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SURFACE IMMOBILIZATION OF PLANT CELLS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

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SURFACE IMMOBILIZATION OF PLANT CELLS

JEAN ARCHAMBAULT

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SUMMARY

A novel technique was developed to immobilize plant cells. The cells are deposited on a surface of a man-made fibrous material which provides for strong binding of the plant tissue biomass growing in the submerged culture. It was shown that the plant cells need to be fully viable for the attachment process to occur. Relatively uniform biomass loadings of up to 20 mg d.w. of plant cells per cm^2 of support material, which corresponds to a wet biofilm thickness of ~ 5 mm, were attained in flask cultures.

The scale up of this technique to specifically designed laboratory size bioreactors was performed successfully. The immobilizing matrix was formed into a vertically wound spiral. This configuration resulted in a high immobilizing area-to-volume ratio (0.8-1.2 cm^{-1}). A modified airlift (riser-to-downcomer area ratio of 0.03 and vessel height-to-diameter (H/D) ratio of 3) and a low H/D (~ 1.5) mechanically stirred vessel delivered a best bioreactor performance characterized by low biomass frothing and highly efficient plant cell attachment and retention (≥ 96%).

The growth of Catharanthus roseus plant cells investigated in these bioreactors was found not to be mass

transfer limited. It required mild mixing and aeration levels ($k_La \sim 10-15\ hr^{-1}$). The biomass formation pattern of surface immobilized plant cells (SIPC) generally exhibited a linear growth phase followed by a stationary phase characterized by remaining carbohydrates in the medium, contrary to suspension cultures. This culture behaviour was found to depend on the plant cell type and/or line cultured as well as on the inoculum age. The culture space restriction and unidirectional growth of the SIPC biofilm combined with the limited intracellular availability of essential nutrients rapidly accumulated from the medium by the stationary phase inoculated plant cells are all likely contributing to the culture behaviour.

The gentle surface immobilization technique developed in this work did not hinder the biosynthesis potential of SIPC. In fact, it appeared to induce a partial secretion of some valuable compounds into the culture medium. The mildness, easiness, efficiency, mass transfer characteristics, scale up potential and biomass loading capacity (11-13 g d.w/L) of the surface immobilization technique make it superior to all other immobilization techniques used to culture plant cells. In addition, the SIPC bioreactor overall biomass capacity compares favourably with suspended plant cell concentrations attainable in conventional bioreactors (15-20 g d.w./L).

SOMMAIRE

Une nouvelle technique a été développée pour immobiliser des cellules de plante. Les cellules sont déposées sur une surface d'un matériel fibreux synthétique qui assure un attachement solide de la biomasse végétale en croissance submergée. Il a été démontré que les cellules de plante doivent être vivantes pour que le processus d'attachement se produise. Des chargements en biomasse relativement uniformes jusqu'à 20 mg de matière végétale sèche par cm^2 de matériel de support, ce qui correspond à un biofilm d'une épaisseur de ~ 5 mm, furent obtenus par culture en flacons.

La mise à l'échelle de cette technique dans des bioréacteurs de laboratoire spécifiquement conçus à cet effet a été réalisée avec succès. La matrice d'immobilisation était formée en spirale verticale. Cette configuration a permis d'offrir une grande surface d'immobilisation par rapport au volume de médium du réacteur (0.8-1.2 cm^{-1}). Un réacteur de type airlift modifié (rapport des surfaces d'écoulement ascendante/descendante ~ 0.03 et rapport hauteur/diamètre (H/D) de 3) et un réacteur agité mécaniquement d'un faible rapport H/D (~ 1.5) produisirent les meilleures performances de culture. On y observa de faibles moussages

de la biomasse et son attachement efficace et prolongé (≥ 96%).

La croissance de cellules de la plante Catharanthus roseus étudiée dans ces bioréacteurs n'était pas limitée par le transfert de masse lorsqu'agités et aérés à des niveaux d'intensité moyenne (k_La ~ 10-15 h^{-1}). La formation de la biomasse végétale immobilisée en surface (BVIS) était constituée d'une phase de croissance linéaire suivie d'une phase stationnaire sans consommation complète des carbohydrates du médium contrairement aux cultures en suspension. Il a été déterminé que ce phénomène dépend du type de cellule ou de la souche particulière cultivée ainsi que de l'âge de l'inoculum. Ceci a été attribué à la structure particulière de croissance d'un biofilm (restreinte en espace et unidirectionnelle) ainsi qu'à la disponibilité intracellulaire d'éléments nutritifs essentiels rapidement accumulés du médium par les cellules végétales affamées de l'inoculum stationnaire utilisé.

Cette technique d'immobilisation douce n'a pas nui au potentiel biosynthétique de la BVIS. En fait, cette technique semble stimuler une sécrétion partielle de certains composés précieux dans le médium de culture. La douceur, la facilité, l'efficacité, les caractéristiques de transfert de masse, la possibilité de mise à l'échelle et

le chargement en biomasse possible (11-13 g m.s./L) de la technique d'immobilisation en surface en font une approche supérieure à toutes les autres techniques d'immobilisation utilisées pour cultiver des cellules végétales. De plus, ce chargement en biomasse immobilisée se compare avantageusement aux concentrations en biomasse qui peuvent être atteintes lors de la culture de cellules végétales en suspension dans des bioréacteurs conventionnels (15-20 g m.s./L).

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NOMENCLATURE.

a: Length of matrix spiral configuration (m).

aSP: After stationary phase.

A: Immobilizing surface area (cm^2).

A/V: Immobilizing surface area-to-culture volume ratio (cm^{-1}).

APM: Alkaloid Production Medium (see Appendix 2).

A_R/A_D: Area of riser-to-downcomer ratio of an airlift bioreactor.

A07: Surface immobilizing material.

A09: Surface immobilizing material.

A12: Surface immobilizing material.

b: Growth rate of surface immobilized plant cells (mg d.w./cm^2.d).

b': Growth rate of the surface immobilized plant cell biomass (g d.w./L).

b_E: Estimated growth rate of SIPC (Eq. 3.32) (mg d.w./ cm^2.d).

b_M: Measured growth rate of SIPC (Eq. 3.18) (mg d.w./ cm^2.d).

b_s: Spacing between two immobilizing layers for liquid flow (m).

bSP: Before stationary phase.

BEPP: Breakeven producer price of indole alkaloids ($Cdn 1987).

B_i: Biot number (Eq. 3.22).

B_{i1}: Factor of Equation A1.5.

BVIS: Biomasse végétale immobilisée en surface.

cps: Centipoise (10^{-2} g/cm.s).

C: Concentration of carbohydrate in the biofilm (g/L).

C_m: Concentration of carbohydrate in the medium (g/L).

C_s: Concentration of carbohydrate at the biofilm/medium interface (g/L).

C': Concentration of reacting species in the biofilm (g/L).

C'_s: Concentration of reacting species at the biofilm/medium interface (g/L).

C'_m: Concentration of reacting species in the medium (g/L).

C_i: Concentration of the product inducing compound in the biofilm (g/L).

C_{io}: Minimum contact concentration of the elicitor to the biomass (g/L).

C_{is}: Concentration of the product inducing compound at the medium/biofilm interface (g/L).

C_{mi}: Medium concentration of the inducing compound (g/L).

C_{O_2} : Dissolved oxygen concentration in the liquid phase (g/L).

$C^*_{O_2}$: Dissolved oxygen concentration in the liquid phase at saturation in equilibrium with the gas phase (g/L).

C_D: Drag coefficient (Eq. A5.2).

C_x: Macromolecular compound secreted by SIPC and accumulating at the matrix/cell interface (TEM).

CC: Capital cost of a project ($Cdn 1987).

CW: Cell wall.

CHO: Residual carbohydrate concentration in the medium (g/L).

d: Days.

d.w.: Biomass dry weight (g).

du/dr_1: Shear rate (s^{-1}).

D: Bioreactor internal diameter (m).

D_c: Internal airlift vessel diameter (m).

D_D: Total drag exerted on a flat plate (Eq. A5.3) (N).

D_{eff}: Average effective diffusivity in the biofilm (cm^2/s).

D_L: Average diffusivity in the liquid phase (cm^2/s.)

D_R: Airlift riser diameter (m).

DO: Dissolved oxygen concentration (g/L).

DO^*: Dissolved oxygen concentration in the liquid

phase at saturation in equilibrium with the gas phase (g/L),

DO_f: Final dissolved oxygen concentration in the liquid phase (g/L).

DP: Flow diverting plate.

DPH: Height of flow diverting plate (m).

DW: Net immobilized biomass dry weight attached to an immobilizing structure (g).

e_v: Friction effects to liquid flow at the riser entrance.

f: Proportion of biosynthesized compounds secreted in the biofilm.

f': Friction factor of liquid flow in the riser tube.

g: Gravity constant (m/s^2).

G_L: Liquid mass flux in the riser tube ($kg/m^2.s$).

H: Height of bioreactor (m).

H_L: Liquid height in bioreactor (m).

HPLC: High Performance Liquid Chromatograph.

I_o: Inoculated biomass (g).

IA: Designation of spiral wound matrix/airlift bioreactor.

IA-i: Experiment i performed in Bioreactor IA.

IAA: Auxin hormone Indole-3-Acetic Acid.

ID: Inside diameter (m).

IP: Designation of spiral wound matrix magnetically stirred bioreactor.

IP-i: Experiments i performed in Bioreactor IP.

IPC: Immobilized Plant Cells.

j_G: Gas superficial velocity (m/s).

j_L: Liquid superficial velocity (m/s).

k_L: Medium to biofilm mass transfer coefficient (cm/s).

k_La: Oxygen transfer capacity of a bioreactor (h^{-1}).

K: Consistency index ($g/cm.s^{2-n}$) (Eq. 2.1).

K_f: Model parameter (Eq. 3.9).

l: Total immobilizing matrix plus biofilm thickness (m).

L: Riser length (m).

$(L/D_R)_{eq}$: Equivalent friction length.

mg d.w./cm^2: Units of SIPC measurement: mg of dried biomass per cm^2 of immobilizing area.

M: Immobilizing material.

M_w: Immobilizing material liquid absorptivity (g of liquid/cm^3 of material).

MCR17: Catharanthus roseus cell line.

n: Flow behaviour index (Eq. 2.1).

n^1: Integer number (Eq. 3.37).

n_1: Liquid phase aeration efficiency index (Eq. 4.1).

N_e: Number of experimental points used for a

correlation.

NAA: Auxin hormone α-Naphthalene Acetic Acid.

NB5: Standard solid growth medium (see Appendix 2).

N_2/PO_4: Concentrated solution of nitrogenous and phosphate salts added to a SIPC culture.

OC: Operating costs ($Cdn 1987).

O_2CR_c: Estimated oxygen consumption rate of a SIPC biomass (μMO_2/mg d.w..h) (Eq. 3.99).

OD: Outside diameter (m).

ppm: Parts per million.

P_x: Concentration of products biosynthesized in plant cells (g/L).

dP_x/dt: Intrinsic SM biosynthesis production rate of plant cells (g/L.d).

P_b: Concentration of products in the biofilm (g/L).

P_M: Concentration of products in the medium (g/L).

P_m: Concentration of products at the medium/biofilm interface (g/L).

PAL: Phenyl ammonia lyase enzyme.

PC: Plant cell.

PO_4: Phosphate ion.

Q_a: Air flow rate in the riser tube (L/min).

Q_L: Liquid flow rate in the riser tube (L/min).

Q8: Immobilizing material.

r: Linear regression correlation coefficient.

Re_H:	Reynolds number with hydraulic radius.
Re_i:	Impeller Reynolds number.
Re_L:	Reynolds number along a flat plate of length L. (Eq. 3.15).
RM:	Raw material cost ($Cdn 1987).
RPM:	Impeller rotational speed, turns per minute.
s:	Statistical standard deviation.
S:	Sucrose concentration (g/L).
Sc:	Schmidt number (Eq. 3.16).
Sh:	Sherwood number (Eq. 3.13).
S_i:	Reacting species concentration (g/L).
Su582:	Nicotiana Tabacum cell line.
Su1277:	Nicotiana Tabacum cell line.
SB1:	Glycine max cell line.
SC:	Suspension culture.
SIPC:	Average dried surface immobilized plant cell biomass per cm^2 of support area (mg d.w./cm^2).
SM:	Secondary metabolites.
SP:	Stationary phase.
STR:	Stirred tank reactor.
t:	Time (d).
t_a:	Tax rate on profits.
t_d:	Division time of a cell (d).
t_{Ki}:	Immobilizing material thickness (m).
T:	Wet biofilm thickness (Eq. 3.94) (m).
TLC:	Thin Layer Chromatography.

$(u_g)_R$: Superficial gas velocity in the riser tube (m/s).

v: Velocity of the medium in the matrix structure (m/s).

v_a: Actual reaction rate (g/L.d).

v_b: Reaction rate at the fluid bulk concentration (g/L.d).

v_f: Reaction rate in the biofilm (g/L.d).

v_{fs}: Reaction rate at the biofilm surface concentration (g/L.d).

v_{fc}: Reaction rate for immobilized cells (g/L.d).

v_{FC}: Reaction rate for free suspended cells (g/L.d).

$(v_{fc})_{nMT}$: Reaction rate for immobilized cells without mass transfer limitations (g/L.d).

$(v_L)_r$: Superficial liquid velocity in the riser tube (m/s).

V_b: Biofilm volume (m^3).

V_{MA}: Immobilizing material volume (m^3).

V_p: Quantity of fine chemicals produced.

VVM: Aeration rate: volume of gas per volume of liquid per minute.

W: Rotational speed of the viscometer (RPM).

W_s: Weight of the immobilizing structure (kg).

W_{TD}: Total dry weight of a biomass loaded immobilizing structure (kg).

W_{Tw}: Total wet weight of a biomass loaded immobilizing structure (kg).

W_w: Net wet immobilized biomass weight (kg).

W/D: Wet-to-dry weight biomass ratio.

WS: Immobilizing material.

X: Biomass concentration (g d.w./L).

X_{ss}: Biomass concentration of SIPC (g d.w./L).

X_T: Total biomass of the biofilm (kg).

y: Direction perpendicular to the immobilizing surface.

y': Direction perpendicular to the immobilizing surface.

$Y_{x/s}$: Biomass/substrate yield.

$Y_{x/s}$: Proportion of produced biomass which is immobilized.

α: Two phase flow void fraction (Eq. 3.8).

α_{cal}: Viscometer instrument/spindle constant.

δ: Wet biofilm thickness (cm).

ΔP_{fm}: Friction loss of the liquid in the matrix structure ($kg.m^2/s^2$) (Eq. 3.9).

ϕ: Thiele Modulus (Eq. 3.44).

Φ: Observable Thiele Modulus (Eq. 3.49).

η_e: Effectiveness factor (Eq. 3.42).

η_i: Efficiency factor (Eq. 3.41).

η: External effectiveness factor (Eq. 3.45).

ρ_L: Liquid density (kg/m^3).

ρ_g:	Gas density (kg/m³).
τ:	Shear stress (Eq. 2.1) (g/cm.s²).
τ_o:	Surface shear stress exerted on the SIPC biofilm (Eq. A5.4) (N/m²).
θ:	Viscometer reading.
θ_c:	Circulation time (s).
θ_i:	Adimensional concentration (Eq. 3.36).
θ_m:	Mixing time (s).
μ:	Specific growth rate (d^{-1}).
μ_a:	Apparent viscosity (cps).
μ_L:	Liquid phase viscosity (cps).
µg/g d.w.:	Unit of indole alkaloid concentration measurement µg of SM per g.d.w. of biomass.
µg/L.med:	Unit of indole alkaloid concentration measurement µg of SM per litre of medium.
Ω:	Observable external mass transfer efficiency modulus (Eq. 3.46).
1B5:	Standard growth medium (see Appendix 2).

1.0 INTRODUCTION.

1.1 Situation in Plant-derived Biochemicals.

Vinblastine (Figure 1.1) is one of the few drugs obtained from plants that is approved for cancer treatment. It is biosynthesized by the plant Catharanthus roseus at a concentration of 0.0005% (w/w of plant biomass). Its low concentration, its structural complexity precluding any chemical synthesis, and the plant supply sources unreliability explain its exorbitant price of $5 x 10^6 (US 1982)/kg (1).

Plants are a unique and versatile, but barely explored, source of such high value/low volume fine chemicals (2,3,4). These compounds are not synthesized through the primary life sustaining biochemical pathways of the plant cell. They originate from the secondary metabolism of the organism and are believed to play some kind of ecological, rather than physiological, role for the plant (1,4). They are mainly used as pharmaceuticals, flavours, fragrances and dyes (2). In fact, 25% of all US prescriptions contain plant derived products (1) and 90% of all natural products are found in plants (5). This represents more than $9 x 10^9 (US 1984) of annual retail drug sales and $1.5 x 10^9 (US 1984) of annual aroma product sales (6).

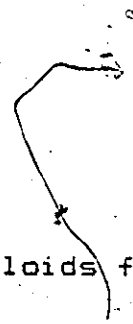
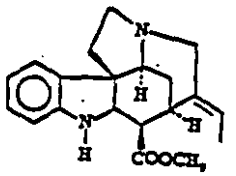
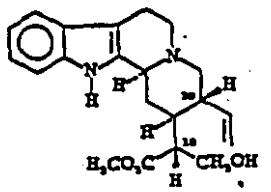


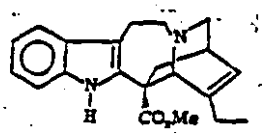
Figure 1.1 – Alkaloids from plants.



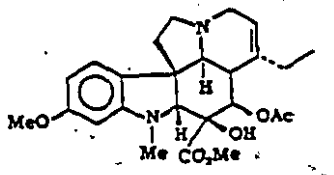
Akuammicine
(Strychnos type)



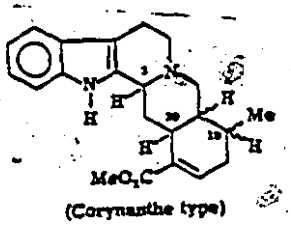
(16*R*)-Sitsirikine



Catharanthine
(Iboga type)

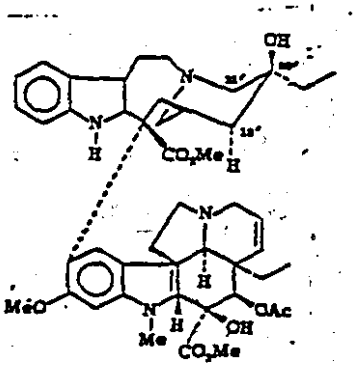


Vindoline
(Aspidosperma type)



(Corynanthe type)

	19–H	20–H
Ajmalicine	β	β
19-Epiajmalicine	α	β
Tetrahydro-alstonine	β	α



Vinblastine

Plant secondary metabolites cannot be economically synthesized chemically by man (7). Their structural complexity implies biosynthesis through very elaborate biochemical pathways involving multiple interacting genes and enzymatic steps. This also rules out biosynthesis using isolated enzymes and genetic engineering, at least for many years when considering our limited kowledge of the control of plant genetics, as means of producing these chemicals (7-9). Consequently the culture in vitro of viable plant cells remains the only practical alternative to plant vegetative growth for the production of these important compounds (1,4).

In fact, this type of cell culture has been suggested as an alternative source for these chemicals since the 1960's (10,11). However, research in this field has resulted in only two recent industrial applications (2), with the production of Shikonin from cells of the plant Lithospermum erythrorhizon having received some publicity (1). This process is based on traditional batch suspension culturing and extensive and delicate biomass extraction. At an estimated production volume of 5 kg of Shikonin per three weeks and a selling price of $4000 (US 1982)/kg, relative to $4500/kg when produced from the plant, the profitability of this process appears marginal. In addition, the many factors which made this production

successful are believed not to be easily applicable to the culture of other types of plant cells (1).

Major basic plant physiology and biochemical engineering advances are still required for this technology to be used widely in industry (2,3,7). The main problems to be solved include

- the genetic instability of the selected high producing plant cell lines (11-13)
- the uncontrolled biosynthetic behaviour of cultured plant cells (7,8)
- the heterogeneity of the plant cell culturing milieu (2,8,14) and
- the low concentration of the produced chemicals, mostly retained within the plant cells (2,6,7,12).

1.2 Literature Review.

1.2.1 The Technique of Plant Cell Tissue Culture.

1.2.1.1 Plant Cell Physiology.

Plants are formed of highly compartmented eukaryotic cells (Figure 1.2). Their physiology is much more complex than that of microorganisms. Some of their important physiological characteristics relevant to this project include

- morphological peculiarities,
- cellular dimensions,
- growth behaviour,
- oxygen requirements,
- nutrients uptake and
- genetic potential.

In addition to the usual eukaryotic organelles, plant cells have three unique elements: the plastids, the vacuole and the cell wall. Proplastids are organelles that differentiate into various storage compartments, into biochemically active organelles and/or into

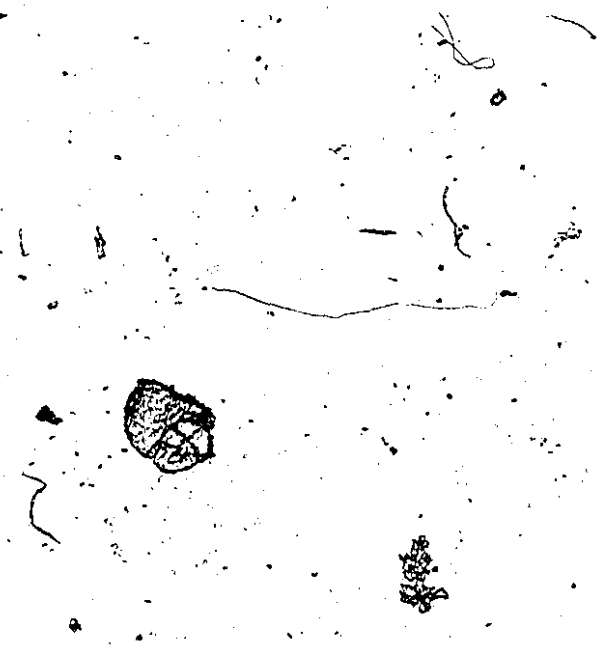
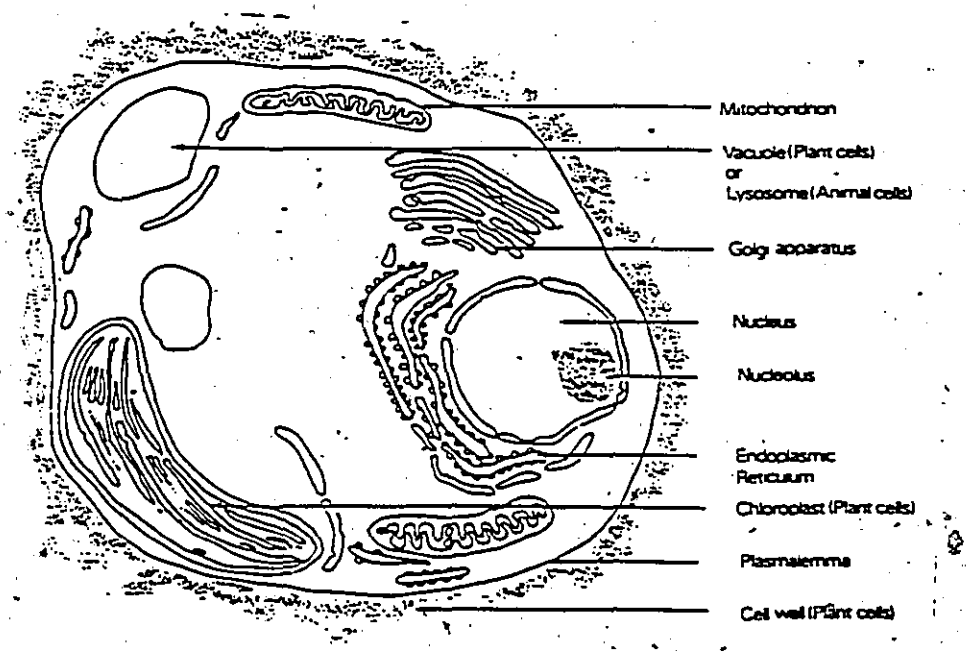


Figure 1.2 - Typical eukariotic cell.


Mitochondrion
Vacuole (Plant cells)
or
Lysosome (Animal cells)
Golgi apparatus
Nucleus
Nucleolus
Endoplasmic
Reticulum
Chloroplast (Plant cells)
Plasmalemma
Cell wall (Plant cells)

photosynthetically active chloroplasts under suitable conditions (15).

The vacuole is an important, complex, highly active and multi-functional organelle. Developing with age, it becomes the prominent structure of the mature plant cell (> 50% of its volume) (15). Its main functions include (16-18)

- control of the cell volume and osmotic potential,
- lysosomic activities,
- enzyme compartmentation,
- location of other organelles,
- control of the cell ion and metabolite balances and
- storage of nutrients and primary and secondary metabolites.

The plant cell is particularly sensitive to its osmotic environment. The vacuole controls the internal osmotic pressure (turgor pressure) of the cell, which is mechanically equilibrated by the cell wall, relative to the external medium osmoticum. This regulatory mechanism is involved in certain aspects of the plant cell metabolism, for example in its growth process (19-22) and in morphogenetic differentiation (23). This phenomenon may represent an interesting approach to stress induced SM

expression (24-27) and to forced osmotic secretion of stored compounds.

The wall of plant cells is a more intricate and dynamic structure than that of other types of cells. It is composed of an elaborate mixture of polysaccharides, structural and enzymatic (invertase) proteins and metal ions (27-29). The cell wall composition evolves with growth (30-31) and eventually differentiates into a lignified rigid primary wall, an internal cellulosic secondary wall and an extracellular middle lamella composed of gluing pectin substances in the plant (32). Cultured plant cells generate mainly a primary unlignified cell wall (33). There is evidence of the secretion of macromolecular compounds (mainly polysaccharides and proteins) by older cultured plant cells in their culture medium (27,32,35 and others) making them sticky (2). It is important to underline this last point for its potential in assisting in the plant cell immobilization.

This normally rigid wall protects and contains the cell efficiently. The cell wall plasticizes during growth under hormonal (auxin) induced local acidification and rigidifies after expansion (27,32). It is freely permeable to species of molecular weight 1600 and lower (36). It forms with the cytoplasmic membrane a physiological unity relative to

environmental exchange and growth (37). Finally, it is interesting to note evidence of a recognition role associated with the cell wall (38,39). This is involved in the elicitation of plant cells for the production of SM. This subject will be discussed in Section 1.2.2.

Plant cells are much larger (20 - 100 µm) than microorganisms (1 µm) and animal cells (10 - 20 µm). They are natural tissue formers and grow by division and expansion, without detachment from the parent cell. This tendency towards aggregated growth leads to the formation of large heterogeneous clumps of cells. These cell aggregates break up easily when cultured in suspension upon excessive mixing shear (3,15). Cultured plant cells are voluminous and highly hydrated (95%).

Plant cells grow slowly (division time of 20 to 60 hours) (40-42) and perpetually when cultured in vitro in their undifferentiated state and as long as nutrients are available. This fact is reflected in low biomass production rates ($\leq$ 7 d.w./L/day (2,8)) and extended cultivation times ($>$ 10 days) implying strict sterility requirements for the culture system. This is only emphasized by the rich defined growth media required (43). The growth follows the well known sigmoidal pattern, even though few generations occur.

The oxygen requirements of plant cells are lower than for microorganisms (10 to 50 times lower) (2). and for animal cells (44). Maximum oxygen consumption rates of 5 to 85 µL O_2/mg biomass dry weight/h have been reported (24,36,45,46) for various plant cell types and culturing conditions. A minimal dissolved oxygen concentration of 1300 µL O_2/L, or 16% of saturation, has also been mentioned, at which the growth of carrot cells was still not affected (45). The oxygen requirements for plant cells immobilized in calcium alginate are also within this range and do not seem to differ significantly from that of plant cells cultured in suspension (25,46-48). This is reflected in the low aeration rate required (0.3 VVM or $k_La \sim 10$-25 h^{-1}) to culture plant cells in suspension (49-52).

The cell wall, with its weakly acidic ion exchange properties (29), its limited porosity (36) and two active membrane systems, the cell's plasmalemma and the vacuole's tonoplast (Figure 1.2), control the uptake of nutrients by the cell (53,54). The plant cell constantly accumulates nutrients from the environment either to fulfill metabolic requirements, for storage and/or for turgor pressure stabilization (55). The cytoplasm regulates this flux of species by means of its metabolism and of the active plasmalemma, relative to the ever changing culture medium and of the actively accumulating vacuole.

The primary transport mechanism of plant cells is active H^+ excretion from the cytoplasm. This makes the cytoplasm alkaline and acidifies the vacuole, the cell wall and the culture medium (54,55). This mechanism is involved in the growth process and, more specifically, in the cell's volumetric expansion. It is highly sensitive to the growth hormone auxin and to the medium osmoticum. Auxin addition or increased medium osmoticum lowers the medium pH from its normal equilibrium level of 5.7 to 4.8-5.0. Other exchange processes between the plant cell and the medium include either passive or osmotic diffusion and active and selective secondary transport of nutrients closely coupled to H^+ secretion (57-59).

Finally, this review of the physiology of plant cells need to be completed by a brief discussion of its complex and poorly controlled genetics (15). Each plant cell contains all the genetic material to generate a complete plant (totipotency). The required parts of this enormous genetic potential are expressed during the normal growth pattern of the cell in the plant, from its appearance at division in the meristemic mass, up to its elongation and differentiation into the various organs of the plant (15). This differentiation results from the switching of gene expression for morphological and functional specialization. It is induced by a combination of

- a change in growth pattern,
- spontaneous plant cell self-assembly,
- intercellular interactions,
- tissue requirements,
- various environmental factors, etc.

This differentiating behaviour, which may be reversed, is particularly important for the culture of plant cells and their induced biochemical specialization for SM production (2,8). In fact, one of the main barrier to the industrial exploitation of this type of culture comes from our inability to control the biosynthetic behaviour of plant cells (8), which may require some level of morphogenesis (2,50,60) or tissue forming simulation (42), and/or a well controlled, homogeneous and particularly defined chemical and physical (stressing) environment (61). As discussed in Section 1.2.1.2, this culture mode has not been achieved yet. This may partly explain the well known non-reproducibility of plant cell cultures (2,8,42 and others). Another major genetic problem associated with this technology involves the tendency towards polyploidy of cultured plant cells with increasing cell line age and the observed concomitant loss of biosynthetic potential (8,13,62-65).

1.2.1.2 The Culture of Plant Cells In Vitro.

Cells are obtained from a plant by excising, sterilizing and placing a tissue on a solidified and well defined medium containing various growth regulators. Within 4 to 6 weeks, a solid mass of dedifferentiated plant cells are generated from the original explant. Continued growth of a healthy callus of undifferentiated plant cells is obtained by subculturing monthly onto fresh solid medium. Easier handling and faster growth of plant cells are achieved by transferring the friable biomass to liquid medium, similar to the one used for callus growth, mixed in a shaked flask. Weekly subculturing at an inoculation volume ratio of 5-20% insures continued growth of this suspension.

Suitable scale-up of this type of culture, even only to the level of a laboratory bioreactor, represents a special challenge when compared to most microbial fermentations (42,61,65,66). Major areas of concern include the type of growth, mixing and aeration requirements of the suspension in relation to the biomass characteristics.

