricata* and *C. robusta* appeared to be more sensitive to shear, although they could be cultivated

under moderate stress.

In **Chapter 4** experiments are described to study the effects of long-term hydrodynamic stress on *C. roseus*. This cell line was cultivated in a chemostat modified for the cultivation of plant cells. *C. roseus* was cultivated in this chemostat at an agitation speed of 16.7 s^{-1} during several months. Comparison with experiments performed under considerably lower stress showed no distinct impact on growth or metabolism by long-term hydrodynamic stress.

The experiments described in **Chapter 5** show the feasibility of stimulation of secondary metabolism in *C. roseus* by chemical/osmotic stress exerted by polyethylene glycol 600 and 6000. There appeared to be a discrepancy between experiments carried out in shake flasks and fermenters. Stimulation of production of ajmalicine in shake flasks could not be reproduced in a fermenter. Presumably this is owing to the difference in gas exchange of both culture systems. The plant hormone ethylene that can stimulate secondary metabolism may have accumulated in the shake flasks where gas exchange was primarily by diffusion. In the fermenters that were aerated no accumulation occurred, so no stimulation took place. These results indicate the need to check as soon as possible findings from shake flask experiments by fermenter experiments. Besides, they show the dangers of defining process conditions from shake flask research.

The fresh-weight to dry-weight ratio of the biomass appeared to decrease by a high osmotic value of the medium. This finding could play a role in attaining higher biomass concentrations, which could benefit the economic feasibility of a process.

In **Chapter 6** the results of this study are generally discussed. The main conclusion of this work is that plant cells are less sensitive to hydrodynamic stress than currently has been assumed. Moreover, the methods for maintenance and propagation of cell lines could determine its shear-sensitivity or shear-tolerance. Future research should be aimed at the circumstances determining shear-tolerance as: build-up and properties of cell wall and the ability to recuperate. Besides, the possible relationship between shear-tolerance, ease of cryopreservation and osmo-tolerance should be investigated.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek was gericht op het bestuderen van de effecten van hydrodynamische en chemi/osmotische stress op plantecellen gekweekt in een geroerde bioreactor. Het onderzoek werd uitgevoerd in het kader van het samenwerkingsverband van de Technische Universiteit Delft en de Rijksuniversiteit Leiden op het gebied van de biotechnologie (BDL) binnen de projectgroep Plantecelbiotechnologie. Het doel van dit project is om voldoende kennis en expertise te verwerven voor het opzetten van grootschalige processen voor de productie van waardevolle chemicaliën met behulp van plantenweefselculturen.

In **Hoofdstuk 1** worden een aantal aspecten van de grootschalige kweek van plantecellen voor de productie van secundaire metabolieten behandeld: procesontwerp, regime analyze, en schatting van hydrodynamische stress parameters. Uitgaande van een hypothetisch productieproces worden aan de hand van binnen de projectgroep aanwezige kennis, literatuurgegevens en redelijke aannamen de voor dit proces benodigde fermentoren berekend. Op de grootste fermentor van het proces (25 m^3) wordt een regime analyze uitgevoerd. Hetzelfde wordt uitgevoerd voor een geometrisch gelijkvormig schaalverkleinde 5-l fermentor voor de condities van gelijkblijvende power input en tipsnelheid van de roerder t.o.v. de 25 m^3 reactor. Voor zowel de *productieschaal* als de *labschaal fermentor* worden de *hydrodynamische* stress parameters geschat.

In **Hoofdstuk 2** wordt een overzicht gegeven van de literatuur over de methoden voor het bepalen van de gevoeligheid van plantecellen voor hydrodynamische stress. Het blijkt dat dit onderwerp bij plantecellen zelden uitvoerig bestudeerd is, dit in tegenstelling tot dierlijke cellen en hybridomas. Er wordt geconcludeerd dat experimenten om shear-gevoeligheid te bepalen het best kunnen worden uitgevoerd in een schaalverkleinde versie van de bioreactor die men voor ogen heeft voor een grootschalig proces.

In **Hoofdstuk 3** worden de resultaten van experimenten gepresenteerd die tot doel hadden de effecten van kortdurende hydrodynamische stress op plantecellen in suspensie te bestuderen. *Catharanthus roseus, Nicotiana tabacum, Tabernaemontana divaricata* en *Cinchona robusta* werden gekweekt als batchcultuur in een 3-l fermentor bij verschillende omwentelingssnelheden van de roerder. *C. roseus* en *N. tabacum* bleken ongevoelig voor

hydrodynamische stress opgewekt door een 4.5 cm turbineroerder bij een roersnelheid van 16.7 s^{-1}. *T. divaricata* en *C. robusta* bleken gevoeliger te zijn voor shear-stress. Ze konden echter nog bij een matige stress gekweekt worden.