The slowness of plant cell growth places stringent requirements on the culture system for reliability and sterility (42). A large inoculum (5-20%) is essential to

ensure the presence of sufficient self-growth factors (67) and the stability of the culture (68). This requirement for a large inoculum and the slow growth of plant cells represent a serious process time lengthening for the scale up of such a culture to large volumes (8 weeks to attain 10000 L from a 100 mL inoculum) (69).

When cultured in suspension, the large, gluey and aggregated water balloons that are plant cells create considerable difficulties. They settle within minutes if not agitated (40,70). Their large size and rigid but shear sensitive cell wall (71) limit the mixing rate in stirred tank reactors (STR) to 100-350 RPM which is just marginal for good gas dispersion, mass transfer and maintainance of the culture homogeneity (40,66,72,73).

Plant cell suspensions are limited to biomass concentrations of less than 30 to 50 g d.w./L of suspension because of their high hydration level (95%+) (69). The culture broths behave as viscous shear thinning fluids, at least for biomass concentrations higher than 10 g d.w./L (74). These Non-newtonian fluids obey the Power Law Model.

$$\tau = K(du/dr_1)^n \qquad (1:1)$$

The values of K and n parameters of this model for various plant cell types cultured in suspension were presented by Tanaka (74). For example, values of 0.72 and 20.6 $g/cm.s^{1-2n}$ were experimentally measured (see Section 2.7 and Appendix 1) for the flow behaviour (n) and consistency (K) indices of a 20.7 g d.w./L Catharanthus roseus cell suspension of Line MCR17, the main biological model system of this work (see Section 2.1). These figures are consistent with others (74). The measured apparent viscosity (μ_a) of this suspension at a rotational speed of 50 RPM of the viscometer was 520 cps (Appendix 1). It was shown that n does not depend on the biomass concentration, whereas K and μ_a are dependent on the culture morphology and age. These parameters are proportional to the 6-7th power of the dried biomass concentration (74). Consequently, a C. roseus cell suspension of 30 g d.w./L would have a μ_a (at 50 RPM) of 5800 cps.

This serves to illustrate the difficulties encountered by many researchers (69,72 and others) in trying to achieve homogeneous mixing of such cultures. Many have used microbial fermenters equipped with shear intensive (Rushton turbine or marine) impellers operated at low speeds (80-300 RPM). This equipment and operating conditions are not suitable for achieving uniform mixing of these shear sensitive and shear thinning plant cell suspensions.

Airlift bioreactors are believed to be generally better for culturing plant cells in suspension (2,12,45). They provide for

- efficient mixing and aeration at low shear, because of better flow characteristics (36,73),
- better sterile containment (50),
- more reliable operation (73),
- easier scale up and
- good growth ($\mu \sim 0.38\ d^{-1}$ for C. roseus cells (73)) and SM production (75).

However, even this bioreactor configuration suffers from serious drawbacks. Plant cell suspensions, when they are directly aerated even to the required low rate of 0.3 VVM for sufficient oxygen transfer (49-52), are well known for their excessive foaming. They produce thick froth of secreted macromolecular compounds and agglomerated cells on top of the culture broth (2). This cannot be solved by antifoam addition since it may be deleterious to the culture (71). This culture behaviour limits both the aeration/mixing rate of airlift to 0.4-0.6 VVM and the suspended biomass concentration which can be cultured in such a bioreactor to 15-20 g d.w./L (2,40). Higher aeration rates have also been shown to overgas the culture (high dissolved oxygen concentration) (76) and to hyperventilate the biomass which results in excessive

carbon dioxide stripping from the plant cells (77,78). Both actions lead to lower growth rate (by > 16% (73)) and biomass yields (by ~ 37% (73)) (69,76,78). In fact a 0.6 VVM aeration rate or a k_La of 15 h^{-1} were suggested as best operating conditions for plant cell biomass production in airlift bioreactors without consideration for basic mixing requirements (52). This limited aeration range reduces the mixing and mass transfer efficiency of this type of vessel, especially for shear thinning plant cell suspensions, as compared to the 1.0-1.5 VVM aeration rate suggested for optimum mixing in an airlift (79-82).

Most of the research efforts mentioned above have been mainly concerned with the shear sensitivity, frothing and excessive aeration problems associated with the suspension culture of plant cells in bioreactors. Much less attention has been given to investigating the rheological properties of this type of fluid and their effect on the mixing within the bioreactor to attain a homogeneity level comparable to that in a shake flask. Mass and oxygen transfer to a homogeneously mixed culture may be less critical when considering the relatively slow metabolism of plant cells. In particular, aeration means which tend to induce less foam formation seem not to have been considered to improve the culture system efficiency.

The stickiness of plant cells further complicates the situation described above. This stickiness leads to wall growth and instrument plugging (42,69,70). All these difficulties, which worsen upon scale up, contribute to the heterogeneity of the suspension and to the formation of numerous dead zones leading to premature senescence of the culture (83,84).

In summary, most bioreactor configurations used to grow plant cells in suspension have failed to provide for an apropriate well controlled and homogeneous culture environment. Conventional mechanically stirred tank reactors are too shear stress intensive, while airlift reactors produce too much foam. Batch or continuous operation features either a continuously changing medium or washing out of the precious biomass. Consequently the culture of plant cells in suspension in bioreactors, besides being unnatural to these tissue formers, has resulted in very heterogeneous biological systems of limited biomass density. In view of the high sensitivity of the differentiating behaviour of plant cells to the culture environment, which is so important for SM production, it is easy to understand some of the difficulties encountered in developing this technology.

1.2.2 Biosynthesis of Secondary Metabolites from Cultured Plant Cells.

Secondary metabolites, and more specifically indole alkaloids from the plant Catharanthus roseus, the model system of this project, are not inert end products (85). They are present in the plant in a dynamic metabolic equilibrium which is strongly dependent on the location and developmental stage of the repository tissue as well as on the environment (86). The net product accumulation results from the difference between biosynthesis, storage and either conjugation, interconversion, oxidative polymerization or simply degradation.

The situation for cultured plant cells is much more complicated and obscure because of

- the heterogeneity of the cell population (genetics and developmental stage),
- the poorly controlled physical (and chemical) culture environment,
- the ever changing medium composition and
- the unnatural morphological state of the suspended cells.

Generally, SM are biosynthesized by viable, active, stable and non-dividing plant cells under conditions

promoting some biochemical specialization/differentiation (1,42,61). The requirement for non-growing, stationary plant cells originates from the fact that growth prevents differentiation and specialization and mobilizes all the cell energies towards its primary life proliferating metabolism. In addition, the need for aggregation and intercellular closeness seems important (42,87), whereas morphogenetic requirements are still uncertain (2,59-61,88-90).

Reasonably wide product spectra, including compounds not found in the original plant, have been obtained from cultured plant cells at yields comparable or higher than in the original plant tissue (~ 0.1 to 3.5% of the cultured biomass dry weight) (84,91-95). Most of the yields of interesting compounds are still too low for industrial application, even though a yield of 23% has been reported for the industrial production of Shikonin from cultured plant cells of Lithospermum erythrorhizon mentioned in Section 1.1 (6). The product spectra vary and are not as wide as in the plant (7). They become narrower and the yields diminish with the cell line age (up to 50% in 1 year) (84,92,96). No dimeric alkaloid, such as Vinblastine, has been found in cultured plant cells (91,94).

The higher product yields mentioned above were obtained by culturing plant cell lines, specially selected for their superior biosynthetic capabilities, in media designed to promote the biosynthesis of these compounds (14,88,97). These differ markedly from standard growth media. This scheme implies some growth/production decoupling (87), which may be dependent on the culturing system type (14) and/or on the aggregation level of the cultured cells. This decoupling phenomenon may not be generalized for all types of plant cells (98).

This scheme is not without problems. Production results are variable and often unreproducible (7,8). Depending on the original plant species, the high producing cell lines are generally unstable (7,8). The wide genetic potential of the plant cell, its uncontrolled expression and the heterogeneity of the culture environment are believed to be the main causes for the unreliability of this production scheme (12,13,88,96), with SM turnover (87,88) and retention of the compounds within the cell (99,100) as other possible factors involved. Also, differentiation induced by the production media is tolerated by plant cells only for a short term (101).

It has been said that SM play some ecological role, the plant responding to environmental challenges by some

chemical defense mechanisms (102). Successful production of SM from plant cells requires that such triggering stresses be determined and simulated under close control in the culturing system (103). Three classes of such triggering factors, and some combinations, have been evaluated: chemical, physical and bioengineering factors.

The design of production media mentioned previously has been the approach most studied to improve the production of SM from plant cells. Although results vary with the plant species cultured, some general tendencies, at least applicable to Catharanthus roseus plant cell lines, have been observed. The main features which distinguish these media from growth media are their higher sucrose content, their lower phosphate and nitrate concentrations and the hormones used.

Sucrose, at concentrations of up to 8%, has been shown to promote SM expression (96,103-106). Growth inhibition, osmotic shock and/or induced differentiation are believed to be responsible (23). Low phosphate concentrations (< 0.5mM) that limit growth improve SM production (103,104,106,107). Nitrate is rapidly (2 days) accumulated by plant cells and stimulates growth. Following nitrate assimilation, significant metabolic changes occur including an increase of the enzymatic activity of phenyl ammonia

lyase (PAL) which leads to tryptophan, an essential precursor of indole alkaloids (108). Consequehtly the nitrate limitation per se reduces alkaloids expression (104).

The effects of phytohormones are variable. It was shown that the growth promoting auxin indole-3-acetic acid (IAA) (and naphthalene acetic acid (NAA)) at concentrations lower than 0.5 µM is essential for the culture survival and does not inhibit SM expression. Higher concentrations and the use of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4D) strongly hinder SM production (88,109). This may be related to the fact that the synthetic auxin cannot be metabolized by plant cells (110). The cytokinin hormones, kinetin and 6-benzylaminopurine, have also been shown to promote SM expression. The mechanism of action is believed to involve their protein synthesis enhancing capabilities, mainly directed towards PAL increased activity (111).

The use of these SM biosynthesis promoting media for the production of these compounds requires long cultivation periods (> 2 weeks) and still gives relatively low product yields. Recent development originating from the field of phytopathology may drastically improve this process. It was found that the antimicrobial chemical defense

mechanisms of cultured plant cells can be directly triggered by the addition of some elicitor compound(s) to the culture. This results in the rapid (< 72 hours) biosynthesis of antimicrobial products (SM) and the partial secretion of some of them (up to 50% of the quantity produced) in the medium (112-115).

This technique is difficult, sensitive and must be optimized for each plant cell type cultured. An optimized elicitation system includes a specific cell line, a suitable elicitor preparation, which may be well defined chemically or a dead and sterile microbial or fungal homogenate, and a proper process protocol. This set of operating procedures involves introducing a given amount of the elicitor (≤ 5% V/V) into a culture of a specific age for a time period not exceeding 24 hours in order to maintain the culture viable. The products biosynthesis starts after 6-12 hours of elicitation, with production and medium secretion peaking after ~ 24-36 hours. No special culture medium other than the regular growth medium is required and reelicitation of the viable plant cells is possible. The amounts of SM biosynthesized compare to the quantitites obtained by using a production medium.

This promising technique is still in its infancy, with little reported results in systems larger than shake flasks

(114). Numerous theoritical unknowns and practical problems remain to be solved, including the variability of the restricted chemical response obtained (113) and its scale up in a suitable physical system.

Few physical factors have been evaluated to improve the yield of SM from cultured plant cells. The effect of aggregation has been discussed briefly above and will be further elaborated in Sections 1.2.3 and 1.2.4.1 together with immobilization. Osmotic shock with sucrose at concentrations of up to 8% has been used in most SM production media with success (44,72,97,103-106). Osmotic and/or ionic salt stresses (NaCL at ~ 7.5% w/v) have also been shown to double SM production especially in large aggregates of cells (5-10 mm) (116). This may also affect the cell membrane permeability (117), a subject that will be discussed in Section 1.2.3 together with pH effects (118).

Varying the culture temperature has been examined with mixed success, resulting from no effect (116) to improved biosynthesis (119), chilling stress and SM release by organelle rupture (120). The culture of Catharanthus roseus cells in suspension at 16°C in a suitable production medium resulted in slower growth (by a factor of 4) and biosynthesis but in significant improvement in SM

accumulation (by a factor of 4.5) as compared to 27°C (121,122). This improvement was related to the fewer but larger cells of the culture and the lower degradation of SM at the lower temperature. Another study (119) showed best growth of the culture at 30°C and best SM biosynthesis at 20-25°C. This could be exploited for a process in which growth and production are decoupled.

Light has variable effects on SM production by suspension cultured cells, depending on the original plant species. Its effect is reflected in altered cell membrane permeability (123,124). The few results available show an increased production (by a factor of 3 or more depending on the product), relative to dark culture (83,116,125). Light also affects the SM spectra produced (104).

High aeration rates ($k_La > 15\ h^{-1}$) have been shown to lower biomass growth rate and yields (52) because of hyperventilation, as discussed in the preceeding Section. They also have been found to improve SM biosynthesis of plant cell suspension cultures (75,83,91). The concentration of dissolved oxygen (DO) in the medium has been suggested as a more valuable parameter to assess the effect of this operating condition. A DO concentration of 30% of saturation was found optimum for biomass growth and yield and SM production in this case (76). However, most of

the studies on the effect of the aeration rate on the culture performance were carried out with airlift bioreactors which suffer from the operational problems discussed previously. These problems were shown to limit the efficiency of this type of bioreactor. Consequently these results must be assessed with care.

Besides the de novo biosynthesis capabilities discussed above, plant cells can specifically biotransform complex chemicals into valuable products (126,127) and be fed precursors to improve SM yields (91,97).

1.2.3 Production of Secondary Metabolites from Plant Cell Suspension Cultures.

In this process, one is trying to stimulate and regulate the delicate genetic machinery of plant cells to produce SM within the culture systems discussed in Section 1.2.1.2. The scale-up of these systems further increases

- the suspension heterogeneity,
- mass transfer deficiencies and
- contamination problems.

The limited mixing intensity allowed slows down growth. The culture must be maintained in a sensitive stationary state for long periods (> 2 weeks) under absolute

sterility. The harvesting of the product further complicates the process since SM are generally not secreted in the medium but retained in the cell's vacuole (13,89,122,128).

Consequently, the whole process scheme to produce SM from a suspension culture of a high yielding plant cell line require

1- Inoculation (≥ 10%) of a stabilized and defined process strain in a suitable growth environment,

2- Rapid growth to the maximum biomass concentration,

3- Induction of SM expression, with or without medium changeover or addition of chemical(s) (elicitation),

4- Maintenance of the culture in this production stage for the necessary time,

5- Product harvesting, which may involve either

a) biomass/medium separation and biomass extraction for product recovery, or

b) induced SM secretion in the medium, biomass/medium separation, with possible re-use of the cells and finally medium extraction for product recovery.

This lengthy procedure requires extreme care and sterility as well as delicate liquid/solid handling and extraction operations which are difficult to implement industrially.

Bioengineering studies of this process have paid little attention to

- the heterogeneous and unnatural state of plant cells cultured in suspension, and its' effect on SM expression,
- the close control of the physical and chemical culturing environment,
- the effect of the culturing system configuration and SM retention in the biomass on final product recovery.

Efforts have been directed mainly at handling the growth/production decoupling characteristic of many of these plant cell cultures with their numerous operational restrictions (2,129).

The batch configuration has been preferred because of the handling difficulties and the reduced viability of a recycled biomass as well as the product retention in the plant cells (69). The use of the less efficient stirred tank reactor (STR) has resulted in growth rates comparable or lower than for airlift and in lower (~ 0%) SM yields (40,69,72,126). Recently the use of a spiral stirrer has been reported to mix growing lant cells in suspension (130). Good SM production was reported but aeration means and rates were not disclosed. The fedbatch (131) and multiple continuous STR (26) configurations were evaluated

for the operational flexibility required by this growth/production dissociated process. The first configuration allowed adapting the medium composition to the cell's needs within certain limits. This resulted in improved product yields (150%). However, control over aeration and mixing of this type of system is difficult (69). The second configuration allowed some physical separation of the growth and production phases, with an increase in the culture heterogeneity, some product improvement (~ 30%) and serious contamination problems.

In fact, the industrial production of Shikonin from cells of the plant Lithospermum erythrorhizon mentioned in Section 1.1 is performed in two consecutive batch operated STR. A growing inoculum is prepared in the first vessel (200 L) during 9 days. This biomass is concentrated by filtration in an intermediate small tank and fed with a production medium to the main bioreactor (750 L) where it is retained for 14 days. Thereafter the biomass is separated from the medium and is extracted for SM (1). The reported Shikonin productivity of this system is 0.07 g/L.d (9).

Increases in SM yields of the order of 30 to 100% have been reported when culturing plant cells in airlifts as compared to other suspension systems (68,75,126). This

improvement is ascribed to the superior aeration efficiency of this type of bioreactor. Presently the airlift configuration remains the preferred alternative of many researchers to study the production of SM from suspension cultured plant cells. However the STR seems to be regaining the favor of a few research groups (69,72).

1.2.4 Plant Cell Immobilization.

1.2.4.1 Why to Immobilize Plant Cells?

This technology represents one of the most interesting solutions to the unique problems associated with the culture of plant cells for the production of SM. Its advantages are numerous, especially regarding the physiological, hydrodynamic handling and process engineering aspects of this type of cell culture.

Immobilization promotes the inherent tissue forming tendency of plant cells by taking advantage of their natural adhesiveness (42). The biomass and medium are generally well separated, improving their respective homogeneity (1,129,132). It permits to maintain viable and active non dividing plant cells and to preserve their biosynthesis potential for long periods (up to 7 months as compared to 3 weeks for a suspended biomass) (1,133,134).

This stationary state of plant cells also improves their genetic stability (132,135). The biomass organization level attained upon immobilization, which is intermediate between a suspension and the original plant tissue, favors cell/cell contact. This organization of the plant cell biomass, its polarized and asymmetrical aggregation and its reduced growth rate are precursors of differentiation and secondary metabolism (133,134). The physical and chemical gradients created by, and surrounding the immobilized biomass generate particular microenvironments which are believed to induce some degree of differentiation and favor secondary metabolism (133,136).

The immobilizing matrix and/or the induced aggregation configuration provide this fragile biomass with good protection from mixing shear (132,134).

The confinement of the plant cells makes the whole process simpler and more flexible. Overall control is easier, with uniform mixing of the medium and mass transfer. The biomass is not carried into the foam layer which may form (133). The inherent slowness of the plant cell metabolism may very well not make this process diffusion limited (5). While the solubility of the diffusing species (for example oxygen or hydrophobic secondary metabolites) in the medium, confining matrix

and/or biomass may affect mass transfer and production kinetic (133). Denser plant cell cultures could be attained by immobilization as compared to suspension culturing, especially when considering the limited mass transfer efficiency and the numerous operational problems associated with this last culture mode (see Section 1.2.1.2) (133).

The efficient biomass and medium separation also allows the easy chemical and physical manipulation of the culture, including the possibility of separating the growth and production phases (133,135,137). The volumetric efficiency of the process can be improved by the reuse of the biomass, by the better mass transfer characteristics of the immobilized system, by continuous flow operation and by the positive influence of the rested state of the immobilized plant cells (IPC) on their biosynthesis potential (1,133,135,137). In fact, increased SM yields of 140-176%, as compared to suspension cultures, were observed for C. roseus cells immobilized in calcium alginate gel beads (5). However, this effect may be attributed to the weak eliciting property of the gel immobilization matrix used (138).

The immobilization of plant cells is not without problems. The use of the immobilization technology requires

that the growth of the confined biomass be limited for efficient mass transfer and stimulation of secondary metabolism. This has been difficult to achieve when immobilizing plant cells by the popular gel entrapment technique (see Section 1.2.4.3) (5,133). While the secretion of the products in the medium; a crucial prerequisite to the usefulness of the immobilization technique, remains a particularly restrictive step for plant cells as discussed in Sections 1.2.2 and 1.2.3 (1,133,135).

1.2.4.2 The Whole Cell Immobilization Technology.

The techniques used to immobilize enzymes and whole cells have been relatively well developed. The technology of whole cell immobilization is gaining in popularity. It provides for one-operation biosynthesis of complex compounds from simple substrates without the need for specific and costly enzyme purification, special enzyme environment or cofactors artificial regeneration (139-141).

In addition to the advantages discussed in the previous Section, this technology has been reported to

- improve significantly (10x) the stability of confined cells as compared to free cells (142),

- provide for means of regulating the immobilized biomass physiology by better control over mass transfer, oxygen supply, cell density and microenvironment factors (140,143-145),
- improve the cell resistance to the environment and
- accelerate certain aspects of the cell metabolism (142).

However, the successful application of this technology requires that

1- growth, after full loading, be severely limited to prevent biomass leakage and support matrix breakage (145), even though some growth may be required to maintain biomass stability (142),
2- the product be extracellular, soluble and non-growth associated (145).

In addition, some other restrictions must not be overlooked. These include the loss of activity during immobilization, the reduced mass transfer due to the aggregation of the biomass and the presence of the immobilizing matrix, the difficulties of maintaining an active biomass over long periods (145) and downstream processing requirements.

The basic attachment technique must be sterile, easy, non-contaminating, non-toxic and be performed under mild and non-damaging conditions. It must be of a high biomass capacity, without impeding physiological and mass transfer processes, and be easy to scale up. The support matrix must be mechanically stable and well configured to provide for an efficient interaction between the immobilizing material, the biomass and the medium. Three whole cell immobilization technique have been pioneered.

Solid carrier attachment involving physical and/or chemical bonds between an insoluble support matrix and cells is a mild and simple process. It results mainly in surface immobilization with good mass transfer. It is difficult to control and highly sensitive to the environment. Its main problems are efficient initial biomass attachment and subsequent retention, which is inversely related to the cell-carrier bond strength and to the amount of biomass attached to the support (140,141,145).

Cell aggregation by flocculation and/or chemical crosslinking results in culture micro and macro heterogeneity. Chemicals toxicity, biomass retention and mass transfer restrictions are but a few of the problems that limit the application of this technique (140,141).

Cell entrapment in a matrix, either solid, formed in situ from synthetic polymers (polyacrylamide) or natural gels (agar, agarose, alginate, carrageenan) crosslinked by metal ions, or in a preformed membrane (encapsulation) has been used with some success (139-141,145). Entrapment in a porous solid is easy and mild. Its problems are similar to the surface immobilization technique, with initial biomass attachment in the interior of the matrix and subsequent mass transfer being more difficult.

The popular gel immobilization technique provides for good protection of the fragile biomass. However, it suffers from serious drawbacks. This technique involves mixing a suspension of cells with a suitable polymer solution. This mixture is subsequently treated with a gelling agent (Ex. $CaCl_2$) and formed mechanically into the desired matrix structure (beads, sheet etc.). This technique is difficult to perform and to scale up, especially when carried out under sterile conditions (133,146). The gelling treatment is stressful to the cells (10 to 50°C heat shock or toxic crosslinking agent). The resulting matrix lacks mechanical stability unless chemically crosslinked (147), has reduced porosity and may be sensitive to chelation by medium ions (calcium alginate and PO_4 (145)). Hollow fiber cartridges are expensive. They provide for heterogeneous immobilization and

difficulties in aeration may be encountered (145). The entrapment membrane represents an additional resistance to mass transfer and affects, or may be destroyed (gel beads) by, regenerative growth.

The main bioreactor/matrix configurations experimentally examined include (14)

1- the STR, airlift, packed and fluidized beds and rotating basket scheme for the matrix/biomass particulate form,

2- the wound roll in a cylindrical vessel, the baffled tank and the filter press arrangements when the matrix is available or made in a flat sheet and

3- the circulating loop for the hollow fiber cartridge.

All systems can be adapted to batch or continuous operation.

1.2.4.3 Immobilized Plant Cells for the Production of Secondary Metabolites.

Few research groups have immobilized plant cells for SM production (Table 1.1). Brodelius and coworkers have led the way since 1979 in entrapping plant cells in natural gels, the most successful matrices (136,147-151). They have shown that

TABLE 1.1

EXAMPLES OF IMMOBILIZATION OF CULTURED PLANT CELLS

Plant species	Immobilization method	Polymer	Products	Reference
C. roseus	Entrapment	Agarose Alginate Agar Carrageenan	Ajmalicine isomers	153 148 134 134
D. carota	Entrapment Entrapment	Alginate Alginate	Digoxin 5- -hydroxygitoxi-genin	148 154
G. max	Entrapment	Hollow fiber	Phenolics	42
L. vera	Entrapment Entrapment	Urethane prepolymers Agar, alginate, carrageenan	Pigments Pigments	155 156
C. frutescens	Adsorption	Polyurethane foam	Capsaicin	157
S. aviculare	Covalent linkage	Phenylene oxide	Steroid glycerols	158
A. tricolor	Entrapment	Chitosan	Oxalate	159
A. syriaca	Entrapment	Chitosan	Proteases	159
M. piperatus	Entrapment	Polyacrylamide	Neomenthol	160

- alginate crosslinked with calcium ions is superior to most other gel supports tested for the mildness of the technique, the retained viability and SM productivity (148),
- long term retained activity (6-7 months) is feasible (148),
- up to 50% of the wet incoculum can be easily entrapped (136) and
- SM productivity can be increased up to 140% (or 176% if precursors are fed to the culture) by immobilization as compared to suspension culturing (136). Although this can be ascribed to the weak elicitation properties of alginate (138).

They were successful in applying this technique to immobilize delicate plant protoplasts (cell wall deprived highly osmotic sensitive plant cells) (152).

Others have immobilized a variety of plant cells by this technique with good productivity and long term stability (46,154,156,161-165). Immobilization of plant cells in a gel of polyacrylamide-hydrazide crosslinked with glyoxal has also been examined with relative success (160). The resulting matrix is more stable than calcium alginate, but the immobilization procedure is stressful to delicate plant cells (0°C, glyoxal toxicity).

The serious drawbacks of the gel entrapment technique, are

- the excessive amount of the crosslinking agent required for the matrix gelling: it renders the support sensitive to the environment (PO_4 chelation) or reduces the cell viability, SM yields and secretion (166),
- the limited mechanical stability of the gel matrix which can be chemically crosslinked only to a limited extent because of toxicity problems (167),
- the indirect contact of the cells with the medium resulting in limited mass transfer and oxygen supply, which may cause internal cell death for large aggregates (5 mm) (48,149,162,165) and,
- the bursting of beads during long term operation at limited growth (46,133,150,168).

The preferred process arrangement for this technique is the bead form of the matrix and the bubble column because of the relative ease of preparation and operation. The packed bed cannot be used since it causes compression, mass transfer and death problems (47). Fluidization and STR cause attrition and biomass leakage (47,164). The airlift configuration represents a better compromise, with good mixing at low shear rate and sufficient aeration (24, 164).

In fact, it is reported that calcium alginate IPC of the sensitive plant Papaver somniferum (3[illegible] mm beads) cultured in an airlift bioreactor required 3.75 VVM (!) for survival and production (162). Others have stressed the importance of aeration for SM production by gel entrapped IPC (154). The scale up potential of this technique has been questioned (133,169). Formation of the gel matrix loaded with the plant cells in a sheet form reinforced by cotton and/or stainless steel mesh has been suggested as a stronger configuration easier to handle and to scale up (133). However, this further complicates the initial sterile immobilization process.

Two other particulate semi-entrapment techniques, involving porous polyphenylene oxide particles (250 µm) pretreated with (toxic) glutaraldehyde (158) and reticulated polyurethane foam particles (1 cm^3) (157,170), have been developed. The second approach is particularly attractive for its simplicity, mildness and good biomass (> 95%) and viability retention (> 70 - 80%) after 21 days of culture. It permitted more than a 100 fold increase in SM productivity when compared to suspension culturing under growth limiting conditions (165). However, its scale up appears difficult (171).

Immobilization of plant cells in preformed membrane systems is another interesting alternative. Hollow fibers provide for gentle and complete entrapment without cell leakage. Easy control of the medium and efficient biomass/liquid separation are achieved (135,172). Good growth was obtained for 20 days. However, direct access to the biomass, aeration and mass transfer are restricted. Biomass loading uniformity and efficiency are unknown. In addition, the hollow fiber cartridge is expensive, difficult to obtain and must be installed within an elaborate circulating system subject to contamination.

A flat plate adsorbing membrane has been experimented with success for 110 days (129). The two sided arrangement permitted an efficient physical separation of the medium and biomass. SM productivity of the 3.2 mm wet biofilm over the 125 µm porous membrane was superior to chemostat (by 66%) and batch (by 185%) suspension culturing. However, this method suffers from limited mass transfer and indirect cell/medium contact and may require a contamination prone circulating system.

Finally, purposeful aggregation has been suggested as a new immobilization technique for plant cells (173). The difficulty of controlling such technique, the resulting biomass heterogeneity and the inherent difficutly of

culturing plant cells in an aggregated suspension, as discussed in Section 1.2.1.2, severely restrict the usefulness of this approach.

In summary, most of the research work on plant cell immobilization has centered around the cell entrapment technology that can be scaled-up only with difficulty. It was shown to be a viable alternative for producing SM from cultured plant cells even though there may be some disagreement (169). However, growth control and product secretion, so important to the successful application of IPC (9), have been addressed only to a limited extent. Growth control by medium manipulation has been suggested as a best alternative (149). Solvent permeabilization was, at one time, suggested to promote SM secretion (134,153). This technique limited the long term viability of plant cells since solvents affect their physiology (174). Milder means have since been suggested (9), such as incident light (124), temperature (175), pH (118), osmosis (106), ionic shock (117), hormones (87) and introduction of a fourth product absorbing phase (176,177). The immobilization state itself was found to induce some secretion of the products in the medium (133,163,168).

1.3 Research Objectives.

1.3.1 Defining the Process.

In most cases, the production of secondary metabolites by cultured plant cells will be better effected by using the immobilization technology, even though no such technique has yet been developed that can be easily applied in industry. The economics of this process require maximum productivity which implies maintaining the biomass active for long periods according to a process scheme such as the one that follows.

1- Selection and maintenance of a productive plant cell line.
2- Preparation of a suitable (quality and volume) inoculum.
3- Preparation of the immobilization culturing bio-reactor.
4- Inoculation.
5- Uniform and rapid distribution and immobilization of this inoculum within the matrix configuration.
6- Rapid growth of the IPC.
7- Induction of SM biosynthesis.
8- Harvesting of the products.
9- Regenerative growth of IPC.
10- Final product purification.

Steps 2 to 6 represent the central theme of the process forming the basis of this research project. Step 7 is to be explored only tentatively. Steps 1 and 8 to 10 will not be pursued.

1.3.2 The Objectives of the Research Project.

The main objective of this research project was

to develop a bioreactor system for the culture of immobilized Catharanthus roseus plant cells for the production of secondary metabolites.

This objective is broken down and detailed in the following Sections.

1.3.2.1 Development of a Novel Immobilization Technique for Plant Cells.

Surface immobilization of plant cells (SIPC) seems not to have been studied extensively. The immobilization process based on adhesion is gentle and easy. Mass transfer is only a function of the support configuration and of the medium flow hydrodynamics, two variables easy to manipulate. Efficient initial attachment of the plant cells and control of the biomass leakage from the

immobilizing matrix, the two most obvious problems of this technique, can be ensured by using a suitable inoculum, selecting a proper support material, forming the support into an efficient configuration and designing a culture system for effective and uniform mixing.

Low shear mixing and surface aggregation should provide for sufficient mechanical protection and morphological rest to SIPC.

1.3.2.2 Scale up of the SIPC Technique to a Laboratory Size Bioreactor.

An efficient culture system need to take into account the particularities of the plant cells and the requirements of the immobilization technique and of the process. It must provide for a controlled, well adapted and uniform culture environment. It must ensure sufficient aeration for the survival of the culture and SM production. The bioreactor and matrix must be configured to provide for a high immobilizing area easily accessible to the inoculated plant cells and to the flowing medium. Finally the system must allow the changeover of the medium and a reliable (sterile) operation of the process.

1.3.2.3 Evaluation of the Performance of the SIPC Bioreactor.

The operational procedure must be established for a best and reproducible performance of the bioreactor. The inoculum must be uniformly distributed in the matrix structure for rapid and efficient immobilization. Rapid, uniform but controlled growth of the biofilm must be achieved. High biomass loading of the bioreactor must be attained. The handling of the culture for the various process requirements must be easy. Finally the long term operational reliability of the system must be ensured.

In addition, monitoring, predicting and optimizing the performance of the process will require the development of a suitable model, mostly empirical since the physiology of in vitro surface immobilized plant cells has never been studied nor their SM producing capability. This will be restricted to the growth kinetics of SIPC from inoculation to full biomass loading since the production of secondary metabolites is not within the scope of this research project. This growth kinetics model will permit predicting the SIPC concentration in the bioreactor in relation to operating conditions by monitoring significant measurable variables (carbohydrate consumption rate).

1.3.2.4 Assessing the Secondary Metabolite Production Potential of SIPC.

Finally, the production of secondary metabolites by surface immobilized Catharanthus roseus plant cells under medium stimulation will be assessed with product secretion in the medium.

2.0 MATERIALS AND METHODS.

2.1 The Biological Model Systems.

The main biological model system of this project was the plant Catharanthus roseus which produces a wide spectrum of indole alkaloids (178). Fewer are produced from cultured plant cells. The main alkaloids of interest were the monomers Ajmalicine, Serpentine, Catharanthine and Vindoline (Figure 1.1). Dimerization of the last two compounds gives the expensive Vincristine and Vinblastine which have never been produced by cultured plant cells (14).

Various Catharanthus roseus cell lines were generated at McGill according to the method summarized in Section 1.2.1.2 (43) from plant organs. Line MCR17 was initiated from part of a leaf on 84-09-05. The callus of plant cells produced was subcultured from that time on solid medium NB5 (43) (Appendix 2) every month. The suspension culture of this cell line was initiated on 84-11-13 in liquid medium 1B5 (43) (Appendix 2). This culture has been maintained since that time by weekly transfer (5 to 20% V/V) in this synthetic growth medium.

The SM productivity of line MCR17 cultured in the Alkaloid Production Medium (APM) (43) (Appendix 2) was found superior to other McGill Catharanthus roseus cell lines. It compared favorably to NRC-PBI producing line 953 (Table 2.1). Consequently this line has been selected as the main model system of this project.

Nicotiana tabacum (Tobacco) and Glycine max (Soybean) cell lines Su582 and SB1 were obtained from a callus initiated at NRC-PBI using the solid medium NB5. They were subcultured monthly as line MCR17. The culture in suspension of these plant cell lines was initiated on 86-09-12 in liquid medium 1B5. These cultures have been maintained since that time by weekly transfer (10-20% V/V) in this synthetic growth medium.

The three suspension cultures were grown in 500 mL Delong flasks containing 200 mL of liquid medium B5 supplemented with 2 mM of the growth hormone 2,4-dichlorophenoxyacetic acid and 20 g/L of sucrose (43). The pH value of this medium (1B5) was adjusted to 5.5 before sterilization (120°C, 15 psi, 1 hr). The same growth medium, except for sucrose concentration, were used for all flask and bioreactor SIPC cultures unless indicated otherwise.

TABLE 2.1

CATHARANTHUS ROSEUS CELL LINES ALKALOID PRODUCTIVITY

Plant Cell Line	Origin	Place of Culture	Medium	Alkaloids Content (µg/gr dried biomass)					
				S. Lactam	Tryptamine	Ajmalicine	Catharantine	Tabersonine	Vindolinine
New 953	PBI	PBI	APM(G)	549	-	1220	-	242	Traces
MCR17G	McGill	PBI	APM	158.6	-	1452.7	-	-	-
MCR17O	McGill	PBI	APM	359.2	73.1	616	-	-	-
MCR17	McGill	McGill	APM	158.7	44.5	511.5	-	-	-
MCR17	McGill	McGill	APM	315.7	91.3	310.5	Traces	-	-
MCR17	McGill	McGill	APM	361.9	37.1	400.3	-	117.9	-
MCR17	McGill	McGill	APM	244.3	210.2	350.2	-	92.1	Traces

S. Lactam: Strictosidine Lactam.

The weekly subcultured flask suspension cultures were maintained at 27°C under continuous illumination and agitation on a rotary shaker operating at 150 RPM. Typical growth curves of these suspensions are shown in Figures 2.1 and 3.28.

2.2 Selection of Surface Immobilization Materials.

Strips of materials evaluated for the surface immobilization of plant cells were sterilized in 70% ethanol, washed with sterile medium, placed in Petri dishes, covered with fresh medium 1B5 and inoculated with growing suspensions of either Soybean, Tobacco or Catharanthus roseus plant cells. The Petri dishes were maintained at 27°C and agitated at 50 RPM for 3 to 7 days. Selection criteria included visual assessment of the plant cell culture growth as well as qualitative evaluation of residual biomass attached to the support after a severe water wash.

2.3 Techniques of the SIM Culture in Flasks.

The selected support materials were cut into pieces of 8x8x30 mm. They were suspended in groups of 3 with stainless steel wires in 500 mL Delong flasks, sterilized (120°C, 15 psi, 1 h) with or without medium 1B5 (275 mL),

Figure 2.1 - Typical growth curve of C. roseus cells of line MCR17 grown in shake flask suspension culture under standard conditions.

▼ $\mu = 0.58\ d^{-1}$ ($r = 0.96$)

$Y_1 = 56.2\%$

△ $dCHO/dt = 3.4\ g/L.d$ ($r = 0.95$)

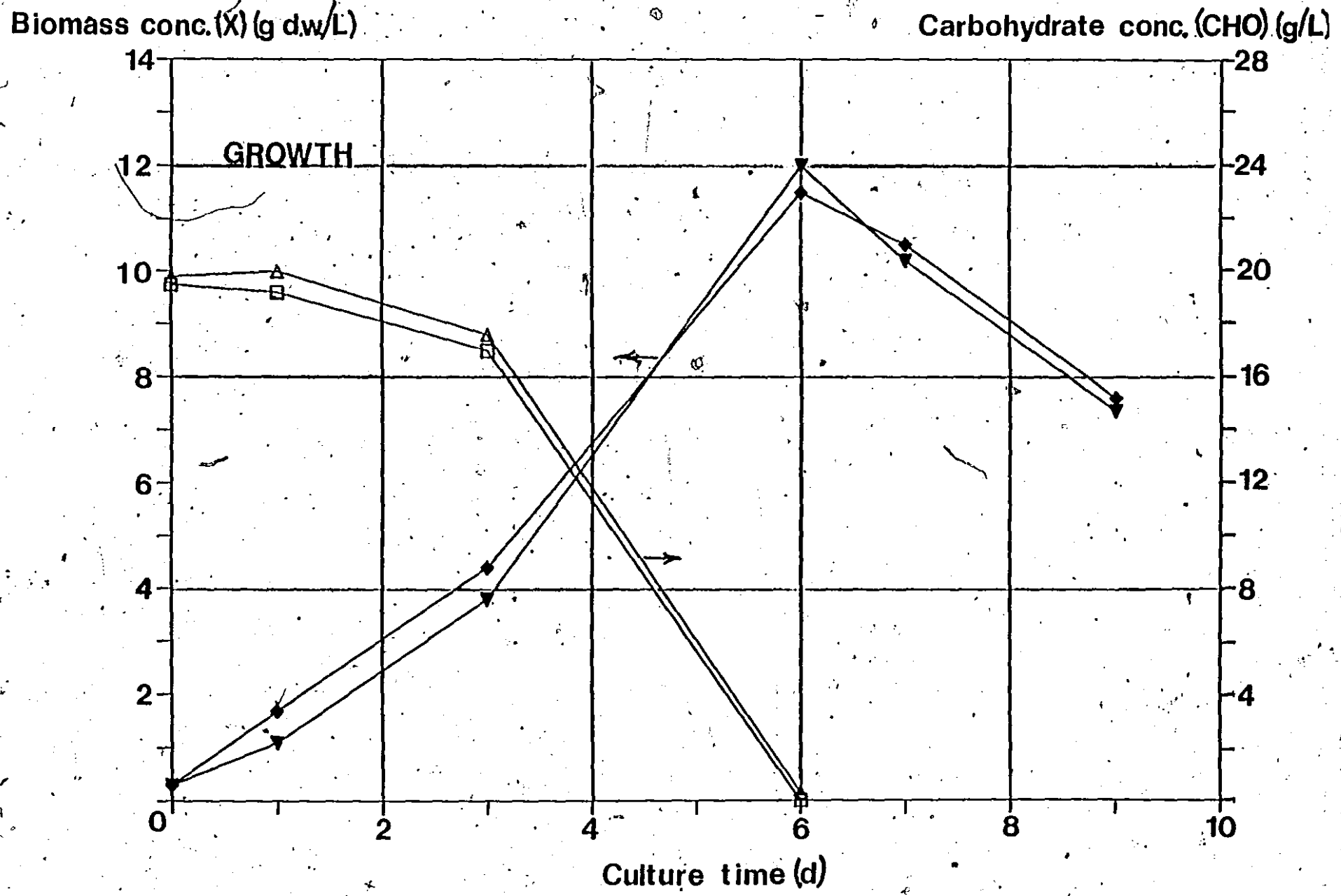
$dX/dt = 1.9\ g\ d.w./L.d$ ($r = 0.98$)

◆ $\mu = 0.53\ d^{-1}$ ($r = 0.94$)

$Y_1 = 54.0\%$

□ $dCHO/dt = 3.3\ g/L.d$ ($r = 0.96$)

$dX/dt = 1.8\ g\ d.w./L.d$ ($r = 0.99$)


Biomass conc. (X) (g d.w/L)
Carbohydrate conc. (CHO) (g/L)
GROWTH
14
12
10
8
6
4
2
0
28
24
20
16
12
8
4
0
2
4
6
8
10
Culture time (d)

which in the latter case was subsequently added. The flasks were inoculated (5-10%) with growing suspensions of plant cells and maintained at 27°C under continuous illumination for periods of 2 to 14 days at mixing speeds of 130 or 150 RPM on a rotary shaker.

In order to increase the available cell immobilizing area relative to the culture volume, a second plant cell surface immobilization flask system was developed. Strips of support material were formed into a spiral configuration of 4.0 cm outside diameter with 100 cm^2 total surface area. The wound support bundle was suspended in a widemouth Erlenmeyer flask (500 mL) containing 300 mL of medium 1B5. This culture vessel was sterilized, inoculated and maintained at 27°C as above. Agitation was by a magnetic bar (1cm×5cm) at 110 to 600 RPM. In certain cases, sterile air was passed through a submerged sintered glass sparger at a rate of ~ 5 mL/min. During the first day of culture, low mixing (110-125 RPM) was generally provided for efficient plant cell attachment. Subsequently the mixing rate was increased to the desired level.

2.4 Evaluation of the Adhesion Strength of SIPC.

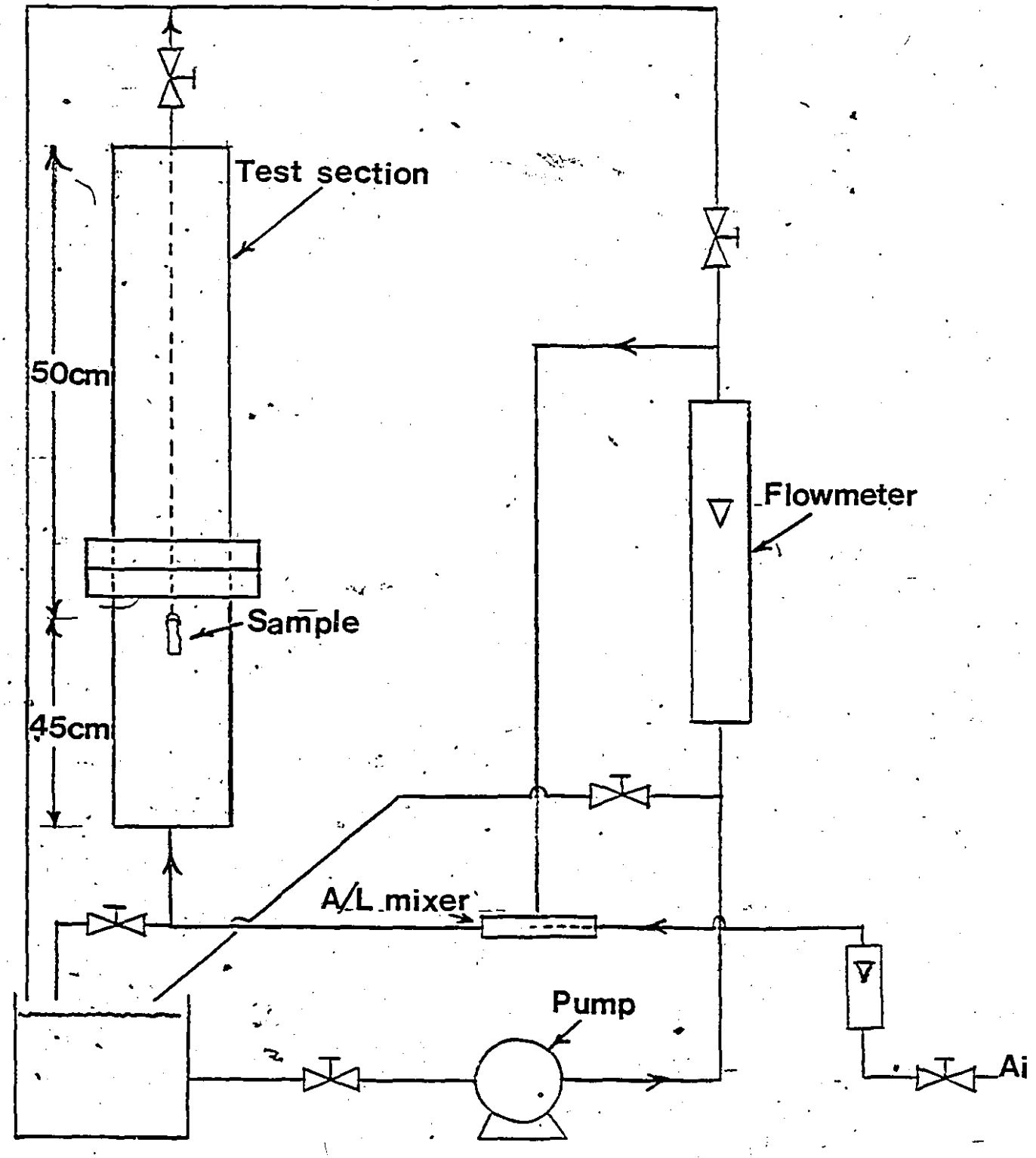
In order to assess the biomass adhesion strength, plant cells surface-immobilized according to the first technique

presented in Section 2.3 were subjected to various hydraulic shear and osmotic stresses simulating the bioreactor culture conditions for periods of 30 to 180 minutes. The test liquids were either water, growth medium 1B5 (sucrose concentration of 20 g/L), or the Alkaloid Production Medium (APM) (sucrose concentration of 50 g/L) (43).

During the first series of tests, SIPC samples (installed vertically) were exposed to each of three test liquids circulating at flows rates of 1.5 to 22 L/min in a 2.54 cm inside diameter column (Figure 2.2). These conditions correspond to flow of Reynolds number 1,000 to 15,000 relative to the column diameter. The second series of tests involved submitting SIPC to two-phase flow in the same apparatus (Figure 2.2) as for the first series. Air was injected at rates of 150 to 600 mL/min (gas superficial velocities of 0.5 to 2.0 cm/s) into each of the three test liquids circulating at rates comparable to the first series of tests.

During the third series of tests, SIPC samples were submerged vertically in a column of an internal diameter of 10 cm (Figure 2.3) containing each of the three test liquids. Air was sparged at rates of 200 to 8300 mL/min (superficial velocities of 0.04 to 1.8 cm/s).

Figure 2.2 — Flow test apparatus.



(Not to scale)

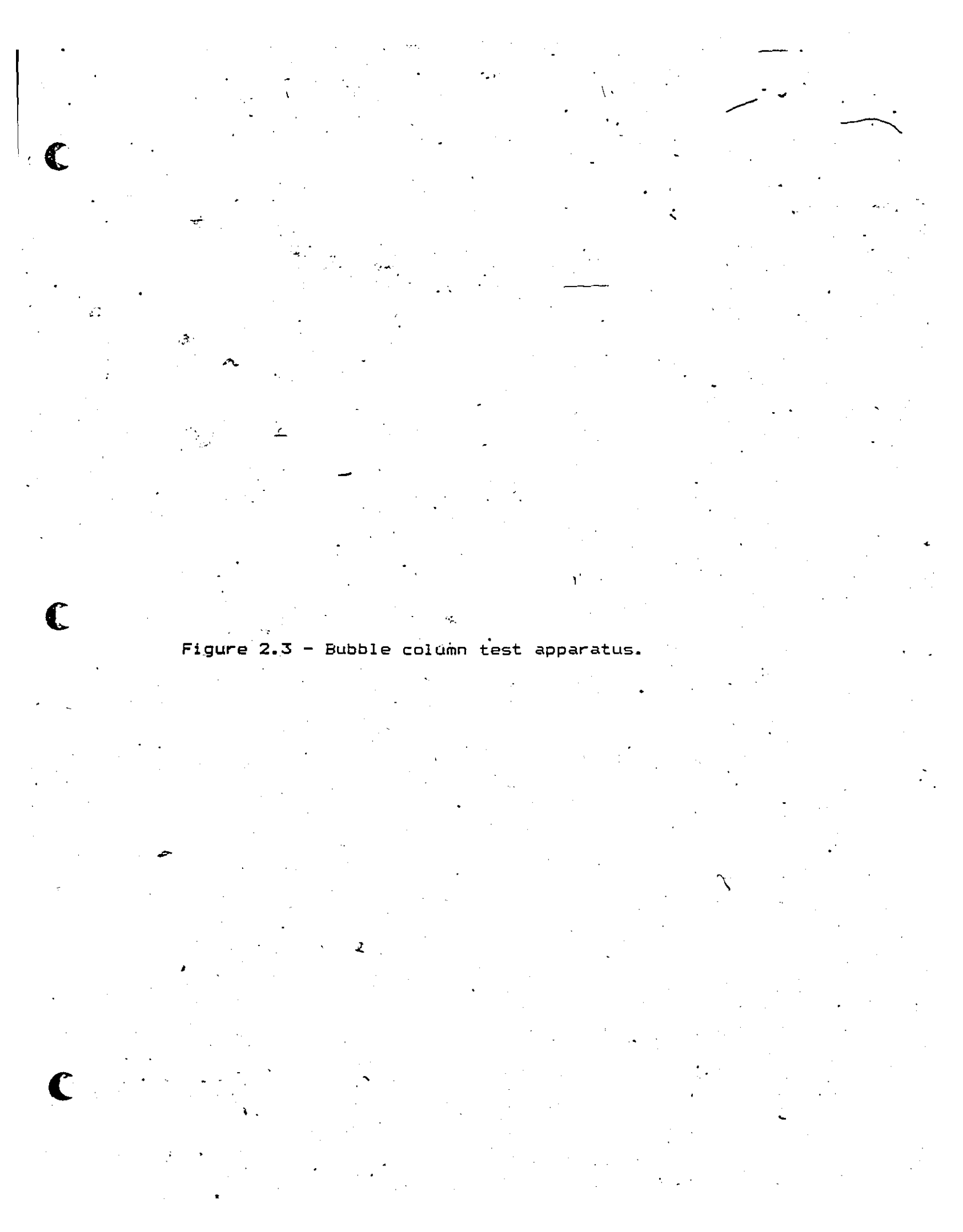
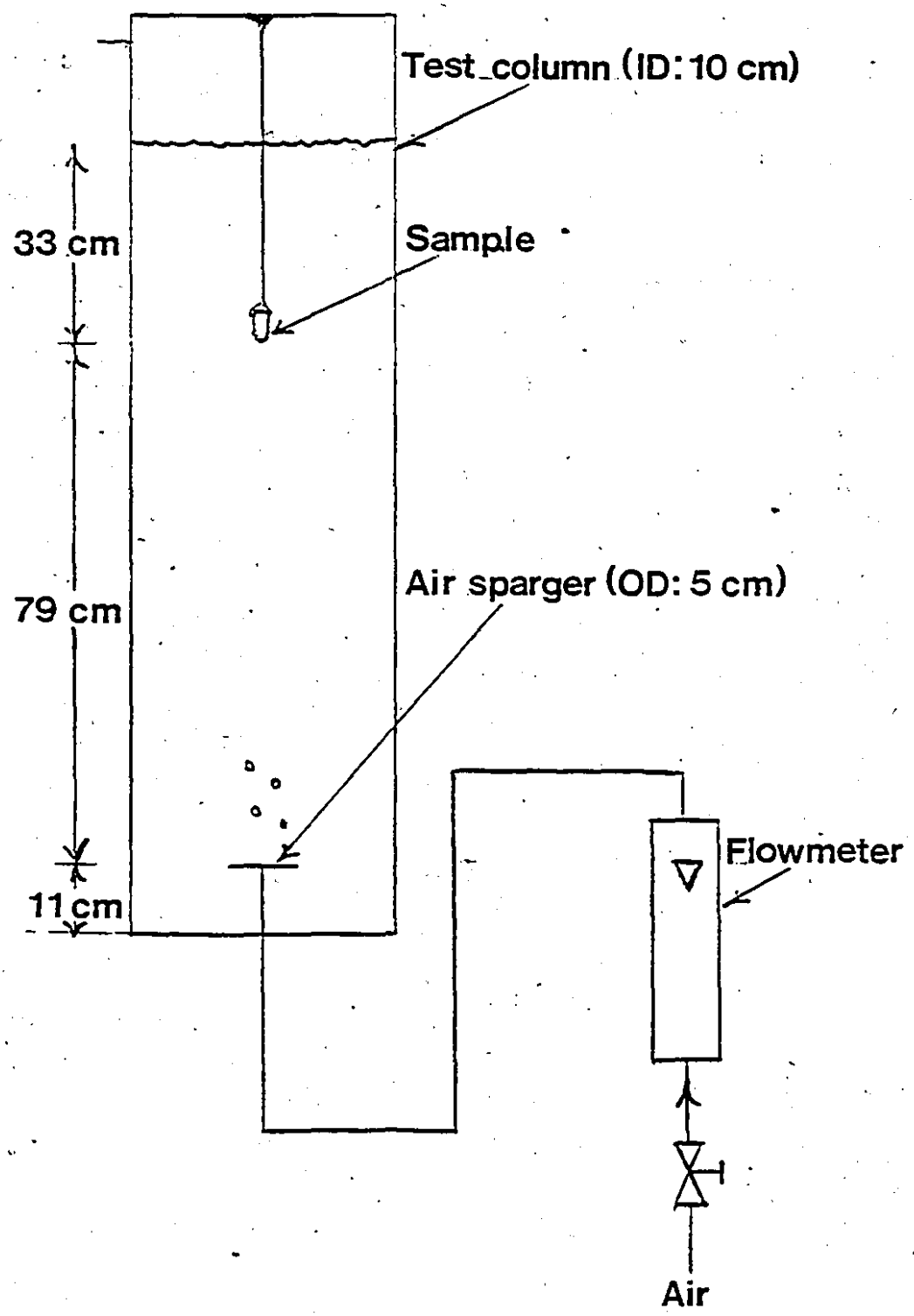


Figure 2.3 - Bubble column test apparatus.


Test column (ID: 10 cm)
33 cm
Sample
79 cm
Air sparger (OD: 5 cm)
11 cm
Flowmeter
Air
(Not to scale)

The strength of adhesion of SIPC is indirectly related to biomass leaking. This parameter was used to assess SIPC attachment to various supports. The residual biomass (dry weight) of stressed SIPC samples (i.e. the immobilized biomass loading of the sample) was compared to the loading of similarly grown but unstressed SIPC samples. These data were fully corrected for any flowing medium sucrose entrapment in individual support matrices. A percentage ratio was calculated that represents the variation of the SIPC loading caused by the hydraulic wash stressing. A 100% figure, for example, represents no significant leaking of SIPC.

2.5 Electron Microscopy Study of SIPC.

Plant cells of Catharanthus roseus, Soybean and Tobacco were immobilized on selected materials by the first technique presented in Section 2.3. Subsequently, they were either submitted to the various shear and osmotic stressing conditions described in Section 2.4 or not. They were fixed with glutaraldehyde, stained with osmium tetroxide, lead citrate and uranyl acetate and embedded in an epoxy resin according to the standard practice for Transmission Electron Microscopy (TEM) observations.

2.6. Analytical.

2.6.1 Plant Cell Viability.

Viability staining of SIPC was performed according to Widholm (179).

2.6.2 Chemical Analysis.

The carbohydrate composition of the filtered (0.45 µm) culture medium was analyzed with a Waters High Performance Liquid Chromatograph (HPLC) model 6000A-U6K equipped with a Refractive Index detector model R401. The separating column used was Biorad Aminex HPX-87. HPLC grade water was the liquid phase. The column was operated at 80 °C and at flow rates of 0.4 to 0.9 mL/min with a back pressure of 500 to 700 psig.

Secondary metabolites were extracted from the biomass according to the method presented in (180). Their identification and quantitative evaluation were performed by Thin Layer Chromatography (TLC) and HPLC. Indole alkaloids standards were obtained from NRC-PBI. The TLC plates used were Sigel 250 µm (Baker Scientific Ltd). Development and staining were done using respectively a 10%

solution of methyl alcohol in ethyl acetate and a 1% ceric ammonium sulfate solution in o-phosphoric acid.

The HPLC used for SM analysis were either the apparatus mentioned above equipped with a Spectra Physics Model 8300 UV detector set at 254 nm or a Spectra Physics Model 8000 system equipped with a variable wavelength UV detector. Separation was achieved with either a RP 3000 Aquapore 10 µm column or a RP8 Spheri5 5µm column supplied by Brown Lee Labs Ltd. The liquid phases were either a constant 60% (Waters HPLC) or a 55 to 68% gradient solution of HPLC grade methyl alcohol in water.

2.6.3 Dried Biomass Measurements.

Suspended plant cells were filtered (10 µm), washed with distilled water and refiltered under vacuum. They were weighed wet and dried overnight in an air circulating oven at 60 °C to assess dried biomass. Immobilized plant cells from flask studies were washed with distilled water, weighed wet and dried to constant weight (24-48 hrs) in the same oven to assess the dried biomass loading of the support material. The immobilized plant cell biomass loaded structure of a bioreactor was removed from the culture vessel at harvesting and drained. It was weighed wet and dried to constant weight (48-72 hours) in the same

circulating oven to assess the immobilized dried biomass loading (Appendix 3). The wet and dry weight of any free biomass was determined as above.

2.7 Measuring the Rheological Properties of a Plant Cell Suspension.

The rheological properties of a line MCR17 plant cell suspension (subculture 75) cultured as per Section 2.1 were measured according to Tanaka (74) with a Brookfield Model RVT rotation viscometer having five spindles. The reading of the viscometer (θ) shows shear stress in terms of the magnitude of the torque at shear rate in terms of the revolution of the rotor (W [RPM]). The equation of a Power Law Fluid is

$$\tau = K(du/dr_1)^n \qquad (1.1)$$

where τ is the shear stress ($g/cm.s^2$), du/dr_1 is the shear rate (s^{-1}), K is the consistency index ($g/cm.s^{2-n}$) and n is the flow behaviour index. This equation can be rearranged (74) as

$$\theta = \alpha_s K W^n \qquad (2.1)$$

where α_s is an instrument/spindle constant (74).

Appropriate log linear regressions of the viscometer readings for various speeds of the rotor and spindles give the K and n parameters of the model (Appendix 1). The apparent viscosity of the suspension was measured at 50 RPM with spindles 2 and 3 (Table A1.1).

All measurements were made at 27°C. For each spindle, a set of readings was rapidly (< 5 minutes) taken for 4 to 7 rotor speeds (0.5 to 100 RPM) from low to high speeds. Between each set of measurements, the suspension was mixed to minimize settling effects. For each speed, three to five readings were taken, which were generally consistent to ± 3%.

2.8 Measuring of the Mass Transfer Characteristics of the SIPC Bioreactors.

A relative mixing time (θ_m) and the oxygen transfer coefficient (k_La) of both SIPC bioreactors (IP and IA, see Section 3.2.2) were measured by the techniques described in the next two Sections. For Bioreactor IA, the B matrix structure (see Table 3.7) was used with Material A07. The resulting reactor downward flow area consisted of 4 matrix spacings (termed A to D, from the exterior to the interior of the structure) and of the peripheral spacing between the vessel wall and the matrix external layer. The measuring

probe (pH or DO) was placed in any of these five spacings at a height of 13.5 cm from the reactor bottom. A similar arrangement (4 matrix plus 1 peripheral spacings) was used for Bioreactor IP. In this case, the pH electrode was placed in any spacing at a height of 7.4 cm from the bottom of the reactor. The small size of Bioreactor IP (2L) prevented installing the DO probe in any matrix spacing. Consequently this probe was installed inclined on top of the matrix structure.

2.8.1 Relative Mixing Times.

A relative mixing time was measured for both SIPC bioreactors by the acid tracer method (81) using an Ingold Model A420 pH electrode, a Cole Parmer Model 5997-20 pH controller with a 0-2 Volts output signal and a Linear Model 1100 chart recorder set at an input signal of 0-2 Volts and at appropriate chart speeds. The pH electrode was calibrated before each series of tests. This calibration was found not to change after each series. The response time of this system was sufficiently fast ($\leq$ 1 s) as compared to the range of relative mixing times measured (10-200 s). All air flow meters were calibrated with a wet test meter. Mixing speeds (Bioreactor IP) were measured with a stroboscope.

For each test, the reactor, arranged in a given configuration (matrix, flow diverting plate level and pH electrode location), was filled to the desired level with tap water (18°C). Operating conditions were set (filtered air flow rate and mixing level for Bioreactor IP). The pH value of the water was adjusted to 5.5 with a 3N HCL solution. At time zero of the test, a given volume (2 mL and 0.5 mL respectively for Bioreactors IA and IP) of the HCL solution necessary to attain a final 3.4 ± 0.2 value of the water pH was rapidly added from the top of the reactor. Simultaneously the chart recorder was started.

A typical response curve is shown on Figure 2.4. A lag preceedes a smooth decreasing curve. Either the measuring system, the speed of the tracer diffusion/mixing and/or the reactors configuration prevented measuring a circulation time. The relative mixing time measured (θm) is defined as the time required to attain 95% of homogeneity (81). Three to five measurements were made for each operating condition with variations of ±5%.