In **Hoofdstuk 4** worden experimenten beschreven waarin de effecten van langdurige hydrodynamische stress op *C. roseus* werden bestudeerd. Deze cellijn werd gekweekt in een chemostat die speciaal voor het kweken van plantecellen was gemodificeerd. Het bleek mogelijk *C. roseus* gedurende ongeveer 2 maanden te kweken bij een toerental van 16.7 s^{-1}. Vergelijking met experimenten uitgevoerd bij een aanmerkelijk lagere hydrodynamische stress laten zien dat er geen duidelijke beinvloeding van de groei of het metabolisme optrad.

De in **Hoofdstuk 5** beschreven experimenten tonen aan dat stimulering van het secundair metabolisme van *C. roseus* door chemi/osmotische stress mogelijk is. Bij de experimenten werden polyethyleenglycol 600 en 6000 gebruikt als stressors. Er bleek een discrepantie te zijn tussen experimenten die in een schudkolf en een fermentor werden uitgevoerd. Stimulering van de productie van ajmalicine, die overtuigend optrad in de schudkolven, kon niet in de fermentorexperimenten worden gereproduceerd. Waarschijnlijk is dit te wijten aan het verschil in gasuitwisseling van beide kweeksystemen. Het plantehormoon ethyleen dat stimulatie van het secundair metabolisme kan bewerkstelligen hoopte zich waarschijnlijk op in de schudkolven. In de beluchtte fermentoren vond geen ophoping plaats en daardoor geen stimulering. De resultaten onderstrepen de noodzakelijkheid om de uitkomsten van schudkolfexperimenten zo snel mogelijk te verifiëren met fermentor experimenten. Bovendien laten ze het gevaar zien van het vaststellen van proces omstandigheden op grond van schudkolf experimenten.

De versgewicht/drooggewicht verhouding van de biomassa werd verkleind door een hoge osmotische waarde van het medium. Dit gegeven zou van belang kunnen zijn voor het bereiken van hogere biomassa-concentraties, hetgeen de economische haalbaarheid van een proces ten goede komt.

In **Hoofdstuk 6** worden de resultaten van het onderzoek besproken. De belangrijkste conclusie van het onderzoek is dat plantecellen waarschijnlijk veel ongevoeliger zijn voor hydrodynamische stress dan wordt aangenomen. Bovendien is de manier waarop plantecelcultures worden aangehouden misschien van invloed op het ontstaan van een shear-gevoelige of een shear-ongevoelige lijn. Toekomstig onderzoek zal zich dan ook moeten

richten op de factoren die de shear-tolerantie van een plantecel bepalen, zoals opbouw en eigenschappen van de celwand en het recuperatievermogen van de cel(lijn). Bovendien moet onderzocht worden of er een verband bestaat tussen shear-tolerantie, cryopreserveerbaarheid en osmo-tolerantie van een cellijn.

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CURRICULUM VITAE

Op 28 juni 1958 ben ik te Den Haag geboren. Na de Gymnasium-β opleiding gevolgd te hebben aan het Christelijk Gymnasium Sorghvliet te Den Haag, ben ik in 1977 Scheikundige Technologie gaan studeren aan de Technische Hogeschool te Delft. In 1982 heb ik mijn kandidaatsexamen afgelegd en ben me gaan specialiseren in de Algemene en Toegepaste Microbiologie met als bijvak Analytische Chemie. Het afstudeerwerk werd uitgevoerd bij de (toenmalige) vakgroep Algemene en Toegepaste Microbiologie onder leiding van dr. J.P. van Dijken, met als afstudeerdocent prof. dr. J.G. Kuenen. Van juni 1982 tot december 1984 was ik studentassistent bij de onderwijswerkgemeenschap Chemie en Maatschappij. Op 20 november 1984 studeerde ik af.

Na mijn afstuderen kreeg ik per 1 december 1984 een aanstelling voor vier jaar als wetenschappelijk assistent bij de vakgroep Moleculaire Plantkunde van de subfaculteit Biologie van de Rijksuniversiteit Leiden. Tijdens mijn onderzoeksperiode was ik gedetacheerd bij de vakgroep Bioprocestechnologie van de faculteit der Scheikundige Technologie en der Materiaalkunde van de Technische Universiteit Delft.