2.8.2 Oxygen Transfer Coefficients.

The volumetric transfer rate of oxygen from the sparged air to the liquid phase is given by (181)

Figure 2.4 – Typical relative mixing time response curve.

Conditions:

Bioreactor IA.

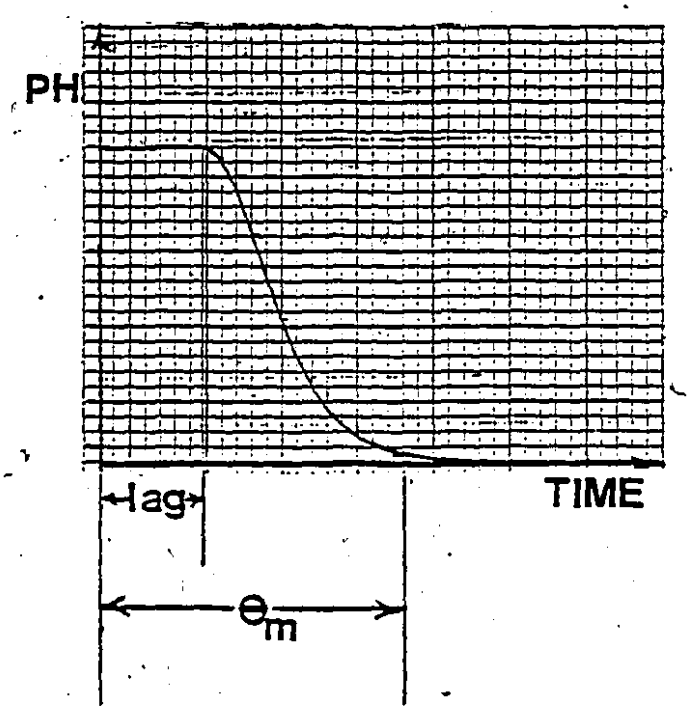
pH electrode at 13.5 cm from the reactor bottom located between the first and second internal immobilizing structure layer.

Liquid volume: 5.2 L.

Liquid level: at the flow diverting plate height (2.54 cm above riser tube or 30.5 cm from bottom).

Air flow rate: 8.1 L/min (1.6 VVM).

Chart recorder speed: 6 cm/min.


PH
TIME
lag
θ_m

$$dC_{O_2}/dt = k_La(C^*_{O_2} - C_{O_2}) \qquad (2.2)$$

where $C^*_{O_2}$ is the dissolved oxygen (DO) concentration (mM/L) in the liquid, C_{O_2} is the dissolved oxygen concentration in in the liquid at saturation in equilibrium with the gas phase and k_La is the volumetric overall oxygen transfer coefficient (h^{-1}). This equation can be rearranged as

$$C_{O_2} = -\left(\frac{1}{k_La}\right)\left(\frac{dC_{O_2}}{dt}\right) + C^*_{O_2} \qquad (2.3)$$

which gives a convenient way of determining k_La by measuring C_{O_2} following a step change in the air (oxygen) supply to the liquid phase. A plot of C_{O_2} against dC_{O_2}/dt (the rate of change in dissolved oxygen concentration) gives a linear relationship with a slope equal to $-1/k_La$.

The k_La of both bioreactors was determined using distilled water (26°C, pH ~ 3) with a Yellow Spring Instrument Co Ltd Dissolved Oxygen Probe and the associated Meter Model 54BP. The meter was linked to the chart recorder used for the mixing time measurements set at appropriate conditions. The DO probe was calibrated in air (8.3 ppm at 27°C) as suggested by the manufacturer. This calibration did not change significantly during the experiments. The response time of this measuring system

was sufficiently fast (~ 1-2 s) relative to the oxygen transfer rates measured ($\leq$ 30 h^{-1}) (80). The DO probe was installed either reversed (Bioreactor IA) or inclined (Bioreactor IP) in the vessels to prevent direct air bubble contact which hinders proper measurement.

For each test, the reactor was arranged in a given configuration (matrix, diverting plate level and DO probe location) and filled to the desired level with distilled water. This water was purged of its dissolved oxygen by oxygen free nitrogen at the required operating conditions. (filtered nitrogen flow rate and mixing level for Bioreactor IP) until a minimal residual DO concentration was reached (~ 0.2 - 0.8 ppm). At time zero of the test, the gas source was rapidly changed from nitrogen to air (maintained at the same flow rate) and the chart recorder was started.

A typical response curve is shown in Figure 2.5. A lag preceedes a smooth rising curve. The k_La values were calculated from these curves according to Equation 2.3. The experimental data of any experiment could be linearly correlated as per (Equation (2.3)) with correlation coefficients (r) higher than 0.85 for 5 to 7 points. The k_La values reported are the averages of 3 to 5 measurements, which varied by ± 7% or less.

Figure 2.5 - Typical response curve of the oxygen absorption by water.

Conditions:

Bioreactor IA.

DO probe at 13.5 cm from the reactor bottom and located in the peripheral spacing.

Liquid volume: 5.2 L.

Liquid level: at the flow diverting plate height (2.54 cm above the riser tube or 30.5 cm from bottom).

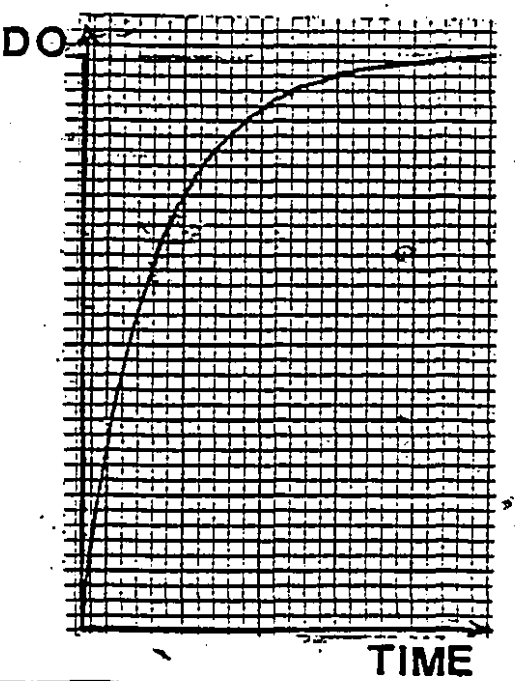
Air flow rate: 5.2 L/min (1 VVM).

DO_o = 0.2 ppm.

DO_* = 6.15 ppm.

Chart recorder speed: 15 cm/hr.

k_La = 23.5 hr^{-1} C^*_{O2} = 5.7 ppm r = 0.85


DO
TIME

3.0 RESULTS.

3.1 Development of the Surface Immobilization Technique.

3.1.1 Selection of Surface Immobilization Materials.

Different selected materials were tested by the technique presented in Section 2.2 for toxicity, growth and surface attachment of C. roseus, Tobacco and Soybean plant cells. They were metals, plastics and ceramics of various natures and surface textures (Appendix 4). Most non-metallic supports were not toxic and allowed cell growth.

The plant cells attached only to three types of materials. A porous but fragile ceramic matrix retained cells efficiently. A rigid and porous matrix made of synthetic fibres bound by polymeric resins (W8 and Q8) and a series of fibrous materials (A07, A09 and A12) were shown to attach plant cells in a superior way. These last two types of supports are characterized by their fibrous and extremely irregular surface texture. The best representatives of the last two types of materials were selected for further study.

3.1.2 Characterizing Flask Cultured SIPC.

3.1.2.1 The Mechanism of Biomass Attachment.

Plant cells culture-immobilized on Material W8 in shake flasks, as described in Section 2.3, were treated for TEM study as described in Section 2.5. Results are shown in Figures 3.1 to 3.7. They show surface-immobilized plant cells to be highly vacuolated and round. Extremely good and close contact between the plant cell wall and the support surface of Material W8 can be observed. At this contact area, the cell wall adapts to the support configuration. There is evidence of some compounds being secreted by the plant cell and accumulating between the cell wall and the support. A substantial layer of this material can be observed at the cell attachment edges where the cell wall is not in close contact with the support. These compounds seem to be acting as a gap filler and/or glueing agent. Closeups of the Tobacco cell wall and support contact area show the arrangement of this compound in strands different from either the cell wall or the W8 Material and parallel to the interface (Figure 3.7).

The surface immobilized plant cells were fully viable as shown by the fluorescein diacetate staining technique of Widholm (179) mentioned in Section 2.6.1. The TEM results

Figure 3.1 - A, B - Catharanthus roseus (PBI Line 953) cells (PC) on support Material W8(M) at a magnification of 3785.
The plant cell wall (CW) is flattening at the contact area with the support material surface. Extracellular material (C_x) is accumulating between the cell wall and the support surface (See Figure 3.3).

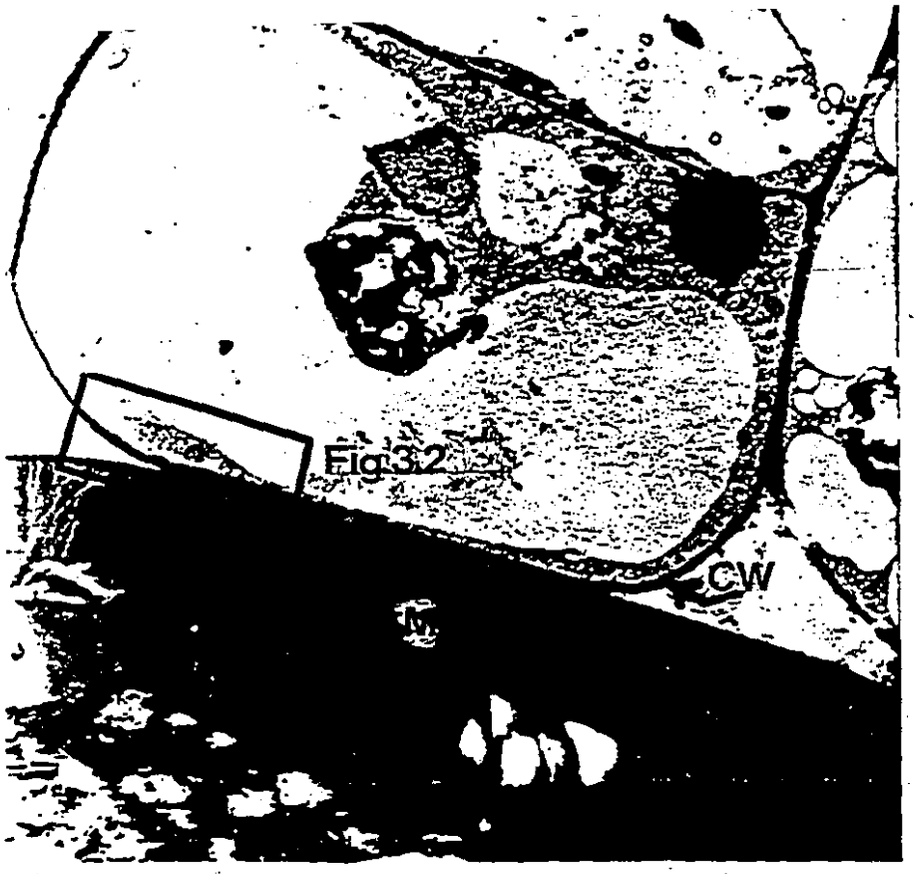


Fig 3.2
CW

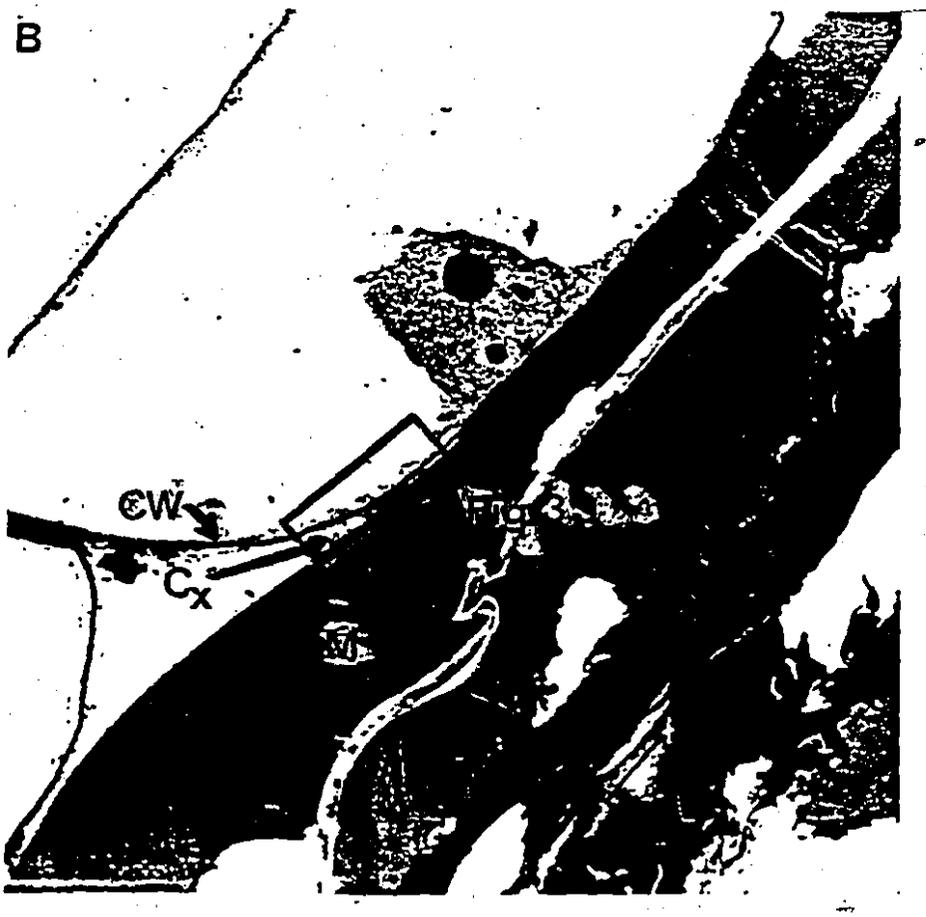

B
CW
Cx

Figure 3.2 - Closeup of Figure 3.1 (A).

Catharanthus roseus (PBI Line 953) cell wall (CW) on support Material W8 (M) at a magnification of 19360.

Intimate contact of the cell and the support material is well seen.

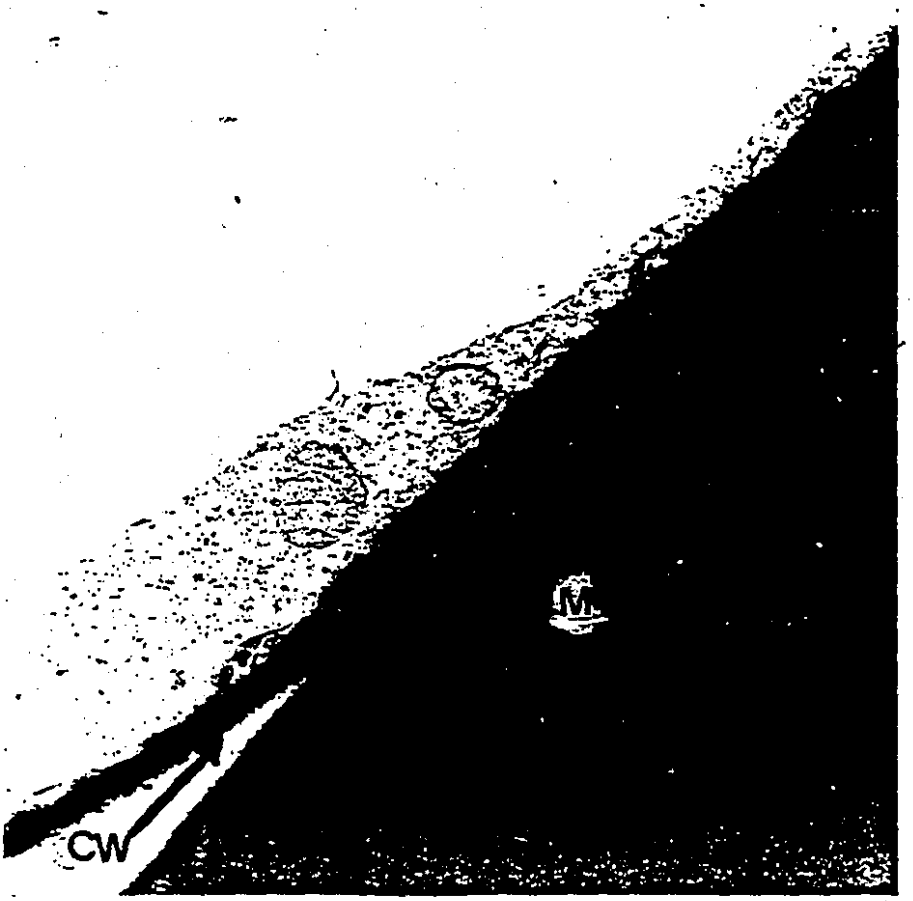

M
CW

Figure 3.3- Close-up of Figure 3.1 (B).

Catharanthus roseus (PBI Line 953) cell wall (CW) on support Material W8 (M) at a magnification of 19360.

The plant cell wall (CW) is seen approaching the support material. The extracellular material (C_x) is accumulating between the cell wall and the support surface.

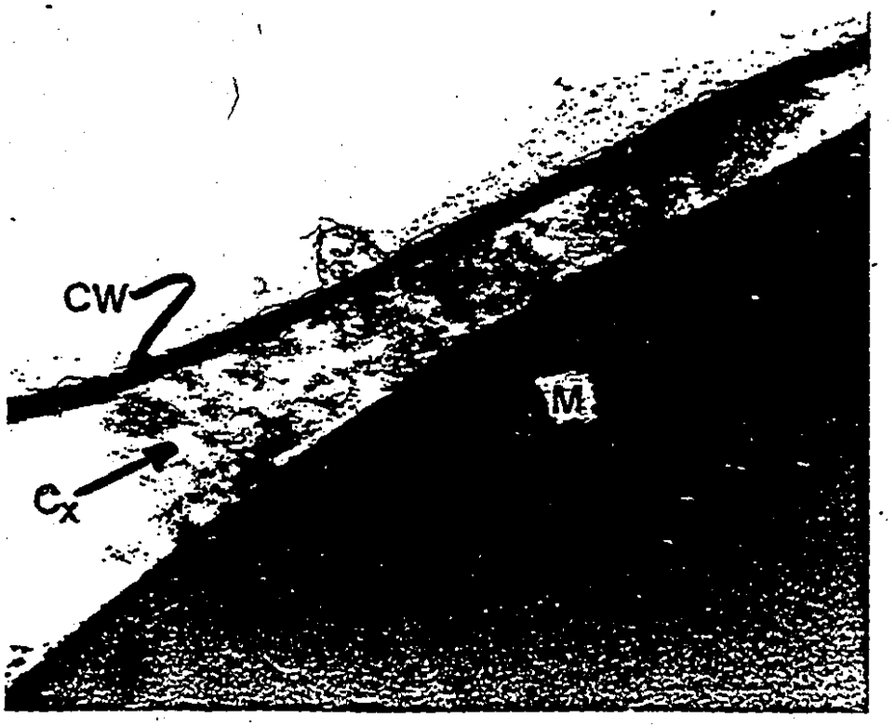

CW
M
Cx

Figure 3.4 - Soybean (PBI Line SB1) cells (PC) on support Material W8 (M) at a magnification of 3785. As in Figure 3.1 for the Catharanthus roseus plant cell, the close contact between the cell wall (CW) and the support surface is noticeable. The cell wall flattened in the contact area and the extracellular material (C_w) accumulated between the cell wall and the support surface (See Figure 3.5).

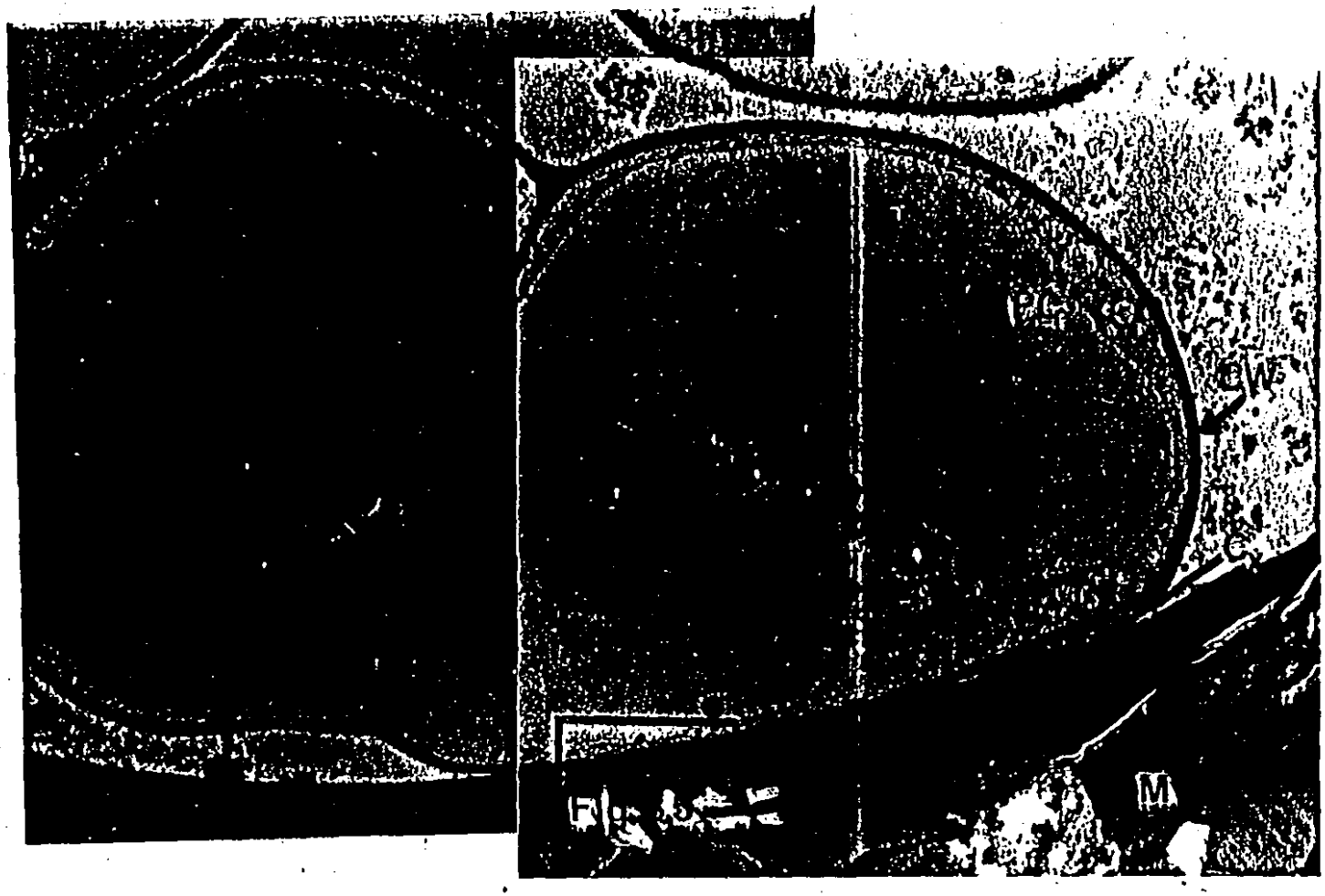


Figure 3.5 – Closeup of Figure 3.4.

Soybean (PBI Line SB1) cell wall (CW) on support Material W8 (M) at a magnification of 19360.

The cell wall (CW) and the support surface are in a close contact. The accumulated extracellular material (C_x) at this interface is of a different nature than that of the cell wall.

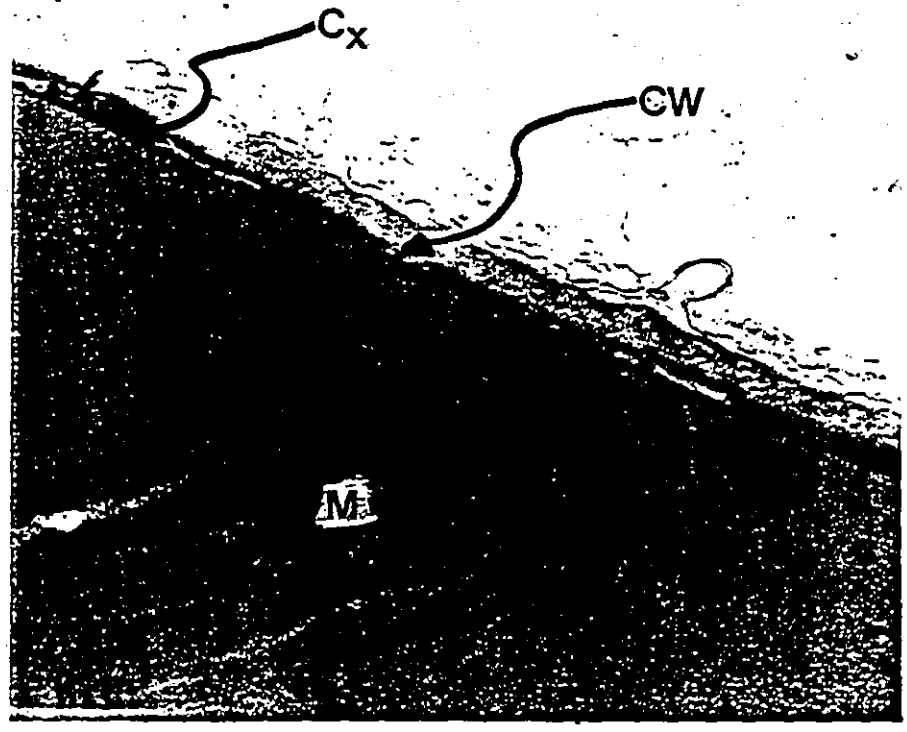

Cx
CW
M

Figure 3.6 - Tobacco (PBI Line Su1277) cell (PC) on support Material W8 (M) at a magnification of 3785. As in Figures 3.1 and 3.2, the close contact between the cell wall (CW) and the support surface can be observed. The cell wall at this contact area is flattened. Extracellular material (C_w) is accumulating between the cell wall and the support surface (See Figure 3.7).

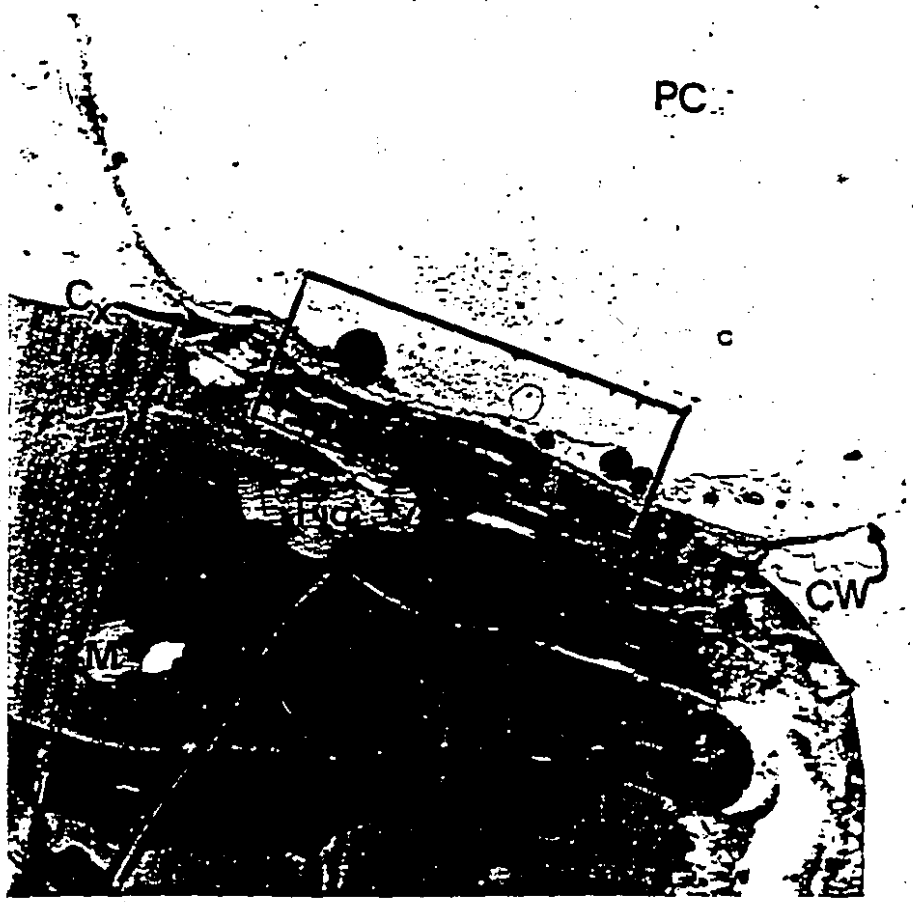
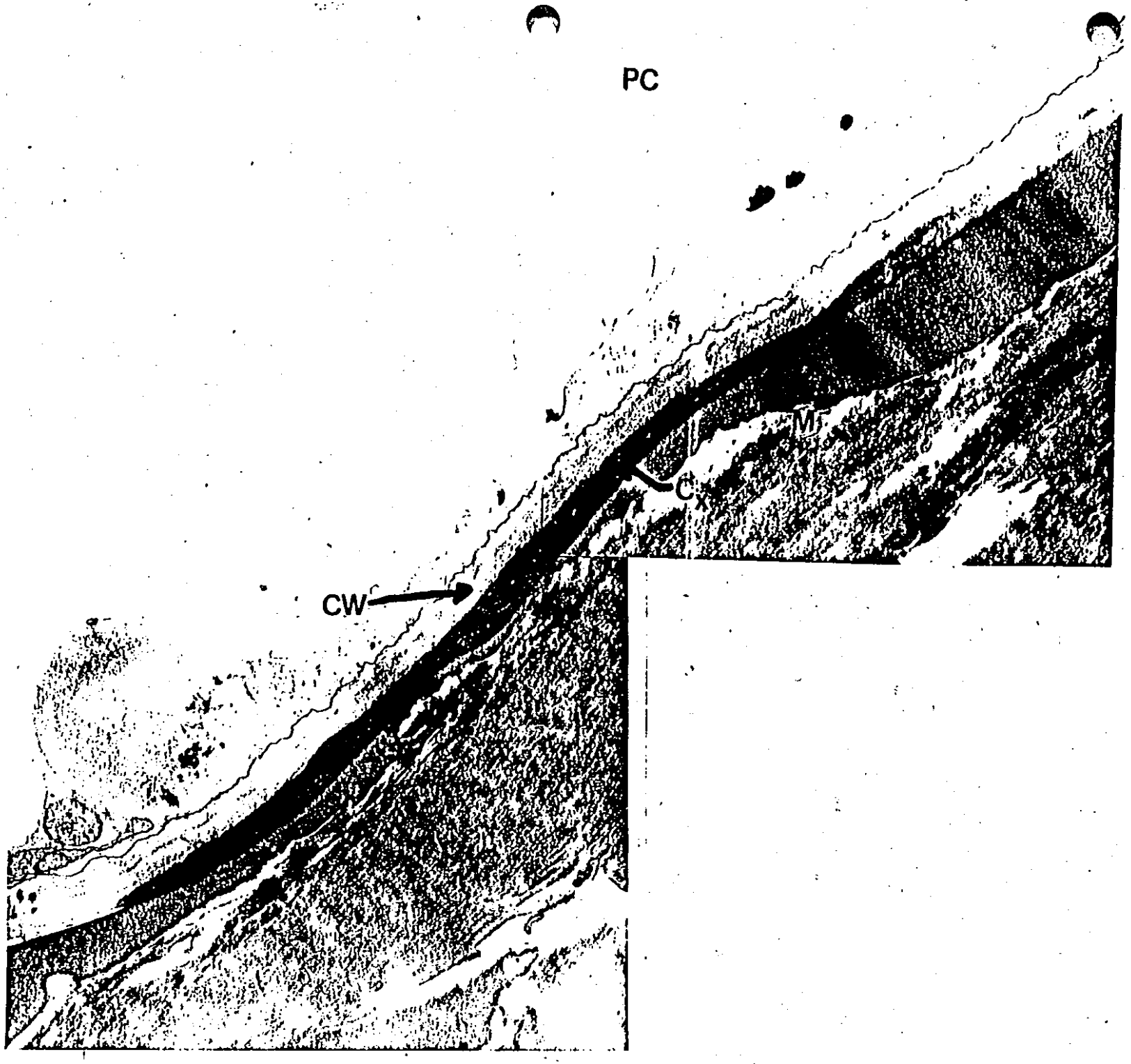

PC
C
CW

Figure 3.7 – Closeup of Figure 3.6.

Tobacco (PBI Line Su1277) cell wall (CW) on support Material W8 (M) at a magnification of 19360.

The closeness of the cell wall-support surface as well as the accumulating extracellular material (C_x) at this interface can be easily seen.


PC
M
CX
CW

of Figures 3.1 to 3.7 show structurally intact plant cells at the surface of the support material underneath the whole biofilm. This indicates that autolysis of these cells has not occured. The viability of SIPC was subsequently confirmed by the numerous growth experiments performed upon scaling up this technique to flask cultures and to laboratory size bioreactors (see Sections 3.1.2.3, 3.3.1 and 3.3.2). In fact, this viability was dramatically confirmed during experiment IA-11 (see Section 3.4 and Table 3.21). A suspension culture of viable plant cells of line MCR17 was introduced in Bioreactor IA and successfully immobilized and grown for 13 days according to the standard procedure developed (see Section 3.3.2.1). At this time, a sterile and dead fungal elicitor preparation was added to the culture (280 g of elicitor preparation or 5% of the culture volume) as per the recommended protocol (114). The characteristic browning reaction of an elicited culture occured within 8 hours after the addition. However, the medium was not changed, contrary to recommendations (114), after 24 hours from elicitation. Consequently all plant cells died (black biomass and medium, cell debris in the medium which became alkaline) after 36 to 48 hours from elicitation. This resulted in complete detachment of the biomass from the immobilization matrix during this period. Two other similar experiments (IA-13 and IP-47, Table 3.21) were carried out with the appropriate medium change. In

these cases, the plant cell biomass remained active (carbohydrate consumption and culture appearance) and attached to the immobilizing matrix.

3.1.2.2 The Strength of the Biomass-Support Bond.

A crucial criterion for selecting a suitable material for plant cell surface immobilization is the strength of the biomass-support material bond. The leakage of biomass out of the immobilizing matrix, the main limiting factor of this type of immobilizing technique, is directly related to this parameter. This was indirectly evaluated by the method presented in Section 2.4 for Soybean, Tobacco and Catharanthus roseus cells immobilized on Material W8 in shake flasks as described in Section 2.3.

The first two series of tests involved submitting SIPC samples to one or two phase flow circulating respectively at 1.5 to 22 L/min and 150 to 500 mL/min (superficial gas velocities of 0.5 to 2.0 cm/s) for the liquid and gas phases in a 2.54 cm column. These conditions correspond to a laminar boundary layer surrounding the SIPC samples (sample surface Reynolds number range of 13,000 to 25,000) and to calculated surface shear stresses of 0.5 to 2.6 N/m^2 (182-185).

Under these conditions, the immobilized biomass loading varied from 35 to 195% with an average of 98% for the Soybean and Catharanthus roseus cells regardless of the exposure time and test medium.

During the third series of tests, SIPC samples were suspended in a stagnant liquid column of a 10 cm diameter in which air was bubbled at 200 to 8300 mL/min (superficial velocities of 0.04 to 1.8 cm/s). The objective of this series of tests was to assess the effect of air bubble attrition on the SIPC biomass.

Under these conditions, the immobilized biomass loading varied from 70 to 200%, with an average of 100% for cells of Soybean and Catharanthus roseus, regardless of air flow rate, exposure time or test medium. Immobilized Tobacco cells performed less satisfactorily during both series of tests.

The spread of these figures come from the variation in the initial loading of SIPC and the absorption by the matrix and biomass of nutrients from the testing medium. However, these results and visual examination permit to conclude that plant cells remain attached strongly to this matrix under the high shear stress and air bubble attrition conditions of these tests.

3.1.2.3 Biomass Loading Performance of SIPC Cultured in Flasks.

The following parameters were investigated for line MCR17 to assess the biomass loading performance of this immobilization technique and to define scale-up design criteria.

1- Maximum biomass capacity of the two types of material selected.
2- Wet volume occupied by the immobilized plant cells.
3- Efficiency of the attachment process.
4- Growth rate of surface-immobilized plant cells.

The surface immobilization of growing plant cell suspensions cultured in shake flasks resulted in relatively uniform biomass loading of the support materials tested.

Various factors were evaluated to maximize the biomass loading. Results are presented in Table 3.1. Loading of plant cells on the support surface depended mainly on the immobilizing material, on the inoculum age and on the mixing speed. The combination of gentle mixing and Materials A07, A09 and A12, which differ only in their thickness, resulted in (200-400%) higher biomass loadings than those observed with Materials W8 and Q8. The A series

TABLE 3.1

SURFACE IMMOBILIZATION OF C. ROSEUS PLANT CELLS IN SHAKE FLASKS

Material	Material Sterilized With Medium	Inoculum		Culture Time (d)	Shaker Speed (RPM)	Suspended Biomass (g d.w./L)	Residual Carbo-hydrates (g/L)	Final pH	Biomass Yield	% Of Biomass Attached	Biomass[1] Loading (mg d.w. /cm^2
		% Of Medium	Age (d)								
W8	No	9%	6	15	130	8.4	0	5.5	38.1%	3.6%	2.7
Q8	No	10%	8	12	130	3.8	11.0	5.7	46.8%	26.0%	10.6
Q8	No	4%	10	16	130	8.7	3.9	5.3	64.0%	13.1%	6.0
Q8	No	7%	13	14	130	5.8	6.1	5.8	55.5%	25.1%	10.5
Q8	No	7%	14	14	150	2.0	12.8	5.5	55.3%	50.9%	12.6
A07	Yes	7%	6	13	130	6.8	6.6	5.5	54.5%	9.4%	8.0
A07	Yes	7%	6	14	150	7.5	0	5.7	38.9%	7.1%	6.0
A07	Yes	7%	12	14	150	8.3	0	5.3	45.7%	7.9%	6.1
A07[2]	Yes	4%	13	14	150	8.1	0	5.4	42.0%	1.7%	1.6
A07	No	4%	12	14	150	7.9	0	5.4	43.4%	7.9%	5.7
A09	Yes	7%	6	13	130	3.5	10.9	5.2	48.7%	19.6%	9.0
A09	Yes	7%	6	14	150	8.2	0	5.7	43.2%	7.9%	5.7

TABLE 3.1 (CONT'D)

SURFACE IMMOBILIZATION OF C. ROSEUS PLANT CELLS IN SHAKE FLASKS

Material	Material Sterilized With Medium	Inoculum		Culture Time (d)	Shaker Speed (RPM)	Suspended Biomass (g d.w./L)	Residual Carbo-hydrates (g/L)	Final pH	Biomass Yield	% Of Biomass Attached	Biomass[1] Loading (mg d.w./cm^2)
		% Of Medium	Age (d)								
A12	Yes	7%	6	13	130	6.8	5.7	5.6	56.0%	22.2%	9.5
A12	Yes	7%	6	14	150	8.4	0	5.7	45.7%	9.5%	6.6
A12	Yes	4%	10	14	130	4.9	8.3	5.3	58.6%	29.9%	16.3
A12	Yes	4%	10	14	130	4.9	7.7	5.3	63.2%	35.6%	20.7
A12[3]	Yes	4%	10	14	130	5.1	6.9	5.4	51.1%	29.8%	17.0
A12	Yes	7%	14	14	150	7.7	0	5.6	41.9%	7.3%	4.7
A12	Yes	7%	14	14	150	7.4	0	5.5	40.6%	8.2%	5.6

(1) SIPC dried biomass loading mg d.w./cm^2 of support area (average of 3 results).
(2) Free floating support.
(3) Culture without illumination.

Materials can also be sterilized in the medium without inhibiting the subsequent growth of plant cells.

The inoculum age is a crucial factor in increasing biomass loading. Inocula 8 to 14 day old gave the best results. Increasing the mixing speed from 130 to 150 RPM improved marginally (20%) biomass loading on the Q8 Material while reducing it substantially on the A07, A09 and A12 Materials.

During the experiments, it was observed that the surface immobilization of plant cells on free floating support is not efficient. Light did not affect the surface adhesion of cells. However, the SIPC culture growth must have encountered some kind of restriction because an incomplete carbohydrate consumption was observed at biomass loadings higher than 3 mg d.w./cm^2 when the mixing speed was 130 RPM. Plant cell suspensions cultured under identical conditions resulted in complete carbohydrate consumption at the 12th day of culture ($\mu \sim 0.24\ d^{-1}$).

The highest loading attained was 20.7 mg d.w. per cm^2 of Material A12. The thickness of this biomass layer was of the order of 5 mm, which gave a wet-to-dry biomass ratio of 25 consistent with the 95% hydration level of plant cells.

The efficiency of biomass deposition on the support material (i.e. the amount of SIPC over the total quantity of biomass present in the culture vessel) reached 25 to 50% in the shake flask system where the immobilizing area was 33 cm^2 relative to a suspension volume of 300 mL (A/V ~ 0.11 cm^{-1}). The large quantity of plant cells produced and not attached to the support has masked some other characteristics of the shake flask SIPC, for example in distinguishing between the adhesion and growth processes.

Much higher attachment efficiencies (75-99%) were attained in the second flask SIPC culturing system employing magnetic bar agitation with a A/V ~ 0.3 cm^{-1} (see Section 2.3). Complete immobilization of the inoculum occurred generally within the first or second day of culture. The medium was completely free of cells until harvesting, with the little free biomass produced either attached to the flask or accumulating in the small foam layer in the air-sparged system.

The superior immobilization efficiency of the technique was later confirmed upon scale-up to the laboratory bioreactors described in Section 3.2. Almost complete immobilization (> 95%) was consistently observed in these larger vessels (Sections 3.3.1.2 and 3.3.2.2). The medium was free of suspended plant cells within 1 or 2 days after

inoculation and until the end of the culture. The little amount of non-attached biomass present in the culture vessel accumulated mainly in the small foam layer on top of the medium.

Results of culture experiments in the magnetically stirred flask system are presented in Table 3.2. High biomass loadings and uniform cell coverage were observed. The final medium pH was lower (Average of 97 experiments: pH = 5.0, s = 3.0) than in normal plant cell suspension cultures (pH ~ 5.6-5.8) and SIPC cultured in shake flasks. Restricted growth and altered metabolism of the SIPC were indicated by the low average growth rates (μ ~ 0.10 d^{-1}) and biomass yields (19-30%) as well as by incomplete carbohydrate consumption, as compared to suspension cultures (μ ~ 0.24 d^{-1} and yield ~ 50-60% at 130 RPM).

An agitation rate of 125 to 200 RPM (Impeller Reynold Number 5200-8300 or a tip speed ~ 33-52 cm/s) seems to represent a threshold mixing level at which mass transfer is sufficient to insure maximum growth rate. This is illustrated in Figure 3.8. Plant cells remained firmly attached to the support material even at a mixing speed of 600 RPM (Impeller Reynolds Number of 25000 or tip speed of 157 cm/s).

TABLE 3.2

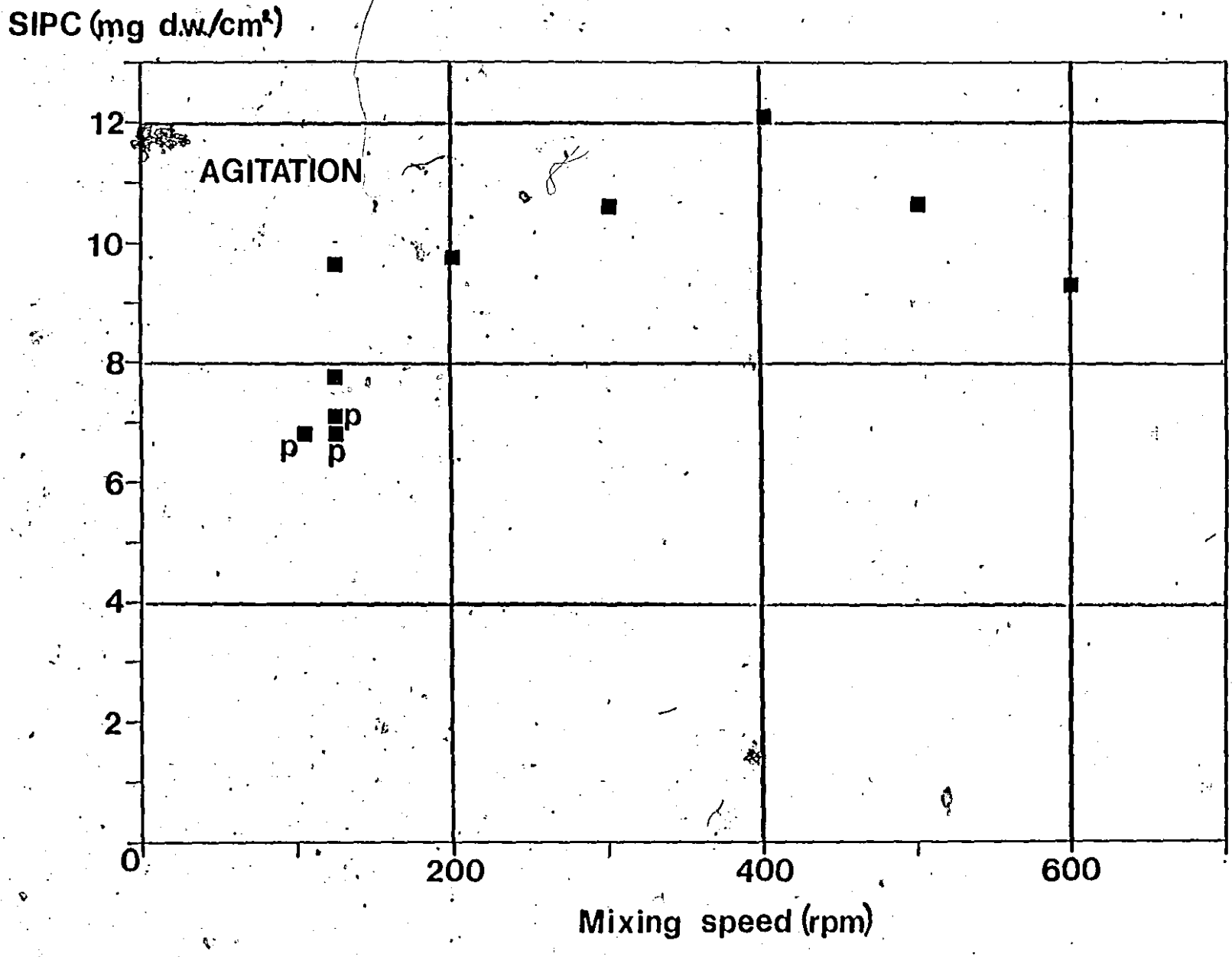
SURFACE IMMOBILIZATION OF C. ROSEUS PLANT CELLS ON MATERIAL A12 IN THE MAGNETIC STIRRER SYSTEM

Inoculum		Aeration	Mixing Speed (RPM)	Culture Time (d)	Residual Carbohydrates (g/L)	Final pH	Biomass Yield	% Of Biomass Attached	Biomass Loading (mg d.w./cm^2)	Average Growth rate	
% Of Medium	Age (d)									(d^{-1})	$(\frac{mg\ d.w.}{cm^2 d})$ [1]
10%	9	Periodic [2]	110	13	16.2	4.8	22.5%	99.5%	6.8	0.05	0.25
7%	10	Sparging	125	14	10.6	5.1	30.6%	99.5%	7.8	0.08	0.38
10%	10	Periodic	125	14	14.3	4.8	18.9%	97.2%	7.1	0.06	0.29
10%	10	Sparging	125	14	10.6	5.0	24.3%	91.8%	9.7	0.08	0.48
10%	10	Periodic	125	14	15.0	5.1	24.9%	96.6%	6.8	0.07	0.29
10%	10	Sparging	200	14	8.8	5.4	29.5%	78.8%	9.8	0.09	0.51
10%	10	Sparging	300	14	8.1	5.4	28.2%	88.6%	10.6	0.10	0.57
10%	10	Sparging	400	14	7.4	5.4	29.2%	89.4%	12.1	0.10	0.67
10%	10	Sparging	500	14	8.1	5.3	27.5%	90.1%	10.7	0.10	0.58
10%	10	Sparging	600	14	6.9	5.0	26.8%	96.3%	9.3	0.11	0.52

(1) Average net growth rate
(2) Flask opening every 2 days
Medium: 1B5, S = 20 g/L

Figure 3.8 – Effect of the mixing speed on the biomass loading of SIPC in the magnetically stirred flask system.

P: Periodic aeration.


SIPC (mg d.w./cm²)
AGITATION
12
10
8
6
4
2
0
200
400
600
Mixing speed (rpm)
p
p
p
p

The growth rate of SIPC cultured in shake flasks was evaluated by periodic retrieval of a flask and measuring the amount of immobilized biomass (SIPC loading [mg d.w./cm^2]). Results are presented in Figures 3.9 and 3.10 for Materials A12 and Q8. These results are compared to the growth of plant cells cultured in shake flask suspensions (SC, X [g d.w./L]).

As shown in Table 3.3, the amount of immobilized plant cell biomass in this system showed a significant linear correlation with time as long as nutrients were not depleted. The carbohydrate consumption of both series of experiments are presented in Figures 3.11 and 3.12. These results are compared to the carbohydrate consumption of plant cells cultured in shake flask suspensions (SC). The effect of the agitation intensity on the growth rate of immobilized cells for each material was as mentioned previously. Increasing the mixing speed lowered the SIPC growth rate on Material A12 to 74% of the original value. The same increased mixing improved significantly (100%) the SIPC growth rate on Material Q8.

The SIPC growth rates presented in Table 3.3 are smaller (by ~ 70%) than for suspension cultures and similar to the average results presented in Table 3.2 for the magnetic stirrer system. A series of similar experiments

Figure 3.9 – Growth curves of C. roseus SIPC on support Material A12 in shake flasks (average of 3 results).

SC: Suspension culture.

▽: 130 RPM.

△: 150 RPM.

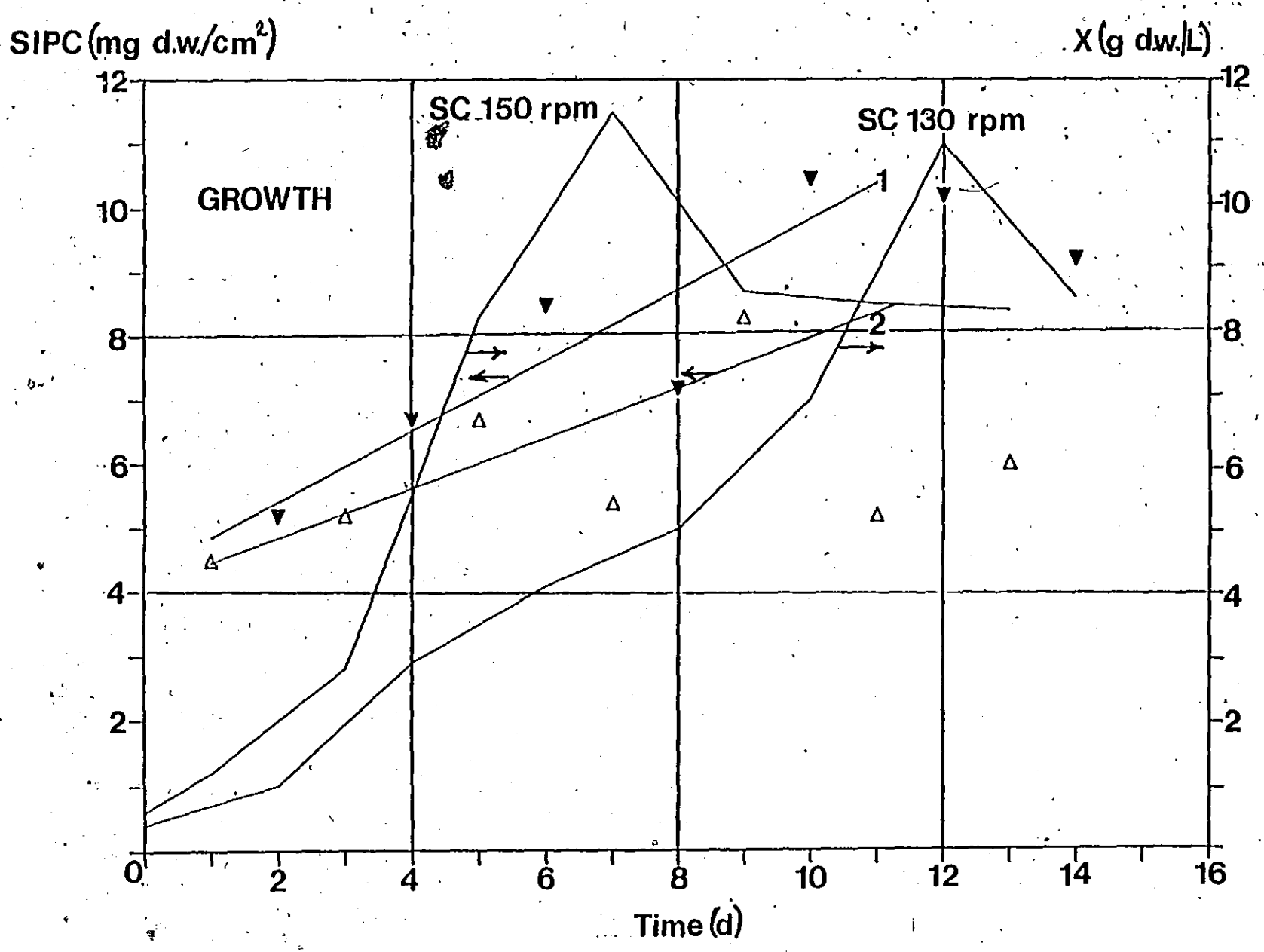

SIPC (mg d.w./cm²)
X (g d.w./L)
SC 150 rpm
SC 130 rpm
GROWTH
1
2
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
Time (d)

Figure 3.10 – Growth curves of C. roseus SIPC on support Material Q8 in shaked flasks (average of 3 results).

SC: Suspension culture.

▽: 130 RPM.

△: 150 RPM.

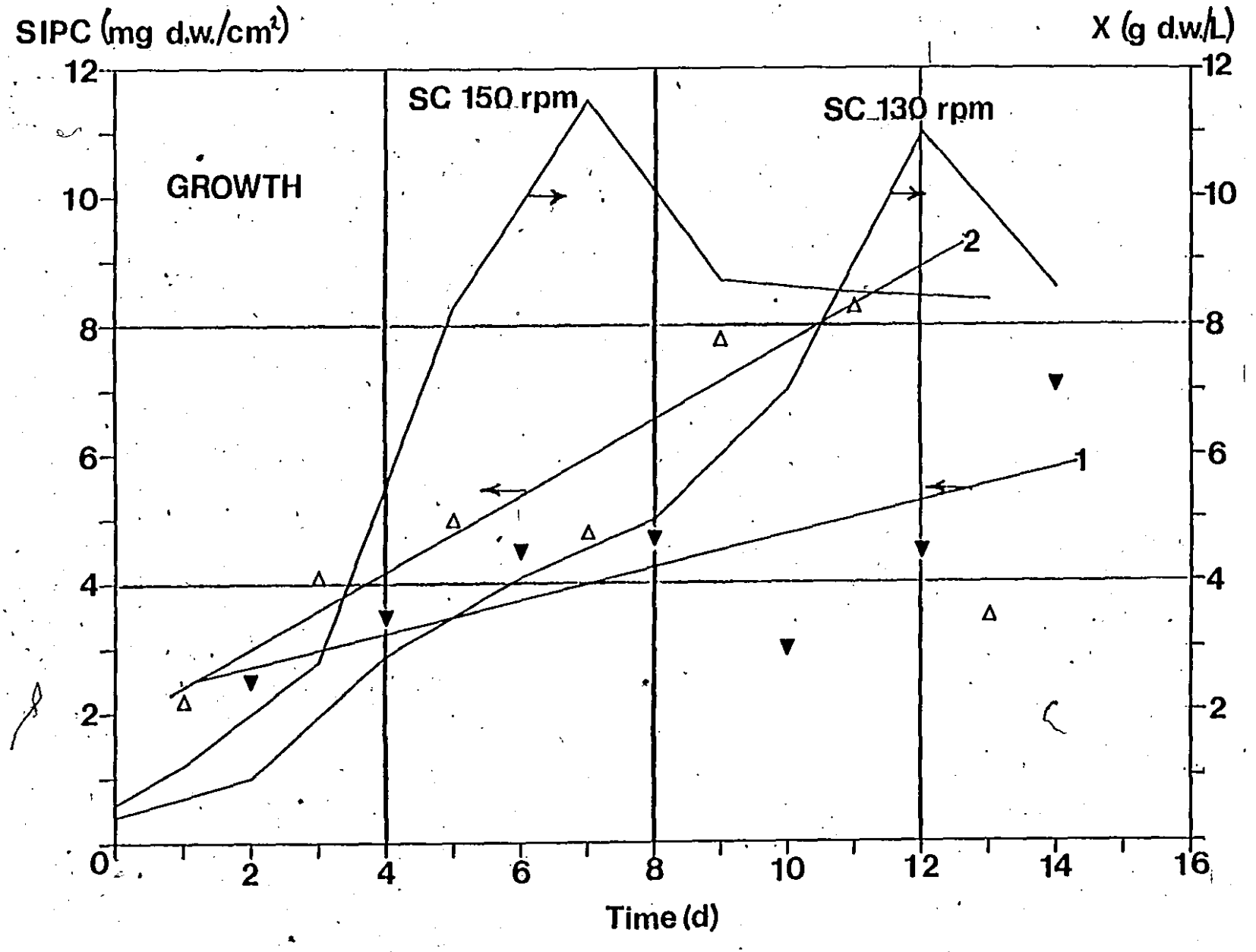

SIPC (mg d.w./cm²)
X (g d.w./L)
SC 150 rpm
SC 130 rpm
GROWTH
1
2
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
Time (d)

TABLE 3.3

CORRELATED GROWTH RATE OF C. ROSEUS SIPC IN THE SHAKE FLASK SYSTEM.

Material	Mixing Speed (RPM)	Data		Time (d)	Biofilm growth rate Correlation	r	Specific growth rate μ (d^{-1})	r
A12	130	Figure3.9 Line 1	○	10	SIPC=0.54t+ 4.3 (3.1)	0.88	0.07	0.89
A12	150	Figure3.9 Line 2		9	SIPC=0.40t+ 4.0 (3.2)	0.84	0.07	0.86
Q8	130	Figure3.10 Line 1		14	SIPC=0.26t+2.2 (3.3)	0.74	0.06	0.73
Q8	150	Figure3.10 Line 2		11	SIPC=0.57t+1.9 (3.4)	0.96	0.11	0.95
Control Suspensions	130	Figures 3.9-3.13		12	X=0.81t-0.36 (3.5)	0.96	0.24	0.95
	150	Figures 3.9-3.13		7	X=1.64t-0.31 (3.6)	0.97	0.41	0.98

X: Control suspension biomass concentration (g d.w/L).
SIPC: mg dried biomass per cm^2 of immobilizing surface.
t: culture time (day).
r: Linear correlation coefficient.
(1) Inoculation: 7% from 10d old inoculum.

Figure 3.11 - Carbohydrate consumption of SIPC on Material A12 in shake flasks.

SC: Suspension culture.

Symbols are as in Figure 3.9.

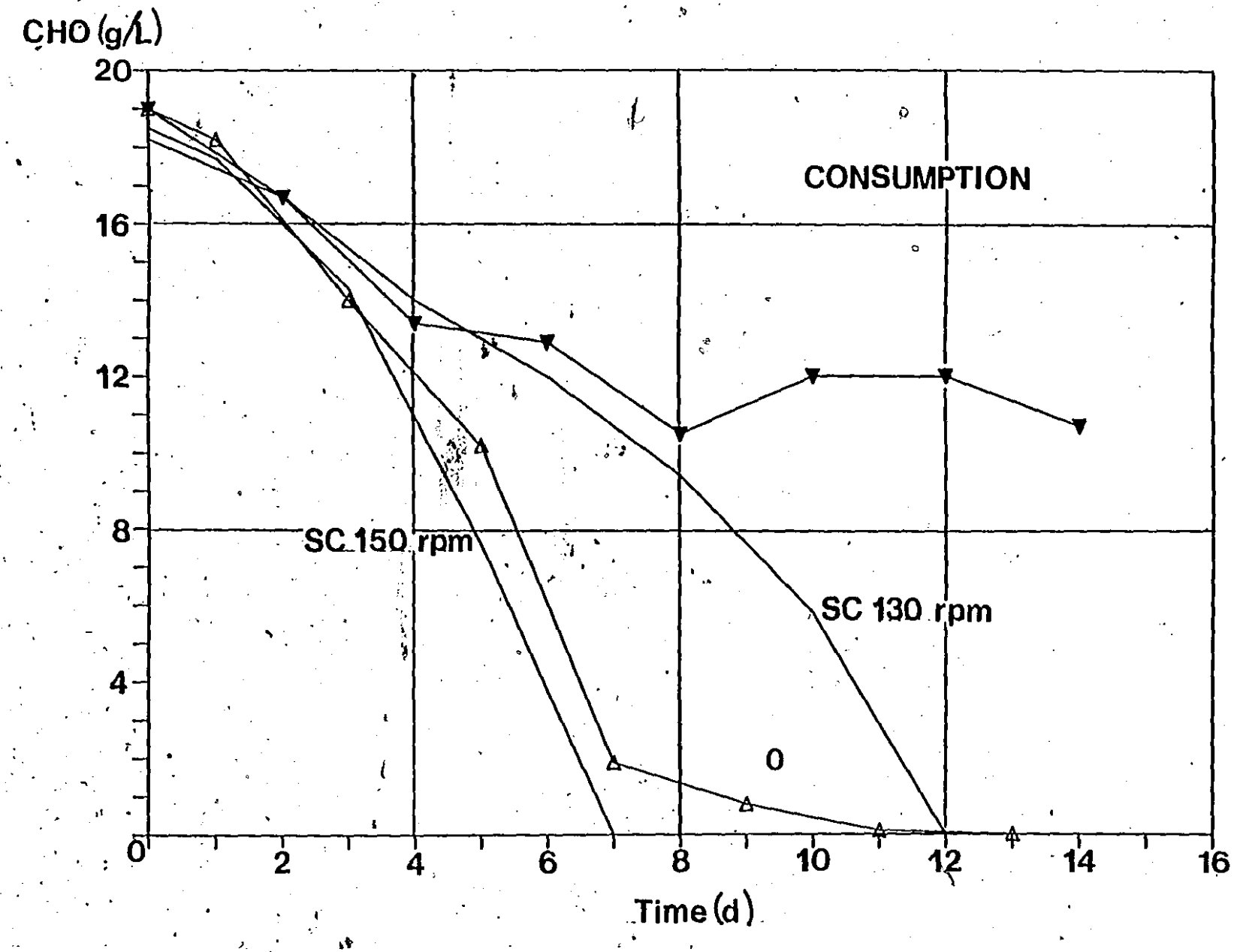
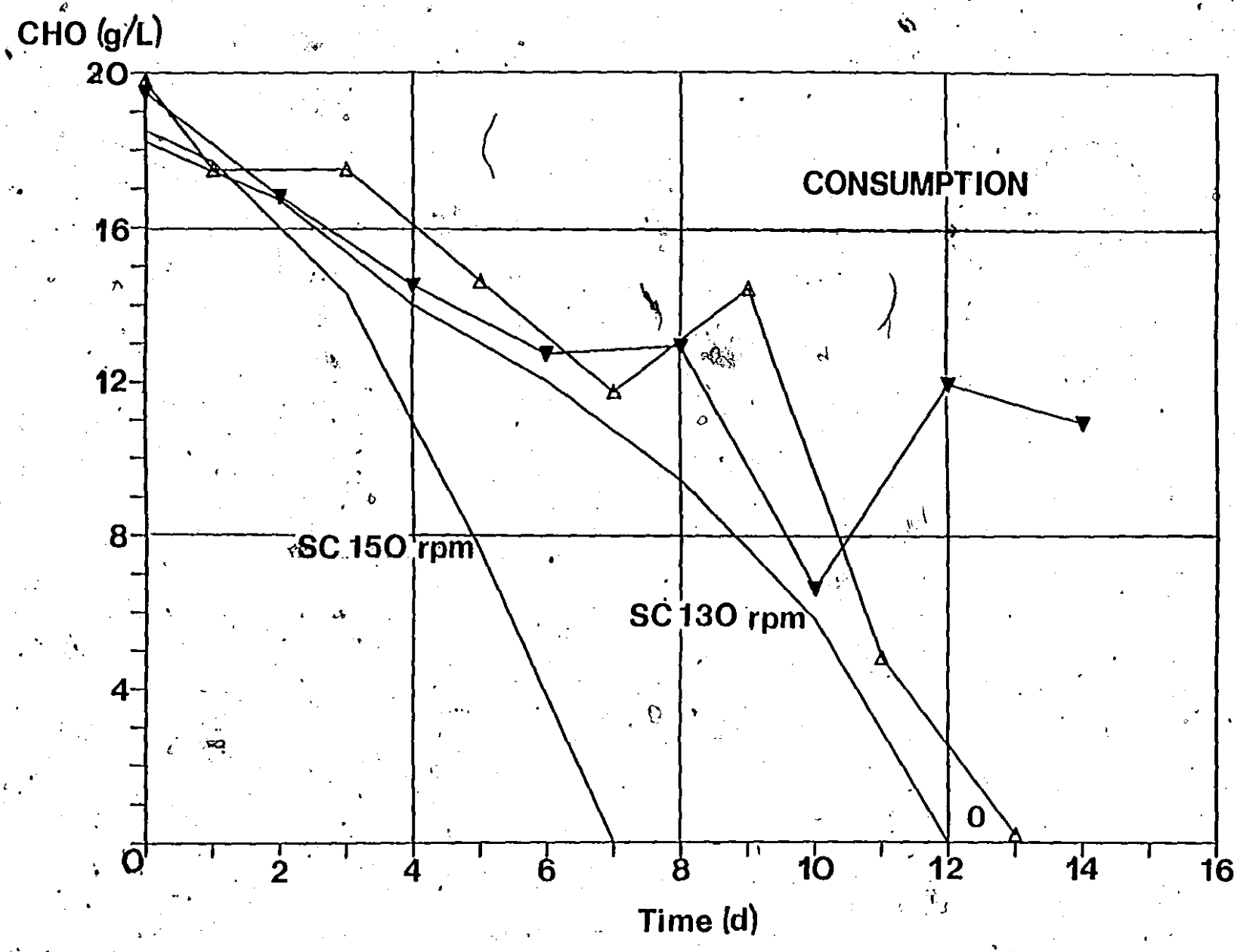

CHO (g/L)
20
16
12
8
4
0
2
4
6
8
10
12
14
16
Time (d)
CONSUMPTION
SC 150 rpm
SC 130 rpm
0

Figure 3.12 - Carbohydrate consumption of SIPC on Material 08 in shake flasks.

SC: Suspension culture.

Symbols are as in Figure 3.10.


CHO (g/L)
20
16
12
8
4
0
CONSUMPTION
SC 150 rpm
SC 130 rpm
0
0
2
4
6
8
10
12
14
16
Time (d)

was carried out with this latter system. Results are presented in Figures 3.13 and 3.14 and are compared to shake flask suspension cultures (SC). These results are summarized in Table 3.4. The relative scatter of the data presented in Figure 3.13 illustrates the sensitivity to operating conditions of this culture technique when carried out in this small flask system ($A/V \sim 0.3\ cm^{-1}$). The results of Figure 3.13 show more distinctly the growth pattern of SIPC. Much less free biomass is present in the magnetically stirred flasks, as compared to the shake flask system, which may mask this growth behaviour.

The results of Figure 3.13 show the same linear growth pattern of SIPC up to approximately the 8th day of the culture. Thereafter the SIPC biomass enters a stable stationary phase. This was confirmed by carbohydrate consumption rates measurements as shown in Figure 3.15. The presence of this stationary phase of SIPC cultures is rather surprising since it occurs without complete carbohydrate consumption by the immobilized plant cells. In fact, less than 50% of the original carbohydrate concentration in the medium was generally consumed by the growing plant cells. This stationary phase of SIPC occurs at the same culture time as that of a plant cell suspension cultured in shake flask agitated at 150 RPM. However, in

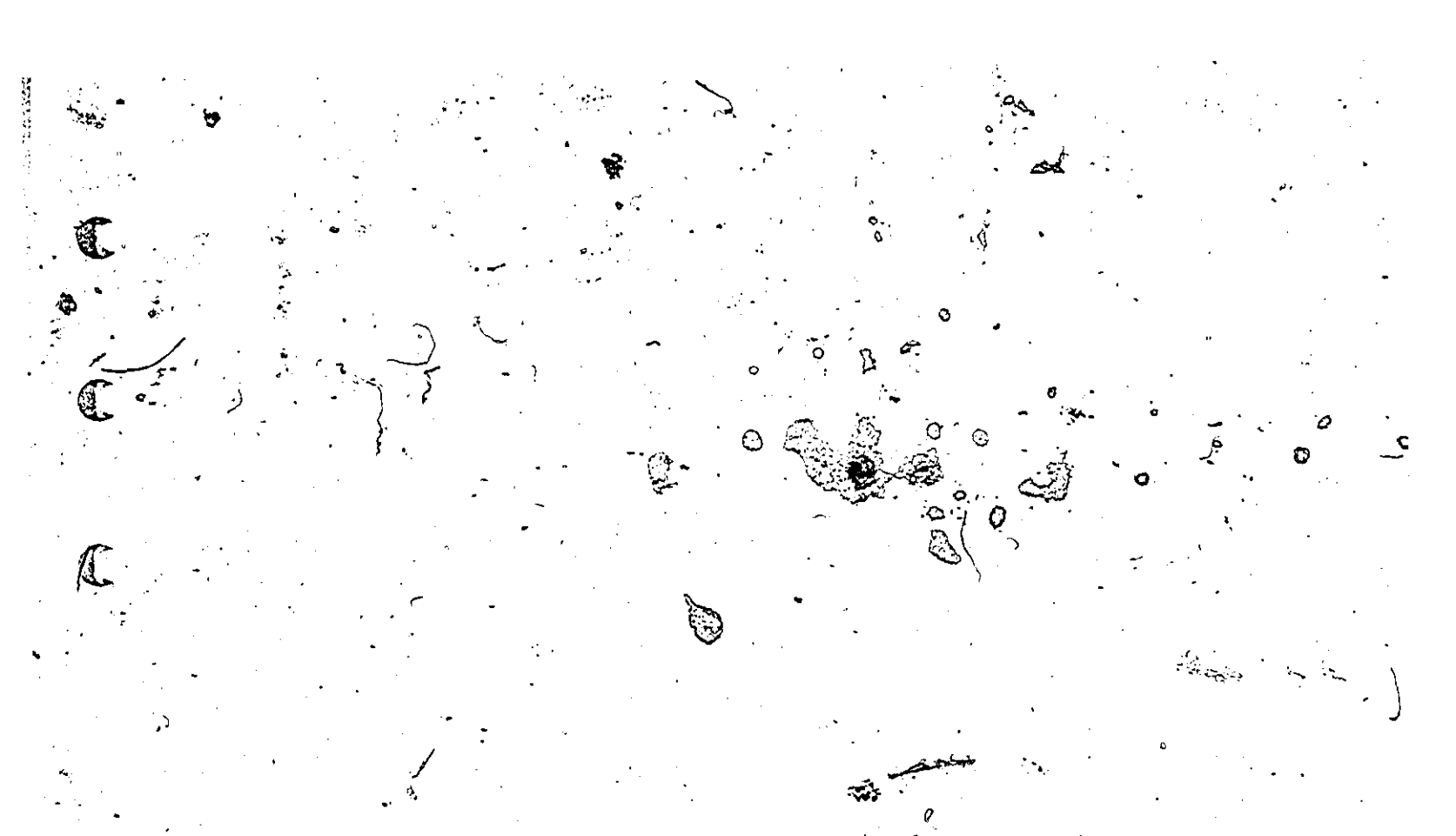


Figure 3.13 – Growth curve of C. roseus SIPC on support Material A07 in the magnetically stirred system (200 RPM, S = 20 g/L, see Table 3.4).

SC: Suspension culture (S = 20 g/L).

▼: Experiment H.

△: Experiment J.

□: Experiment K.

×: Experiment L.

■: Experiment M.

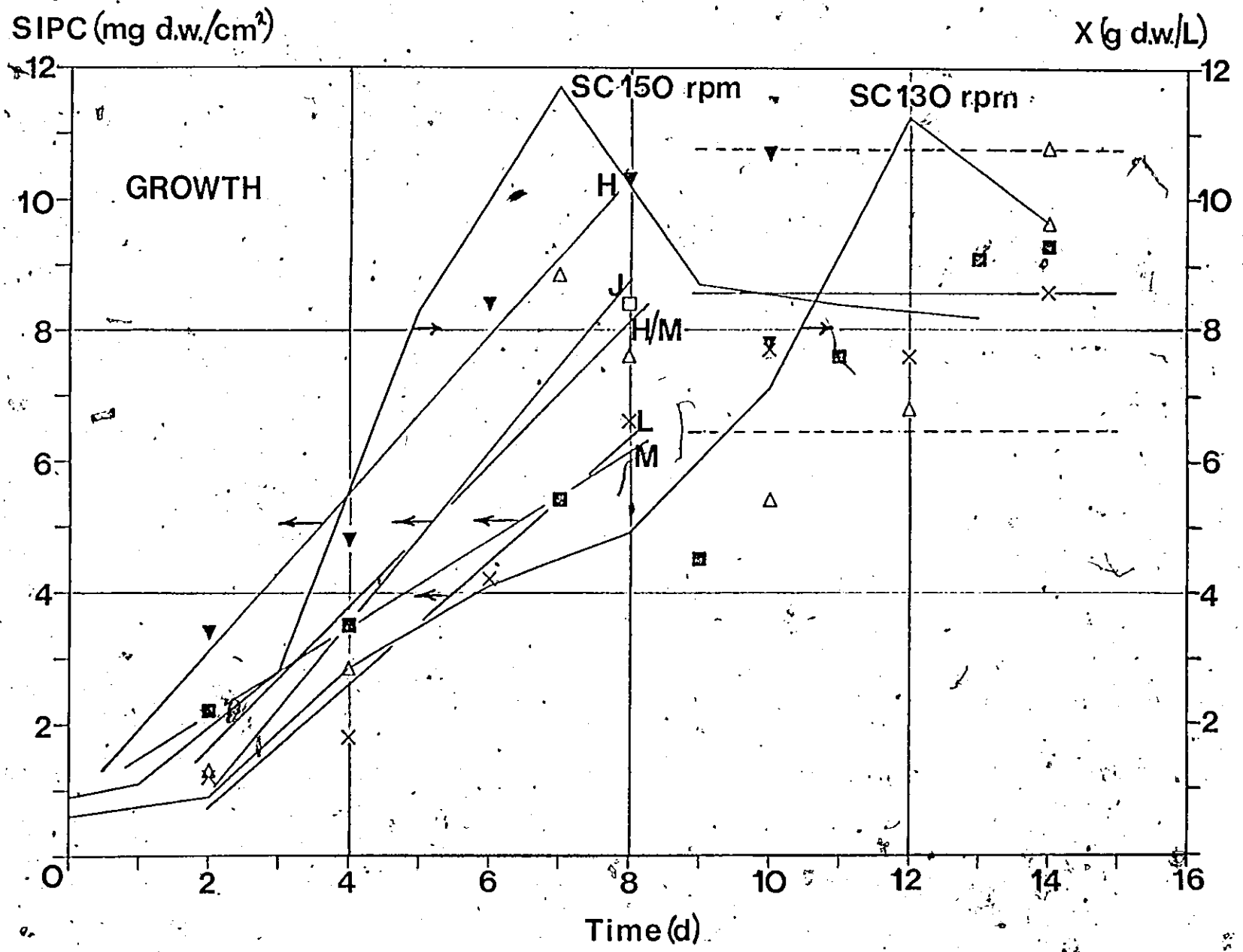

SIPC (mg d.w./cm²)
X (g d.w./L)
SC 150 rpm
SC 130 rpm
GROWTH
H
J
H/M
L
M
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
Time (d)

Figure 3.14 - Growth curves of C. roseus SIFC on support Material A07 in the magnetically stirred system at various mixing speeds and sucrose concentrations (see Table 3.4).

SC: Suspension culture (S = 20 g/L).

▼: Experiment O/T (S = 20 g/L, 400 RPM).

△: Experiment P (S = 40 g/L, 200 RPM).

□: Experiment R (S = 10 g/L, 200 RPM).

■: Experiment T (S = 10 g/L, 200 RPM).

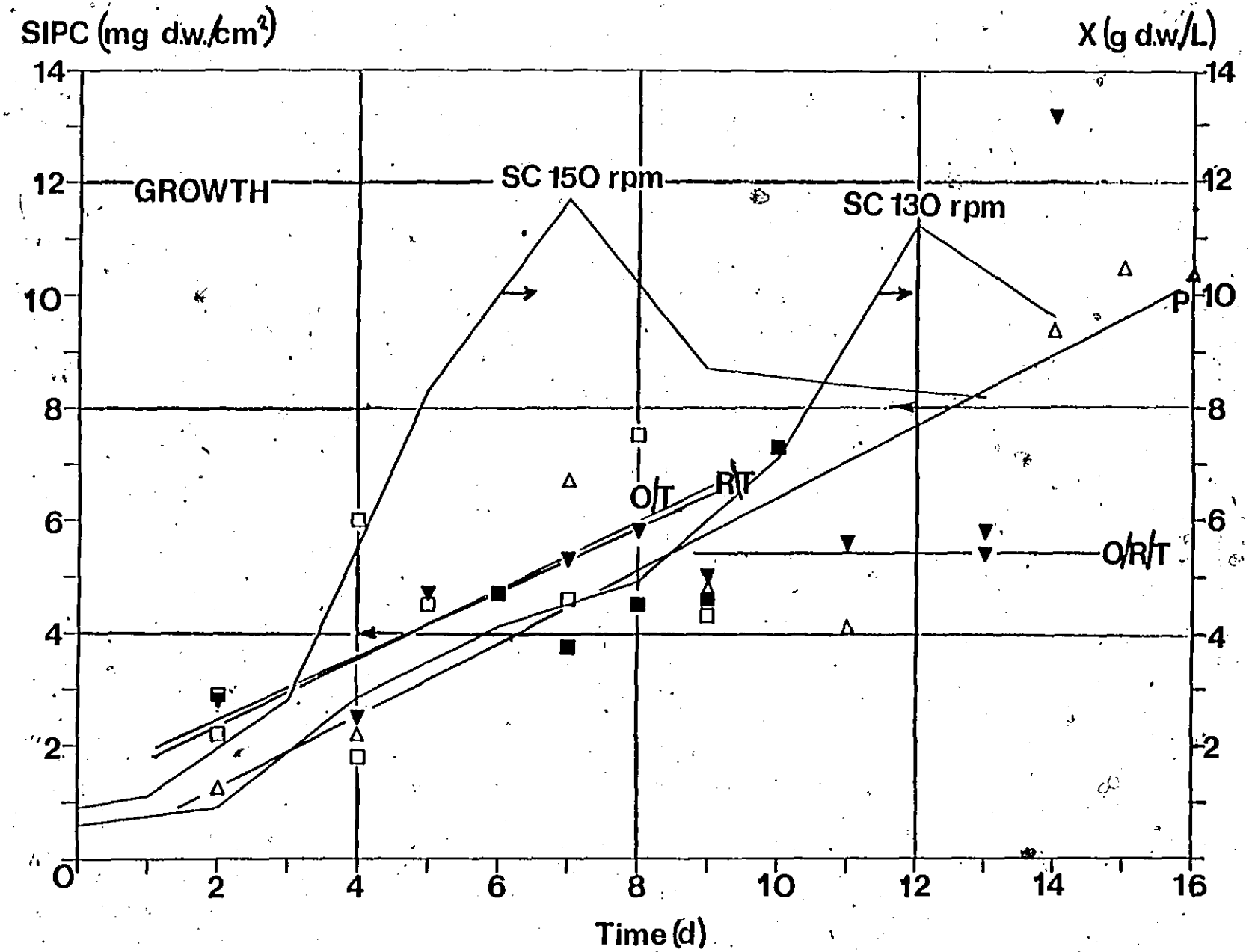

SIPC (mg d.w./cm²)
X (g d.w./L)
GROWTH
SC 150 rpm
SC 130 rpm
O/T
R/T
O/R/T
P
Time (d)
0
2
4
6
8
10
12
14
16

TABLE 3.4

GROWTH KINETICS OF C. ROSEUS SIPC IN THE MAGNETIC STIRRER SYSTEM

Exp.	Med $[CHO]_o$ (g/L)	Mixing (RPM)	Max Time (d)	No. flasks (n)		Time to S.P. $(d)_n$	μ		Biofilm growth rate		$dCHO/dt_{bsp}$		$dCHO/dt_{asp}$		Average Biomass yield	Average W/D	Average $Y_{X/I}$
							(d^{-1})	r	$(\frac{mg\ d.W.}{cm^2 \cdot d})$	r	g/L·d	r	g/L·d	r			
H	20	200	10	6		8_4	0.19	.99	1.21	.99	0.71	0.97	-	-	$24.1_{\pm 8.3}$	$31.6_{\pm 1.9}$	$76.5_{\pm 5.}$
J	20	200	14	8		8_4	0.32	.97	1.25	.95	0.78	0.95	0.25	0.50	$21.2_{\pm 5.9}$	$42.7_{\pm 14.6}$	$78.3_{\pm 13}$
K	20	200	12	2		8_1	0.22	-	1.05	-	1.26	-	0.65	0.35	$31.3_{\pm 4.1}$	$44.6_{\pm 6.1}$	$75.8_{\pm 14}$
L	20	200	14	7		8_4	0.30	0.99	0.93	0.97	0.53	0.99	0.35	0.90	$28.1_{\pm 8.6}$	$52.3_{\pm 16.3}$	$63.5_{\pm 6.}$
M	20	200	14	7		8_3	0.18	0.99	0.64	1.00	0.34	0.85	0.90	0.99	$23.2_{\pm 5.4}$	$41.0_{\pm 5.0}$	$82.7_{\pm 3.}$
H/M	20	200	14	30		8_{16}	0.25	0.87	1.08	0.87	0.84	0.66	0.62	0.46	$24.5_{\pm 7.0}$	$42.9_{\pm 12.5}$	$75.5_{\pm 12.}$
O/T	20	400	14	10		8_5	0.14	0.88	0.56	0.90	0.51	0.80	1.0	0.57	$34.1_{\pm 8.2}$	$36.9_{\pm 3.3}$	$90.2_{\pm 4.}$
P	40	200	16	8		-	0.13	0.91	0.64	0.91	0.47	0.87	-	-	$38.9_{\pm 7.8}$	$36.1_{\pm 13.8}$	$80.3_{\pm 11.}$
					(P')	8_3	0.33	0.99	1.11	0.97	0.26	0.57	0.89	0.93			
R	10	200	9	7		8_6	0.22	0.85	0.72	0.72	0.80	0.70	-	-	$30.7_{\pm 4.8}$	$42.2_{\pm 4.5}$	$73.9_{\pm 13.}$
T	10	200	10	7		8_5	0.10	0.73	0.33	0.55	0.11	0.31	-	-	$37.3_{\pm 12.1}$	$47.7_{\pm 7.8}$	$77.2_{\pm 20.}$
R/T	10	200	10	14		8_{11}	0.16	0.76	0.50	0.64	0.41	0.42	2.45	0.99	$34.2_{\pm 9.7}$	$44.9_{\pm 6.7}$	$75.6_{\pm 16.}$

Conditions: Material A07, Sparged air aeration 28 ±1°C, Inoculum: 10%/10d old

Figure 3.15 - Carbohydrate consumption of SIPC cultured in the magnetically stirred system (200 RPM).

SC: Suspension culture (S=20 g/L).

▼: Experiment H (S = 20 g/L).

△: Experiment J (S = 20 g/L).

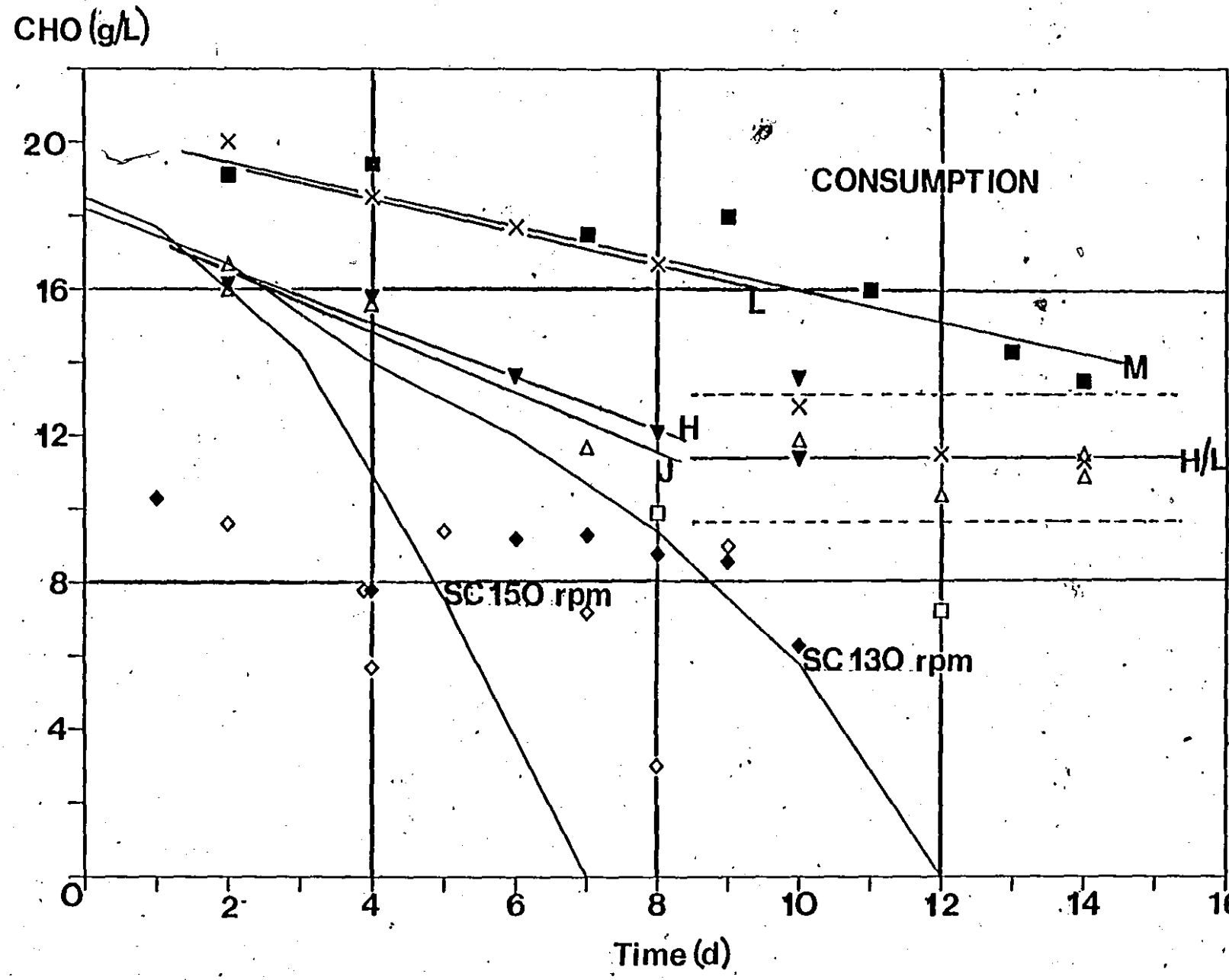
□: Experiment K (S = 20 g/L).

×: Experiment L (S = 20 g/L).

■: Experiment M (S = 20 g/L).

◇: Experiment R (S = 10 g/L).

◆: Experiment T (S = 10 g/L).


CHO (g/L)
20
16
12
8
4
0
2
4
6
8
10
12
14
16
Time (d)
CONSUMPTION
L
M
H
J
H/L
SC 150 rpm
SC 130 rpm

this case this coincided with carbohydrate exhaustion from the medium (see Figures 3.13, 3.14 and 3.15).

The growth rates of SIPC cultured in this system, before stationary phase and at an initial sucrose concentration of 20 g/L, are much larger (1.08 mg d.w./cm^2.d) than the average values presented in Table 3.2. They are also much larger (100 to 200%+) than for SIPC shake flask cultures (Table 3.3). In fact, the specific growth rate of the combined 5 experiments H to M (0.25 d^{-1}) compares to that of a plant cell suspension cultured in shake flask agitated at 130 RPM (0.24 d^{-1}).

As shown in Table 3.4, the growth rate of SIPC on Material A07 is decreased by 44-48% upon increasing the mixing speed from 200 to 400 RPM (Impeller Reynolds Numbers of 8300 and 16600 or tip speeds of 52 and 104 cm/s). A similar negative effect of increasing the mixing speed on the growth of SIPC on this type of material cultured in shake flasks was noticed in Tables 3.1 and 3.3. The effect of the initial carbohydrate concentration on the growth rate of SIPC is not clear. The biomass yields of SIPC cultured in this system is lower (20-39%) than for suspension cultures (50-60%) as observed in Table 3.2. The weight-to-dry weight ratio (W/D) of the SIPC biomass (25-50) is higher than the value presented previously.

3.2 Development of Laboratory Size Bioreactors for Surface Immobilized Plant Cells.

3.2.1 Selection of Bioreactor Configurations.

The numerous factors, either biomass, engineering or immobilization technique related, which have influenced the development this system, were discussed in Sections 1.2.1, 1.3 and 3.1. They are summarized in Table 3.5. These three groups of factors are interdependent and play an important role in determining the mixing and aeration rates and the maximum biomass concentration of the system. These considerations were translated in the development of the culture systems discussed in this Section.

The immobilizing structure had to be fixed for efficient immobilization and to prevent biomass abrasion and attrition. The matrix arrangement had to provide for an easily accessible high immobilizing area per unit of reactor volume. Efficient inoculum attachment and subsequent retention of the immobilized biomass were ensured by using Material A07, which was shown in Section 3.1 to surface immobilize plant cells better than other materials, and stationary phase plant cell suspension inocula. Material A07 was available in thin (1.5 mm)

TABLE 3.5

DESIGN CRITERIA FOR SIPC BIOREACTOR.

Criteria	Objectives	Design Restrictions
1- Biomass		
- Wet biofilm thickness	Minimize diffusion limitations (O_2).	3 mm
- Wet-to-Dry weight biomass ratio	Maximize biomass loading of system.	W/D ~ 30-50
- Sensitivity to - mixing	Prevent biomass disruption, heterogeneity and reduced growth rate.	Low shear controlled mixing.
- aeration	Prevent biomass hyperventilation.	Limited aeration rate (k_La <15 h^{-1}).
- Stickiness	Prevent accessories plugging and heterogeneity.	
2- Engineering		
- Mass transfer	Allow efficient, uniform and controlled mass transfer to and from SIPC favoring productive physiological processes.	Suitable and matched bioreactor hydrodynamics and matrix configuration.
- Culture environment	Allow biomass and medium homogeneity and efficient separation.	
- High biomass concentration	Permit high productivity without hindering mass transfer and physiological processes.	High A/V arrangement. W/D Limitations. Fixed structure. Matrix spacing and bioreactor structure.
- Process requirements	Allow regenerative growth, media change-overs and high reliability.	

TABLE 3.5 (CONT'D)

DESIGN CRITERIA FOR SIPC BIOREACTOR

Criteria	Objectives	Design Restrictions
3- Immobilization Technique		
- Immobilization process	Provide for low shear pumping and uniform distribution of the inoculum in the matrix structure.	Matched bioreactor hydrodynamic and matrix configuration. Initial low mixing and aeration rate.
	Provide for rapid and efficient recruitment of the inoculum.	Use of Material A07 and late stationary phase inocula (8-10d old).
- Biomass retention and growth	Permit a well separated two phase system and high biomass loading.	As above. Controlled mass transfer. High A/V fixed matrix structure.

flexible sheets. One of the most compact configuration is the fixed spiral structure.

The hydrodynamics of the bioreactor had to provide for the pumping and uniform distribution of the sensitive and large inoculum throughout the immobilizing structure with minimal foaming. The resulting flow pattern had to permeate uniformly the whole biomass loaded matrix configuration without creating dead volumes to ensure good mass transfer to the immobilized plant cells. It addition, the system had to provide for an effective aeration of the culture, its reliable sterile containment and good scale up potentials.

In this context, the fixed vertical spiral-wound immobilizing structure installed in an airlift bioreactor, with the riser tube at the center of the spiral, was the alternative selected which offered a best overall arrangement meeting these requirements. A second related configuration was developed which involved the same immobilizing structure installed in a low height-to-diameter mechanically magnetically stirred and air sparged bioreactor.

The main parameter that governed the design of these bioreactors is the thickness of the attached wet biofilm.

The only limiting factor, as long as sufficient mixing is ensured, is diffusion within the plant cells layer. This aspect is different from the cell entrapment immobilization technique. The internal diffusion in the layer of cells is a direct function of the biomass nature, structure and density as well as of secreted extracellular products (186). Diffusion of oxygen and nutrients in microbial biofilms was found to be in the range of 80 to 90% of that in water (186). A critical radius of 80 to 2000 fm for mold pellets has been reported for sufficient oxygen transport (186). Information on the diffusion within a plant cell biomass layer does not seem to be available. However, they have been immobilized with good retained viability

- in calcium alginate beads 3 to 4 mm in diameter (47,187 and others), with 5 mm beads being less efficient (5). Recently a range of bead diameter of 1.4 to 3.8 mm has been suggested that permits sufficient oxygen supply for retained viability of immobilized plant cells (48),
- in polyurethane foam particles 1 cm x 1 cm x 1 cm (170) and
- on a porous membrane at a thickness of 3.2 mm (129).

Also, the metabolism of plant cells being inherently slow, it may probably be less subject to diffusion limitations

within the proposed configuration as compared to other systems (149).

From these considerations, a wet biofilm thickness of 3.0 mm was chosen as the basic design criterion for the current new system. This will allow immobilizing 6-12 mg of dried plant cell biomass (W/D ~ 25-50) per cm^2 of support surface area. Other design characteristics of both SIPC bioreactors are summarized in the two following Sections.

3.2.2 Description of the SIPC Bioreactors.

3.2.2.1 Mechanically Agitated Bioreactor IP.

As shown in Figure 3.16 and Table 3.6, this SIPC bioreactor is a 2 L glass vessel with a conical profile bottom. It is agitated by a magnetic bar. Aeration is provided through a sintered glass sparger. The immobilizing material is formed into a vertical square spiral configuration with a supporting "cage" structure made of stainless steel rods (0.24 cm). This structure is fitted vertically in the bioreactor and rests on the top edge of the inverted conical bottom.

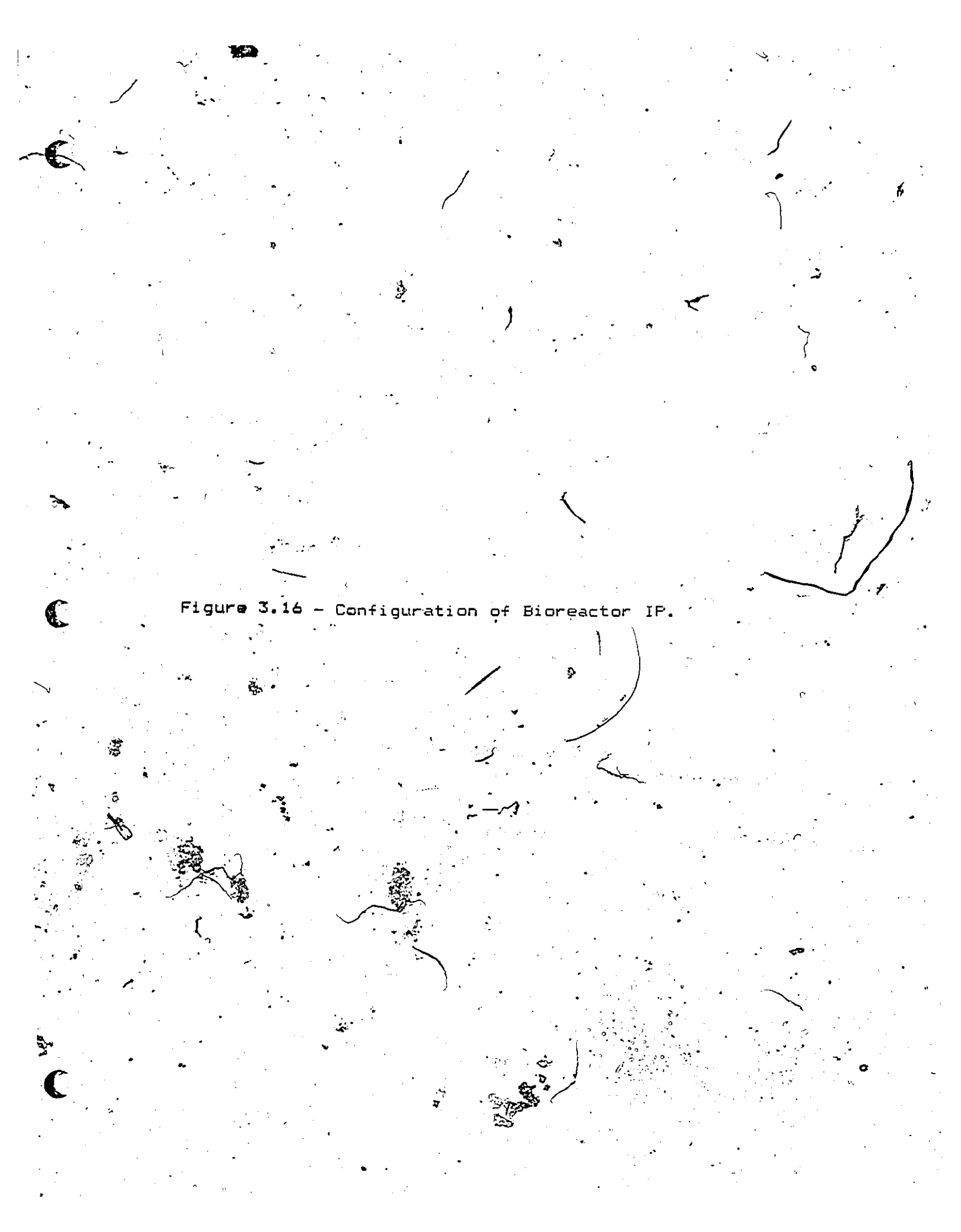


Figure 3.16 - Configuration of Bioreactor IP.

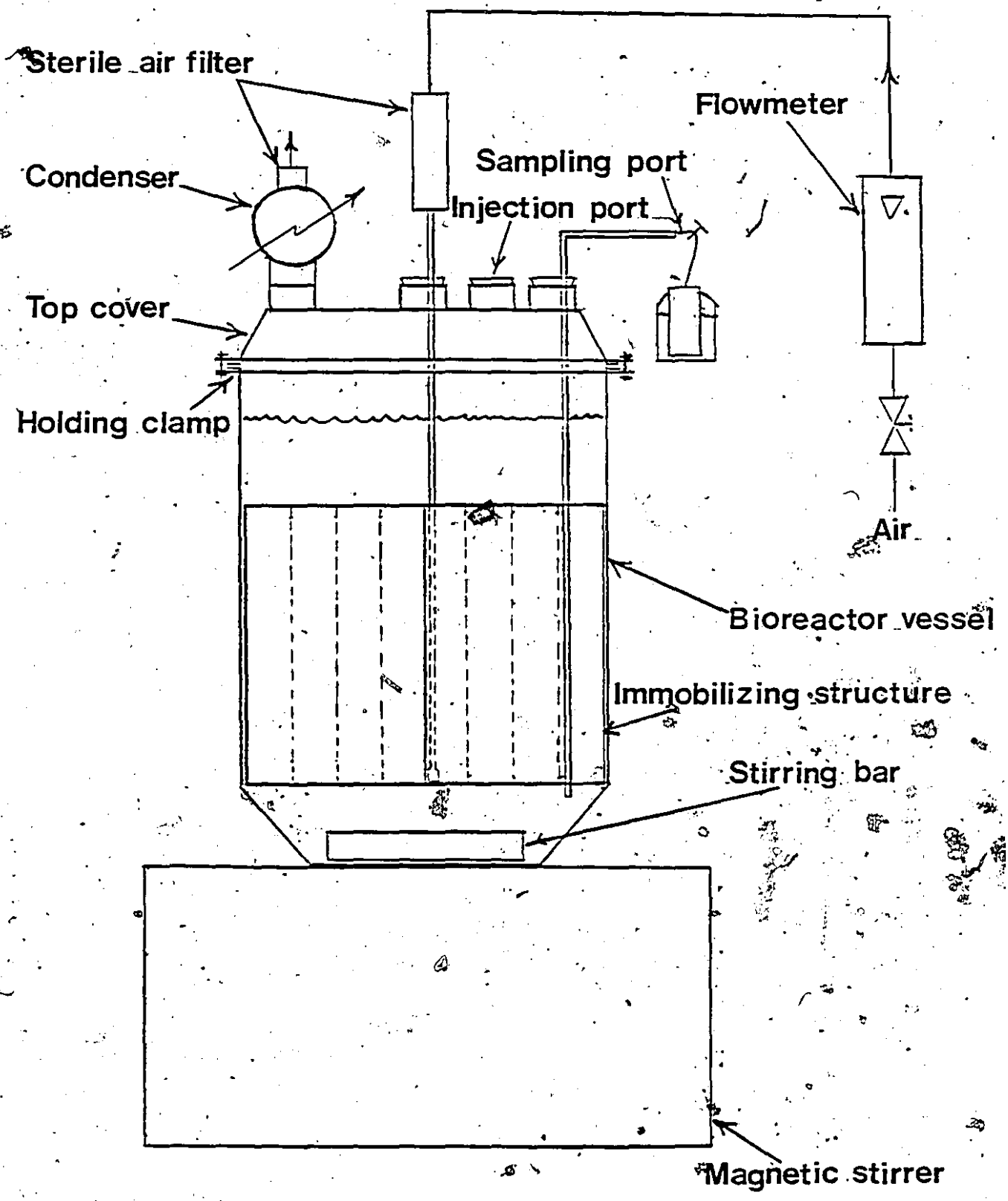

Sterile air filter
Flowmeter
Condenser
Sampling port
Injection port
Top cover
Holding clamp
Air
Bioreactor vessel
Immobilizing structure
Stirring bar
Magnetic stirrer

TABLE 3.6

CHARACTERISTICS OF BIOREACTOR IP

Characteristics	Dimension
1 - Vessel	
Internal height	20.0 cm
Inside diameter	13.0 cm
Conical profile bottom	
- Internal height	3.0 cm
- Smaller inside diameter	8.0 cm
2 - Magnetic Stirring Bar	
Length	7.7 cm
Diameter	1.2 cm
3 - Maximum allowed liquid volume (V_{Lm}) (with matrix)	1.9 L
- Internal height	14.9 cm
4 - Immobilizing structure	
Height	10.0 cm
Net layers spacing at zero loading (Material thickness: 0.15 cm)	1.35 cm
Number of spiral turns	4
Total available immobilizing area (A)	1728.0 cm^2
A/V (Max. Liquid and matrix heights)	0.89-1.03 cm^{-1}

The main characteristics of this bioreactor are its height-to-diameter (H/D) ratio, the spacing between the layers of the immobilizing structure and the total available immobilizing area. The low H/D ratio of this bioreactor (respectively 1.5, 1.15 and 1.0 relative to the total vessel height, to the maximum liquid level and to the immobilizing structure height) were selected to ensure full permeation of the immobilizing structure by the culture broth. This was achieved by the swirling motion imparted by the mixing bar to the liquid phase. The selected layers spacing (1.35 cm) allowed a theoritical flow passage of 0.75 cm at maximum biomass loading of the immobilizing structure. This spacing limited the length of the immobilizing material that could be wrapped around the holding rod structure to 85 cm. The available immobilizing area (1728 cm^2) should permit retention of ~ 15.8 g (W/D ~ 37.5) of dried plant cell biomass at maximum loading in the bioreactor. This quantity corresponds to dried biomass concentrations which can be immobilized in this system of 8.3 to 9.5 g d.w./L of reactor volume at the maximum and immobilizing structure levels respectively.

3.2.2.2 Airlift Bioreactor IA.

The characteristics of this SIPC bioreactor are illustrated in Figure 3.17 and presented in Table 3.7.

Figure 3.17 - Configuration of Bioreactor IA.

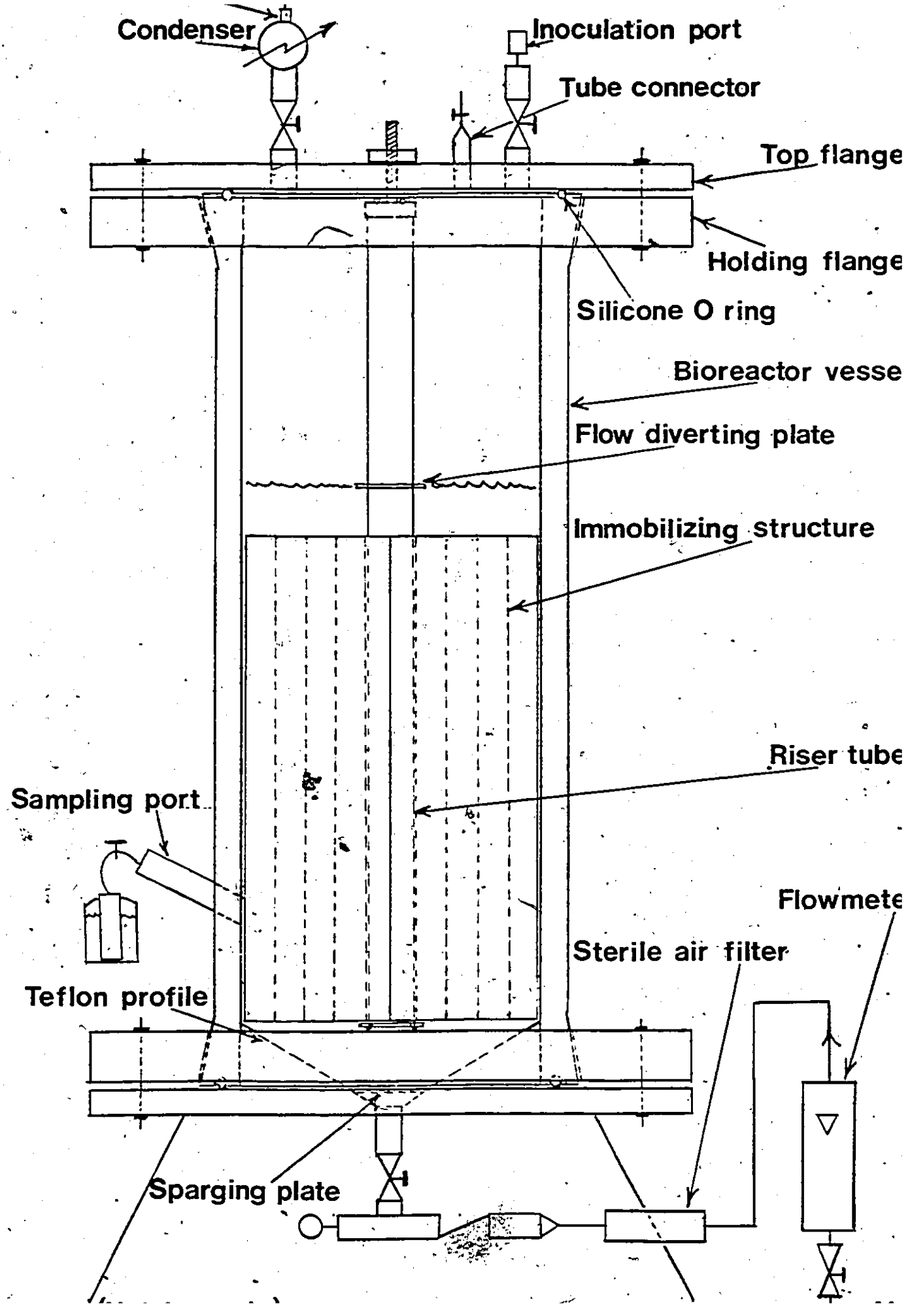

Condenser
Inoculation port
Tube connector
Top flange
Holding flange
Silicone O ring
Bioreactor vesse
Flow diverting plate
Immobilizing structure
Riser tube
Sampling port
Flowmete
Sterile air filter
Teflon profile
Sparging plate

TABLE 3.7

CHARACTERISTICS OF BIOREACTOR IA

Characteristics	Dimension
1 - Vessel.	
Internal height (H)	45.7 cm
Inside diameter (D)	15.2 cm
Slenderness ratio (H/D)	3.0
Inversed conical profile	
- height	3.8 cm
- smaller diameter	3.2 cm
Riser tube	
- length (= I. structure height)	24.7 cm
- inside diameter	2.54cm
- location from bottom	3.2 cm
- riser-to-downcomer ratio (AR/AD)	0.03
Flow diverting plate	
- diameter	3.8 cm
- location above riser	2.54cm
Accessories volume (without immobilizing material)	609 cc

Characteristics	Dimension
2 - Liquid Volumes. (with matrix)	
Maximum (height)	7.6L (45.7 cm)
Maximum allowed (h)	6.5L (39.4 cm)
To diverting plate (V_D)(h)	5.2L (30.5 cm)
To matric level (h)	4.4L (27.9 cm)

3 - Immobilizing structures.

	A	B	C
Layers spacing at no loading (cm)	1.12	1.09	0.92
Immobilizing area (cm^2)	5400	6500	8430
A/V D (cm^{-1})	1.04	1.25	1.62

Bioreactor IA is a modified airlift 6 L glass vessel. It is equipped with a teflon inverted conical profile to ensure no dead space at the bottom of the reactor. The immobilizing material is formed into a vertical square spiral configuration within a structure of stainless steel rods (0.24 cm) welded to a central (vertical) stainless steel tube of a 2.54 cm ID. This structure is fitted vertically in the bioreactor and is attached to the top flange. The central tube of the immobilizing structure is the riser tube of the airlift.

The main characteristics of this bioreactor are the flow diverting plate, the spacing between the immobilizing layers and the total available immobilizing area. The flow diverting plate is located on top of the riser tube at 1 diameter above its higher end. This particular mechanical feature combined with suitable operating conditions (liquid level and aeration rate) provided for uniform and efficient mixing within the immobilizing structure (see Section 3.2.4.1). The selected spiral layer spacings (A: 1.12 cm; B: 1.09 cm; C: 0.92 cm) allowed theoretical flow passages of 0.52 cm, 0.49 cm and 0.32 cm respectively at the maximum biomass loading of the immobilizing structure. These spacings limited the length of the immobilizing material that could be wrapped around each rod holding structure to 117 cm (A), 133 cm (B) and 173 cm (C). The quantities and

concentrations of dried plant cell biomass that could be retained by these immobilizing structures are presented in Table 3.8.

The two SIPC bioreactors were designed without internal accessories other than those required for hydrodynamics control and immobilization to prevent heterogeneity and instrument fouling with the biomass. The control of temperature was ensured by operating the bioreactors in a temperature controlled chamber. The pH of the medium, measured at sampling, was relatively stable and was not controlled. The dissolved oxygen concentration of the medium was not controlled.

3.2.3 Modelling the SIPC Bioreactors.

In this Section, a model is presented that relates the operating conditions, mass transfer characteristics and configuration of the designed bioreactors to the behaviour of the SIPC biofilm. This model will be used to correlate the growth of SIPC to carbohydrate consumption since the immobilized biomass cannot be sampled to measure concentration without dismantling the bioreactor. This substrate is the major contributor to biomass formation and its concentration in the medium is easily measured. The model will be extended to describe some process engineering

TABLE 3.8

BIOREACTOR IA IMMOBILIZED BIOMASS CAPACITY

IA Immobilizing Structure / SIPC	A	B	C
W/D = 25			
Total dried SIPC biomass (g d.w.)	65.4	78.8	102.1
Immobilized Biomass Concentration (g d.w./L of reactor volume at Flow Diverting Plate Level)	12.6	15.1	19.6
W/D = 50			
Total dried SIPC biomass (g d.w.)	32.3	39.4	51.1
Immobilized Biomass Concentration (g d.w./L of reactor volume at Flow Diverting Plate Level)	6.3	7.6	9.8

aspects of the induction of product biosynthesis by elicitation. This last Section is presented to complete the model and has not been verified experimentally.

3.2.3.1 Mass Transfer.

The main operational characteristics of the SIPC bioreactors are the aeration and mixing rates within the immobilizing structure. The low oxygen requirements of plant cells discussed in Section 1.2.1 ($k_La \sim 15\ h^{-1}$) will be met without difficulties by the airlift and stirred tank bioreactor configurations selected, especially in a more mass transfer efficient (surface) immobilization system. This has been verified experimentally and will be presented in Section 3.2.4.

Mixing represents a more critical operational parameter of the SIPC system. The mixing rate and uniformity and the mass transfer capacity of both bioreactors are closely related to their liquid pumping capability. This pumping rate for a stirred tank reactor is difficult to evaluate. Correlations were developed relating the mixing time to geometrical and operational parameters. The unique configuration of Bioreactor IP makes the analysis of its hydrodynamics even more difficult while comparison to

existing correlations are not relevant. Consequently the mass transfer characteristics of Bioreactor IP were evaluated only experimentally. They were optimized for the culture of SIPC.

A theoritical hydrodynamics analysis of the airlift Bioreactor IA can be more easily developed and was found more useful considering the scale up potential of this system. The liquid pumping rate of an airlift vessel can be correlated with its air flow rate. However, this is difficult since it depends on the geometry of the system and on two phase flow hydrodynamics. Few literature correlations exist that relate these two variables and that can be applied to varied geometries (82,188).

A system geometrically similar (riser-to-downcomer area ratio, $(A_R/A_D) \sim 0.023$) to Bioreactor IA ($A_R/A_D \approx 0.03$) was studied (82). It featured a more complex and restrictive air injection device and did not include the immobilizing structure in the downcomer section such as built into Bioreactor IA. The following equation was originally developed (82) based on earlier work (182,189). It was modified to take into account the above exceptions. It relates the induced liquid flow rate to the airflow rate in Bioreactor IA.

$$\alpha L g(\rho_L - \rho_g) = G_L(\frac{J_L}{1-\alpha} - J_L) + \frac{1}{2}\frac{G_L^2}{\rho_L}$$

$$[1 + e_v + 4f'(\frac{L}{D_R})(1 + \frac{J_g}{J_L}) + 2 + 4f'(\frac{L}{D})_{90°}] \qquad (3.7)$$

$$+ \Delta P_{fM}$$

where the void fraction α is given by

$$\alpha = J_g/(1.2(J_L + J_g) + 0.35\sqrt{gD_R}) \qquad (3.8)$$

ΔP_{fM}, the friction losses of the liquid flow in the immobilizing structure for laminar flow (190) is given by

$$\Delta P_{fM} = \frac{J_L L \pi (D_R/2)^2 K_f \mu_L}{ab_1^2} \qquad (3.9)$$

and

G_L: liquid mass flux ($kg/m^2.s$),

L: length of open tube (m),

g: gravity constant (9.81 m/s^2),

ρ_L: liquid density (kg/m^3),

ρ_g: gas density (kg/m^3),

J_L: liquid superficial velocity (m/s),

J_g: gas superficial velocity (m/s),

e_v: entrance effect in a tube ~ 0.78,

f': Fanning friction factor for turbulent flow in a pipe (~ 0.008),

D_R: riser tube internal diameter (m),

$(L/D_R)_{90°}$: equivalent friction length associated to the flow diverting plate on top of the riser tube,

K_f: model parameter equal to 12 (190),

μ_L: liquid viscosity (kg/cm.s),

a: spiral length (m),

b_1: spiral spacing for liquid flow (m).

Equation (3.7) was used to estimate the liquid pumping capacity (Q_L) of the bioreactor for air flow rates (Q_G) varying from 1.0 to 10.0 L/min. This range gives superficial gas velocities lower than 0.4 m/s, where reduced pumping efficiency and onset of semi-annular two phase flow regime were observed (182). The experimental results of (82) fitted very well the model and can be correlated as follows for:

$j_G \leq 0.236$ m/s:

$$J_L = 1.9J_G + 0.42 \qquad (3.10)$$

with a linear correlation coefficient r of 0.98.

The calculated pumping capacity of the bioreactor can be correlated similarly for $j_G \leq 0.33$ m/s by

$$J_L = 0.78J_G + 0.26 \qquad (3.11)$$

or

$$Q_L = 0.78Q_G + 8.02 \qquad (3.12)$$

with a linear correlation coefficient of 0.95. The model shows very little effect (~1%) of the immobilizing structure, with or without attached biomass, on J_L or Q_L.

As mentioned in Section 1.2.1.2, a 1.5 VVM aeration rate (7.8 L/min for Bioreactor IA) provides for best mixing in airlift vessels (81). Under these circumstances (Q_L = 14.1 L/m), the model predicts a circulation time of 22 s. This compares to mixing times of 22 to 28 s (81) and 52 s (79) reported for systems nearly similar. This will be verified for Bioreactor IA in Section 3.2.4.1.

Equation (3.12) predicts laminar flow mixing through the matrix (Re_H ~ 240-325 based on the average liquid velocities and the hydraulic radius concept) for all aeration rates lower than 1.5 VVM and for the three matrix structures used, regardless of biomass loading. Turbulent flow would require excessive liquid (and air) circulation rates (> 50 L/min).

The bulk mass transfer coefficient k_L for a flat plate geometry under laminar flow mixing ($Re_L < 5 \times 10^5$, in our case $Re_L \leq 11000$) is related to the system hydrodynamics and the liquid physical properties by (185,191)

$$Sh = 0.664 * Re_L^{1/2} * Sc^{1/3} \qquad (3.13)$$

with

$$Sh = \frac{k_L L}{D_L} \qquad (3.14)$$

$$Re_L = \frac{\rho v L}{\mu} \qquad (3.15)$$

$$Sc = \frac{\mu}{\rho D_L} \qquad (3.16)$$

where

k_L: bulk liquid mass transfer coefficient (cm/s),

L: flowing lenght of plate (cm),

D_L: liquid diffusion coefficient (cm^2/s),

v: liquid velocity (cm/s).

In our case, Equation (3.13) reduces to

$$k_L = 1.0128 * 10^{-4} * v^{1/2} \qquad (3.17)$$

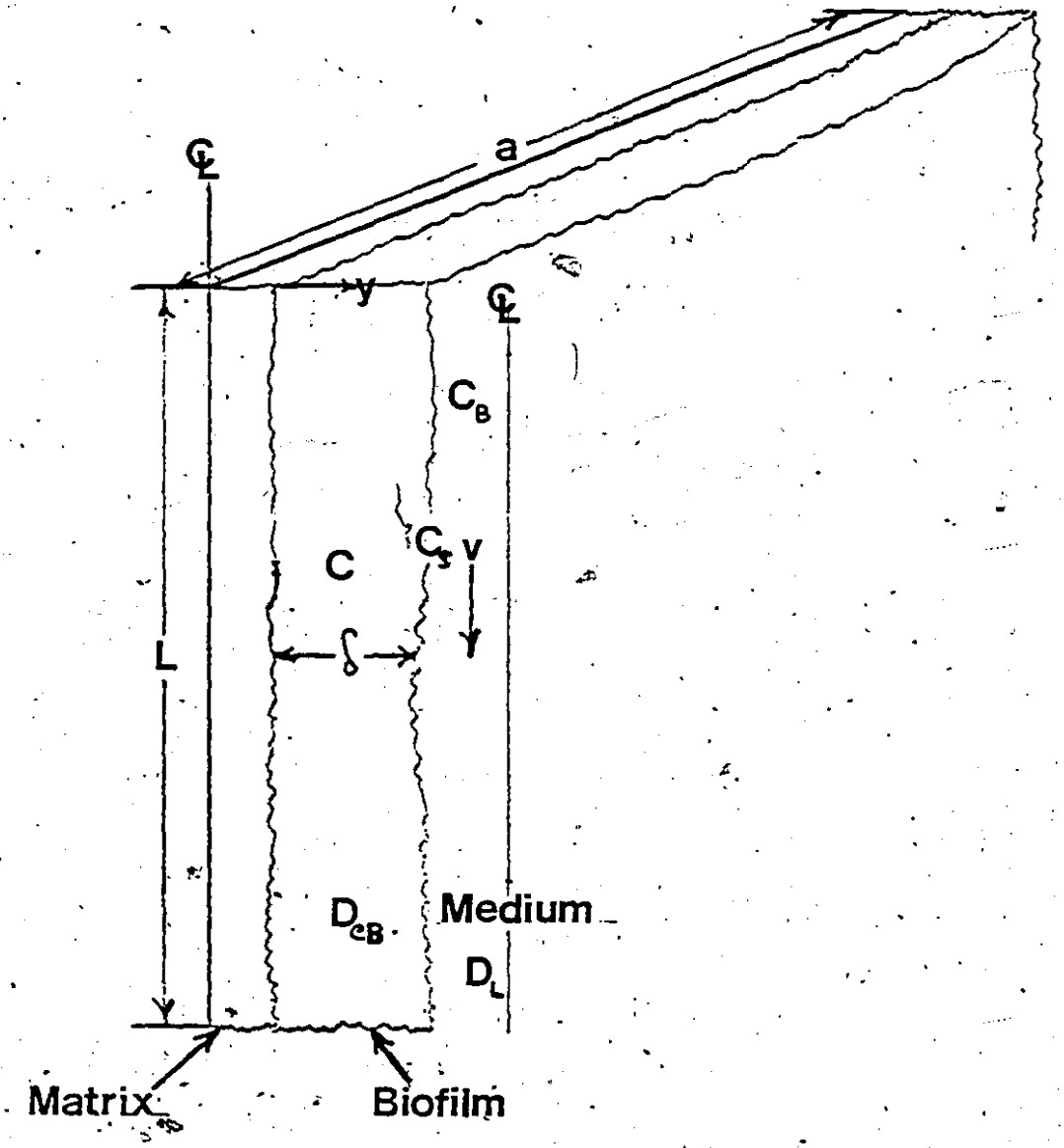
which gives a range of possible k_L values of 1.01 to 2.12 * 10^{-4} cm/s.

3.2.3.2 SIPC Biofilm Growth.

The central theme of the model involves relating the growth behaviour of a SIPC biofilm to the operational mass transfer characteristics of the bioreactor and to the carbohydrate consumption by the biomass. The basic physical model is shown in Figure 3.18. A plant cell biofilm attached to a vertical flat immobilizing surface is submitted to downward laminar flow mixing. This biofilm is either growing or maintained in a stationary productive phase. The model is semi-empirical since the growth (and secondary metabolite production) pattern of a SIPC biofilm is unknown. The model links macro mass transport to macrokinetics phenomena.

This model is different from others presented in the literature for immobilized whole cells (186,192-197) and for biofilms (198-200). It considers the slowness of plant cells biological reactions (days) as compared to microbial kinetics (hours). It involves a thick (3.0 mm against 0.03 to 1.2 mm) and dynamic biofilm attached to a flat plate and not covered by an entrapment membrane.

Figure 3.18 – Surface immobilized plant cells physical model.


a
y
C_B
C
C_s
v
L
δ
D_{eB}
Medium
D_L
Matrix
Biofilm

The following assumptions, partly taken from a model presented for immobilized whole cells (186), define the basis of this model.

A1- The biomass generates no significant heat and is isothermal.

A2- The macro mass transport mechanism in the biofilm involves only fickian molecular diffusion, without convective or electrostatic effects.

The diffusion of oxygen and nutrients through microbial films (186) and in 3.3 mm calcium alginate beads (201) has been found comparable to that of water. An effective diffusion coefficient $D_{eff} \sim 6.6 \times 10^{-6}$ cm^2/s will be used for the SIPC biofilm (48,201-203).

A3- The immobilized biomass is an homogeneous phase.

Active plant cells are assumed uniformly distributed in the biofilm. Its D_{eff} is constant throughout and is independent of the physiological state of the biomass.

A4- A transfer coefficient (k_L) defines mass transport between the medium and the biofilm.

A5- Mass transport to and from the biofilm occurs in one direction (y).

A6- The biofilm is at steady state.

This is justified by comparing the maximum characteristic time required to establish a steady-state concentration profile in the biofilm (δ^2/D_{eb} ~ 3.8 hours) to plant cells biological rates (division time > 1d). This implies that

- the medium is uniform throughout the vessel under sufficient mixing and
- the concentration profiles in the biofilm are nearly uniform.

A7- Growth of the biofilm is polarized in one direction (y).

Space restrictions within the biofilm confine growth to its outer layer. The metabolism of the internal biomass evolves progressively towards maintenance and differentiation.

A8- The problem is reduced to depend on the concentration of one species. This species will be the total carbohydrates at growth, maintenance and regeneration. It would be the inducing compound during elicitation and the group of characteristic alkaloids considered together during production/secretion.

A9- There is only one major reaction and few limited side reactions occuring at each stage of the process (see Section 1.3.1). They include growth and maintenance during the growth stage. They would include elicitation at the induction stage and biosynthesis/secretion, maintenance and negligible growth during the production stage.

It was shown in Section 3.1.2.3 that the growth rate of SIPC cultured in flasks was constant with time (< 8 d) as long as nutrients were abundant. In addition, high immobilization efficiency (>80-99%) was observed especially upon scale up. This growth pattern can be represented empirically by

$$SIPC(t) = \frac{I_o}{A} + bt \qquad (3.18)$$

where SIPC(t): average dried plant cell biomass immobilized per cm^2 of support surface area (mg d.w./cm^2),

Io: quantity of inoculated plant cells (mg d.w.) attached to the support material, which corresponds to all the inoculated

biomass at high immobilization efficiency as observed,

A: available support surface area for immobilization (cm^2),

t: culture time (d),

b: growth rate of the SIPC biofilm on the support material (mg d.w./cm^2.d).

This growth pattern was also observed upon scale up of the SIPC technique in the bioreactors described in Section 3.2.2 (see Sections 3.3.1.3 and 3.3.2.4) which confirms the usefulness of Equation (3.18).

During the growth phase, the bulk and biofilm diffusion fluxes of carbohydrates at the biomass/medium interface are at equilibrium with the slow consumption rate. The biofilm concentration profile is uniform (assumption A6). This is represented by

$$k_L A(C_B - C_S) = \frac{D_{eB}}{\delta} A (C_S - C) = \frac{d(V_B C)}{dt} \qquad (3.19)$$

where A: surface area of the biofilm (cm^2),

δ: wet biofilm thickness (cm),

$V_B = A * \delta$

V_B: volume of biofilm (cm^3),

C: concentration of carbohydrates in the biofilm (C), at its surface (C_s) and in the bulk liquid phase (C_b) (g/L).

The solution of the mass transport part of this double equation gives

$$C_s = \frac{B_i C_b + C}{1 + B_i} \qquad (3.20)$$

or

$$C = C_s(1 + B_i) - B_i C_b \qquad (3.21)$$

where Bi, the Biot number, is defined by

$$Bi = \frac{k_L \delta}{D_{eb}} \qquad (3.22)$$

The carbohydrate consumption rate in the biofilm can be developed as follow.

$$\frac{d(V_b C)}{dt} = \frac{1}{y_i} \frac{d(X_T)}{dt} \qquad (3.23)$$

where y_i is the immobilized biomass (X_T) yield relative to carbohydrates. Equation (3.24) results from combining Equations (3.21) to the empirical growth model of Equation (3.18).

$$\frac{d(V_b C)}{dt} = bA/y_i \qquad (3.24)$$

The combination of Equations (3.24), (3.20) and (3.19) gives

$$C \simeq C_b - \frac{b}{y_1} (1 + B_1)/k_L \qquad (3.25)$$

If appropriate values of the parameters are introduced in Equation (3.25), it can be shown that C, the concentration of carbohydrates in the biofilm is not very much different from the bulk medium concentration.

$$C \simeq C_b - 0.2 - 1.4 \text{ g/L} \qquad (3.26)$$

This is explained by visualizing the growth of a SIPC biofilm as follows. During the inoculation and immobilization phase of the process, the biofilm thickness is small ($B_1 \sim 0$). Growth occurs at $\sim C_b$. As the biofilm thickens ($B_1 > 0$), growth is concentrated mainly at the biofilm outer layer since no space is available internally for expansion and division (assumption A7). The growing biomass layer sees mainly the bulk medium carbohydrate (and other nutrients) concentration. The internal biomass experiences progressive growth decline and adapts its metabolism to a stationary behaviour. This internal biomass is exposed to nutrients diffusing from the medium and not used up by the external growing layer of the

biofilm.

The biomass yield of a SIPC culture may somewhat decline with time as the proportion of stationary to growing biomass increases. This is related to the immobilizing area available relative to the culture volume.

Some important conclusions can be drawn from these considerations.

1- The growth rate of a SIPC biofilm is not mass transfer limited above some minimal mixing level insuring a sufficient supply of critical nutrients (oxygen) to the plant cells and possibly the removal of inhibitory metabolites.

2- The optimum mixing rate of a SIPC culture will be determined to fulfill satisfactorily other process requirements, for example the transfer of sufficient oxygen from the sparged air to the liquid phase (k_La, see Section 3.2.4).

3- The rate of carbohydrate consumption by the biomass can be estimated by the rate of carbohydrate disappearance from the medium according to Equation (3.25).

The growth rate of the SIPC biofilm can be estimated from the rate of carbohydrate disappearance from the medium

according to the following equations.

$$b = \frac{y_1}{A} \frac{d(V_B C)}{dt} \qquad (3.24)$$

$$b = \frac{y_1}{A} \frac{d}{dt} \left(A\delta\left(C_B - \frac{b}{y_1} \frac{(1 + B_1)}{k_L}\right)\right) \qquad (3.27)$$

$$b = \frac{y_1}{A} \left[A\left(C_B \frac{d\delta}{dt} + \delta \frac{dC_B}{dt}\right) - \frac{Ab}{y_1 k_L D_{wB}}\left(D_{wB} \frac{d\delta}{dt} + 2k_L\delta \frac{d\delta}{dt}\right)\right] \qquad (3.28)$$

$$b = y_1 \left\{\delta \frac{dC_B}{dt} + \frac{d\delta}{dt} \left[C_B - \frac{b}{y_1}\left(\frac{1 + 2B_1}{k_L}\right)\right]\right\} \qquad (3.29)$$

The biomass yield (y_1), the mass transfer parameters (k_L, D_{wB}) and the biofilm growth rate (b) were assumed constant. By introducing in Equation (3.29) the value of the results and parameters presented previously, it can be shown that its second term on the right hand side is relatively smaller ($\leq$ 30%) than the first term during the growth phase. Consequently the SIPC biofilm growth rate can be estimated by

$$b \simeq y_1 \delta \, (dC_B/dt)_B \qquad (3.30)$$

This is consistent with the growth mechanism of a SIPC biofilm proposed previously (Equation (3.26) and assumption A7). Equation (3.30) was derived relative to the carbohydrate concentration in the biofilm $(dC_B/dt)_B$. Whereas the carbohydrate disappearance rate from the medium $\left(\frac{dC_B}{dt}\right)_m$ is the measured parameter. These two rates are

related by their respective phase volume. Equation (3.27) must be modified accordingly

$$b \tilde{=} y_1 \delta \left(\frac{dC_B}{dt}\right)_m \frac{V_m}{V_B} \qquad (3.31)$$

$$b \tilde{=} y_1 \frac{V_m}{A} \left(\frac{dC_B}{dt}\right)_m \qquad (3.32)$$

The empirical model of Equation (3.18) can be rearranged in the simple following equation.

$$SIPC = \frac{1}{A} \left(I_o + y_1 V_M \left(\frac{dC_B}{dt}\right)_m t\right) \qquad (3.33).$$

This equation allows estimating the amount of plant cell biomass produced and immobilized in the bioreactor during the growth phase from known parameters (A, I_o, V_M and t) and one measured variable C_B. The biomass yield factor (y_1) of the SIPC biofilm is the only parameter which needs to be determined empirically and seems to decline during growth (Figures 3.29 and 3.37).

3.2.3.3 SM Production.

The product elicitation and biosynthesis phases of the process may be more subject to mass transfer restrictions than the growth phase because of the higher reaction rates involved ($\leq$ 6-72 hours) and/or of the limited solubility of

the moving compounds. These steps involve a thick (3 mm) and stable wet SIPC biofilm and sequential transport processes.

At the product elicitation stage, no real reaction takes place. Some signalling to the plant cells by the inducing compound(s) occurs. The main concern is the minimal degree of contact ($C_{i,e}$) achieved between the elicitor compound(s) and the plant cells after the 24 hours of the elicitation period. This is essentially a transient passive diffusion problem through a biofilm of constant wet thickness δ which has been solved (204).

The situation is equivalent to dipping a flat slab of an absorbing biofilm (total thickness of $2*\delta$ + the immobilizing material thickness (t_{ki}), with coordinate $y' = y - t_{ki}/2$) into a well agitated solution of the elicitor compound(s) (concentration C_{bi}). The transfer area is much larger than the slab thickness (6500 cm^2/0.75 cm). Consequently the transport of the elicitor compound(s) occurs in one direction (y'). The initial concentration of the elicitor compound(s) in the biofilm (C_i) is zero. The biofilm volume (V_b) is smaller than the medium volume ((1.95 L < 3 L) and is largely occupied by the wet biomass. These two conditions make C_{bi} approximately constant. Transport in the biofilm is mainly by diffusion.

This situation is described by Fick's second law

$$D_{eb} \frac{\delta^2 C_i}{\delta y'^2} = \frac{\delta C_i}{\delta t} \qquad (3.34)$$

or

$$D_{eb} \frac{\delta^2 \theta_i}{\delta y'^2} = \frac{\delta \theta_i}{\delta t} \qquad (3.35)$$

upon writting concentrations in the following dimensionless form

$$\theta_i = \frac{C_{ie} - C_i}{C_{ie}} \qquad (3.36)$$

with C_{ie}, the concentration of the elicitor compound(s) at the biofilm surface. Equation (3.35) can be solved by separation of variables with the following boundary conditions (BC).

BC1: $\frac{\delta \theta_i}{\delta y'} = 0$ at $y' = 0$ for all t.

BC2: $\theta i = 0$ at $y' = 1$ for all $t > 0$ with $1 = \delta + t_{ki}/2$.

BC3: $\theta_i = 1$ at $t = 0$ for all the biofilm.

The general solution of Equation (3.35) is

$$\theta i(y',t) = \frac{4}{\pi} \sum_{n=0}^{\infty} \frac{(-1)n^1}{(2n^1+1)} \cos\left[\frac{(2n^1+1)\pi y'}{21}\right] e^{-\left(\frac{(2n^1+1)^2\pi^2}{41^2} D_{eb}\right)t} \qquad (3.37)$$

with n^1, an integer number.

A graphical solution of Equation (3.37) is presented (204) which shows complete saturation of the biofilm with the

elicitor compound(s) after ~ 10 hours of contact.

After this elicitation stage, the medium is changed and biosynthesis of secondary metabolites is assumed to start uniformly in the biomass (P_x). A fraction (f) of these products is secreted in the biofilm ($P_b = fP_x$) under stimulation if required. Subsequently the secreted products diffuse in the biofilm towards the biomass/liquid interface (P_s) where they are swept away by the flowing medium (P_M). The mechanism(s) controlling this stage of the process may be

- the inherent biosynthesis kinetics of plant cells,
- the requirement for some kind of continuous stimulation for biosynthesis,
- the secretion of the SM in the biofilm,
- the removal of these compounds from the biofilm,
- the saturation, either chemical or mass transfer, of the medium with the compounds,
- some other bioreaction or
- a combination of these factors.

The few reports available on the elicitation of plant cells cultured in suspension show best productivity figures of 0.0025-0.0003 g SM/L.h and f $\lesssim$ 10-50% (114,205). These figures indicate that this process is more biologically

rather than mass transfer limited. Equilibrium of fluxes at the liquid/biofilm interface (as Equation (3.19)) gives

$$k_L A(P_B - P_M) = \frac{D_{eB} A}{\delta} (P_B - P_b) = V_B f \frac{dP_x}{dt} \qquad (3.38)$$

or

$$\frac{k_L}{1+B_i} (P_B - P_M) = \delta f \frac{dP_x}{dt} \qquad (3.39)$$

or

$$\frac{k_L}{1+B_i} (fP_x - P_M) = \delta f \frac{dP_x}{dt} \qquad (3.40)$$

Equation (3.40) represents the production stage of this process. It contains numerous unknowns. The inherent kinetics of product biosynthesis by surface immobilized plant cells under medium induction or elicitation and product secretion has not be studied and was not part of the scope of this project. This equation is the starting point of the engineering evaluation of this production process.

3.2.3.4 Process Efficiency.

The metabolism of immobilized whole cells is generally sensitive to mass transfer. Growth and production bioreactions are closely coupled to external mixing and internal diffusion. The controlling process, and its effect on the productivity of the system, can be assessed by using the effectiveness factor concept. This approach can be used to evaluate the validity of the process model presented in the previous Section. There is some confusion about the application of this concept to whole cell immobilization (194,196). This can be resolved by distinguishing the following two factors.

An Efficiency Factor can be defined as follows.

$$\eta_i \equiv v_{ic}/v_{fc} \qquad (3.41)$$

where v_{ic}: rate of reaction for immobilized cells (g/L.d)

v_{fc}: rate of the same reaction carried out under identical conditions by free suspended cells (g/L.d).

This factor is not an effectiveness factor, as defined in traditional chemical engineering (186,196). It includes

mass transfer effects (mixing, reduced transfer area and diffusion through a biofilm) and physiological effects (morphological rest, close cell packing, reduced growth, exposure to a microenvironment different from the medium and influence of the immobilizing matrix material). In fact, this last series of effects may enhance certain favourable biochemical reactions and make η_i larger than 1.

The Effectiveness Factor used in traditional chemical engineering is defined as

$$\eta_m \equiv v_{rc}/(v_{rc})_{nmt} \qquad (3.42)$$

where $(v_{rc})_{nmt}$: rate of the same reaction for immobilized cells (g/L.d) carried out under identical conditions but without mass transfer limitations.

This factor depends on external mixing and internal diffusion. This is generally represented (186,196) by

$$\eta_m = f(Sh, \phi, S_i) \qquad (3.43)$$

where ϕ, the Thiele modulus is defined by

$$\phi = v_{rs}\delta/(2D_{ms}\int_{C'}^{C'_S} v_r dC')^{1/2} \qquad (3.44)$$

with v_{rs}: reaction rate at the surface concentration C'_s (g/L.d),

v_r: reaction rate in the biofilm (g/L.d).

The effect of both mass transfer operations are assessed as follows.

1- External mass transfer.

When the condition $1 << B_i$ (Equation (3.19)) $<< \phi$ is observed, the effect of the rate of external mass transfer on the process can be evaluated by defining an external effectiveness factor $\bar{\eta}$ as

$$\bar{\eta} = v_a/v_b \qquad (3.45)$$

where v_a: actual reaction rate (g/L.d) and

v_b: reaction rate at bulk concentration (g/L.d).

This can be further represented by an observable modulus Ω defined as

$$\Omega = v_a\delta/k_L C'_b \qquad (3.46)$$

Rapid external mass transfer ($k_L C'_b >> v_a\delta$) implies that

$\Omega << 1$ and the reaction rate controls the process. While rapid reaction rate means $\Omega >> 1$ and external mixing controls the process.

2- Internal mass transfer.

When external mass transfer resistance is negligible ($B_i >> \phi$), η_s is given by

$$\eta_s = f(\text{kinetics}, \delta, D_{sb}) = f(\phi) \qquad (3.47)$$

This can be expressed by an observable modulus ξ.

$$\xi = \eta_s \xi^2 = v_s \delta^2 / D_{sb} C'_b \qquad (3.48)$$

At this condition, and as per Equation (3.26), $C'_s \sim C'_b$ and

$$\xi = \eta_s \xi^2 = v_s \delta^2 / D_{sb} C'_b \qquad (3.49)$$

When the effect of diffusion becomes negligible, for example when the biofilm thickness is small or when the reaction is slow,

$$\phi << 1, \ \eta_s \rightarrow 1 \text{ and } \xi << 1 \qquad (3.50)$$

and the process is bioreaction controlled. Whereas diffusion restrictions imply

$$\phi >> 1,\ \eta \sim 1/ \quad \text{and} \quad \delta >> 1 \qquad (3.51)$$

and the reaction may be mass transfer controlled. Product inhibition coupled to low diffusion rate could also produce a similar result.

Within the context of this research project, evaluation of the immobilization efficiency factor (η_i as defined by Equation (3.41)) would be particularly difficult. The culture of a plant cell suspension in a bioreactor at identical conditions as that of an immobilization system would be difficult to achieve. A comparison will be made relative to shake flask suspension culturing.

The slowness of the bioreactions associated with the metabolism of plant cells relative to the rate of the mass transport processes involved suggests $\eta \sim 1$. This can be verified by the following equations.

1- External mass transfer.

$$\text{Equation (3.46):} \quad \Omega = v_m \delta / k_L C'_b << 1 \qquad (3.52)$$

This equation gives.

$$v_m << 57.6\ C'_b \quad (g/L.d) \qquad (3.53)$$

2- Internal mass transfer.

Equation (3.49): $$\xi = \frac{v_m \delta^2}{D_{mb} C'_b} << 1 \qquad (3.54)$$

This equation gives

$$v_m << 6.5\ C'_b \quad (g/L.d) \qquad (3.55)$$

The model of the SIPC bioreactors presented in Section 3.2.3 and the effect of mass transfer on this type of culture (η_i and η_m (Equations (3.53) and (3.55)) will be discussed in Section 4.0 in relation to the experimental results presented in Sections 3.2.4 and 3.3.

3.2.4 Mass Transfer Characteristics of the SIPC Bioreactors.

The uniform distribution of the inoculum and the homogeneous mixing of the liquid phase within the immobilizing structure and the supply of sufficient oxygen to the culture were significantly influenced by the mass transfer characteristics of the bioreactors. The objective

of this Section is to determine suitable operating conditions of these systems to meet these process requirements.

3.2.4.1 Bioreactor IA.

The relative mixing time (θ_m) and the oxygen transfer capacity (k_La) within the immobilizing structure of Bioreactor IA are mainly a function of the immobilizing layers spacing, flow diverting plate location, A_R/A_D ratio and operating conditions (liquid height and aeration rate). The immobilizing layers spacings were determined in Section 3.2.2.2 for high biomass loading and fluid access to the SIPC.

The flow diverting plate is a significant structural feature of Bioreactor IA. The importance of its position above the riser tube to the system's mixing efficiency is illustrated in Figure 3.19. The θ_m in the C layer (see Section 2.8) was selected to represent the relative mixing time within the immobilizing structure. This was the layer of the most difficult fluid access which could be fitted with the pH electrode. The θ_m in all other layers were found identical (42 ± 2 s) and compared to 14 s in the reactor peripheral spacing (at an aeration rate of 1.56

Figure 3.19 – Mixing time (θ_m) in Bioreactor IA as a function of the flow diverting plate height (DPH) above the riser tube.

Q_a = 8.1 L/min.

H_L = DPH:

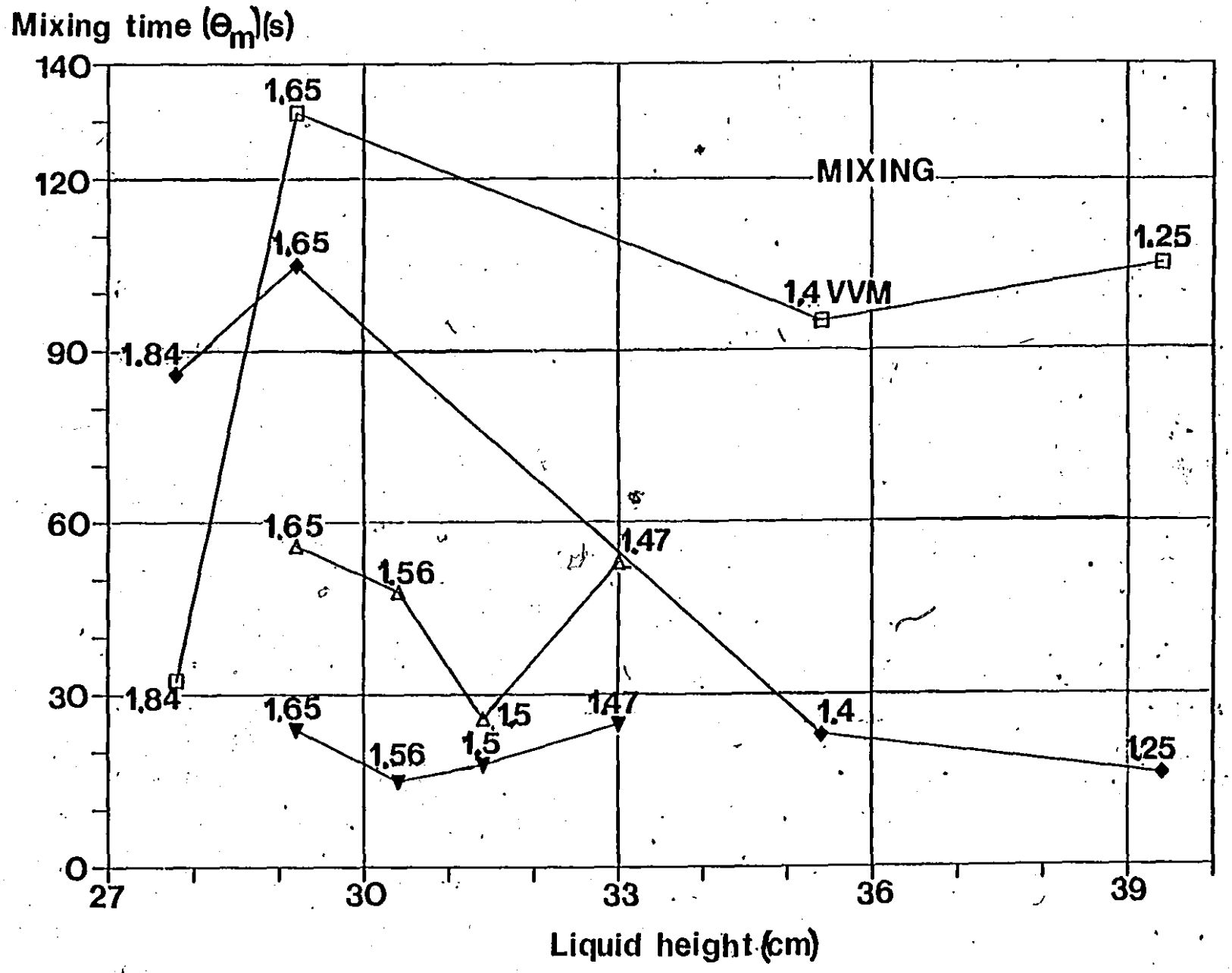
▼: pH electrode in the external layer of the immobilizing matrix.

△: pH electrode in the C layer.

No diverting plate:

◆: pH electrode in the external layer.

□: pH electrode in the C layer.


Mixing time (θ_m)(s)
140
120
90
60
30
0
27
30
33
36
39
Liquid height (cm)
MIXING
1,4 VVM
1,65
1,25
1,84
1,65
1,65
1,47
1,56
1,84
1,65
1,47
1,5
1,4
1,56
1,5
1,25

VVM, a V_L = 5.2 L and with the flow diverting plate located at 2.54 cm above the riser tube).

Mixing within the immobilizing structure (θ_m ~ 100 s) was inefficient as compared to mixing in the peripheral space (θ_m ~ 20 s) at liquid levels (H_L) higher than 35 cm (or higher than 7 cm above the immobilizing structure height) without the flow diverting plate. In fact this shows high flow by-pass (~5/1) above the immobilizing structure and in the peripheral spacing of the reactor. Below this H_L of 35 cm, mixing is comparatively less efficient everywhere in the reactor (θ_m ~ 130 s against 105 s) even if the mixing rate (VVM) increases inversely to H_L decline. However, at H_L ~ immobilizing structure height (27.9 cm), this trend is reversed, with better mixing inside the structure (θ_m ~ 30 s) as compared to the peripheral spacing (θ_m ~ 85 s) since the fluid is uniformly distributed over the spiral structure vertical flow area.

The flow diverting plate located above the riser tube improves significantly the mixing efficiency and uniformity across the whole bioreactor vertical flow area (θ_m ~ 15-25 s in the peripheral space and 25-55 s in the immobilizing structure). A best location of the flow diverting plate (and of the liquid height as shown in Figure 3.20) is observed at 2.5-3.5 cm above the riser tube. A location of

2.5 cm was selected to minimize the required medium volume for a suitable operation of the bioreactor.

The effect of the operating conditions on θ_m are summarized in Figure 3.20 for the best flow diverting plate position. The liquid level relative to the flow diverting plate location affects significantly the uniformity of mixing in Bioreactor IA. At H_L higher than the flow diverting plate location (30.5 cm from the reactor bottom or 12.5 cm above the riser tube), mixing in the immobilizing structure (θ_m ~ 110-200 s) is less efficient than at the vessel periphery (θ_m ~ 10-20 s). At H_L lower than 30.5 cm, this is reversed (θ_m inside ~ 45-70 s against θ_m ~ 20-160 s at the periphery). As a result, a relative mixing uniformity across the whole immobilizing structure vertical flow area is achieved by maintaining the liquid level between the top of the structure height (27.9 cm) and the flow diverting plate height (30.5 cm). This corresponds to an effective liquid reactor volume used of 4.4 to 5.2 litres. Little improvement in θ_m occurs above an aeration rate of 0.9-1.0 VVM.

The measured relative mixing times presented in Figure 3.20 compare to the literature values of 20-50 s mentioned in Section 3.2.3.1 for similar systems. These

Figure 3.20 – Mixing time (θ_m) in Bioreactor IA as a function of the liquid volume (H_L) and aeration rate.

DPH = 30.5 cm.

Liquid Levels: 1- 6.5 L/39.4 cm.

2- 5.8 L/35.4 cm.

3- 5.2 L/30.5 cm (DPH).

4- 4.7 L/29.2 cm.

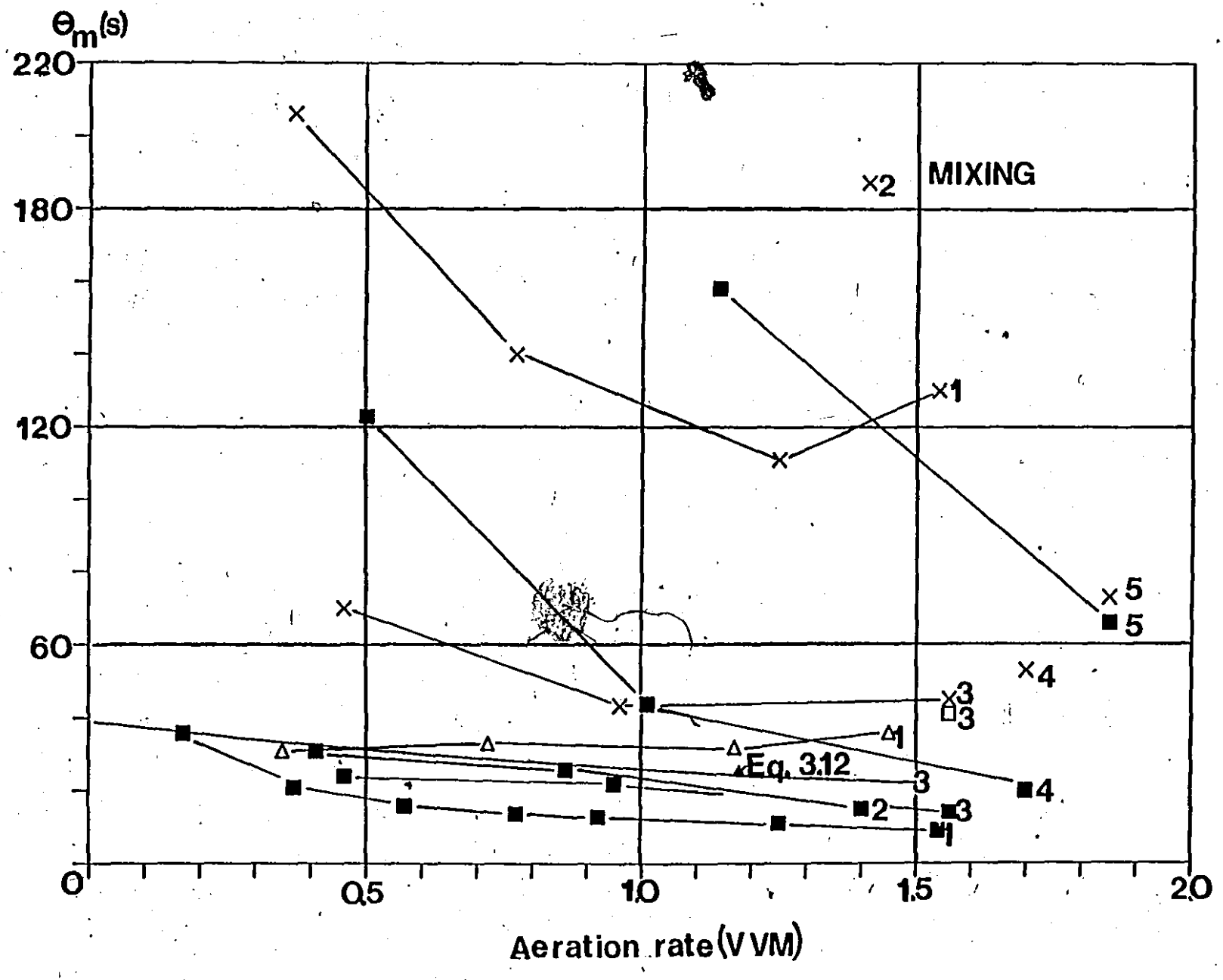
5- 4.4 L/27.9 cm (Immobilizing structure level).

■: pH electrode in the external layer.

×: pH electrode in the A layer.

□: pH electrode in the B,C,D layers.

△: no immobilizing structure.


θm(s)
220
180
120
60
0
0.5
1.0
1.5
2.0
Aeration rate(VVM)
MIXING
Eq. 3.12
×1
×2
×3
×4
×5
■1
■2
■3
■4
■5
□3
Δ1
Δ3

relative mixing times compare also well to the average circulation times predicted by the hydrodynamic model of Equation (3.12) when the immobilizing structure was removed from the bioreactor and when the pH electrodes was located in the peripheral spacing of the bioreactor at $H_L \geq 5.2$ L.

As mentioned in Section 3.2.3.2, mixing should have little effect on SIPC bioprocesses as long as some uniformity is achieved. The results presented above show that the flow diverting plate and proper level control will provide for the degree of mixing uniformity required. In particular, the uniform distribution of the plant cell suspension inoculum within the immobilizing structure can be achieved easily and efficiently by maintaining the liquid level at the structure height (4.4 L or 27.9 cm) during the immobilization period.

The oxygen transfer capacity (k_La) of Bioreactor IA was measured as per Section 2.8.2. Results are summarized in Figure 3.21 and Table 3.9. The results of Figure 3.21 show fairly linear correlations between $k_La(h^{-1})$ and the aeration rate (VVM) according to the following equations.

Line 1: DO probe in the peripheral space.

$$k_La = 13.5(VVM) + 3.5 \qquad (3.56)$$

Figure 3.21 - Oxygen transfer capacity of Bioreactor IA as a function of the aeration rate.

V_L = 5.2 L.

DPH = 30.5 cm.

▼ : DO probe in the external layer.

△ : DO probe in the immobilizing structure.

□ : without the immobilizing structure.

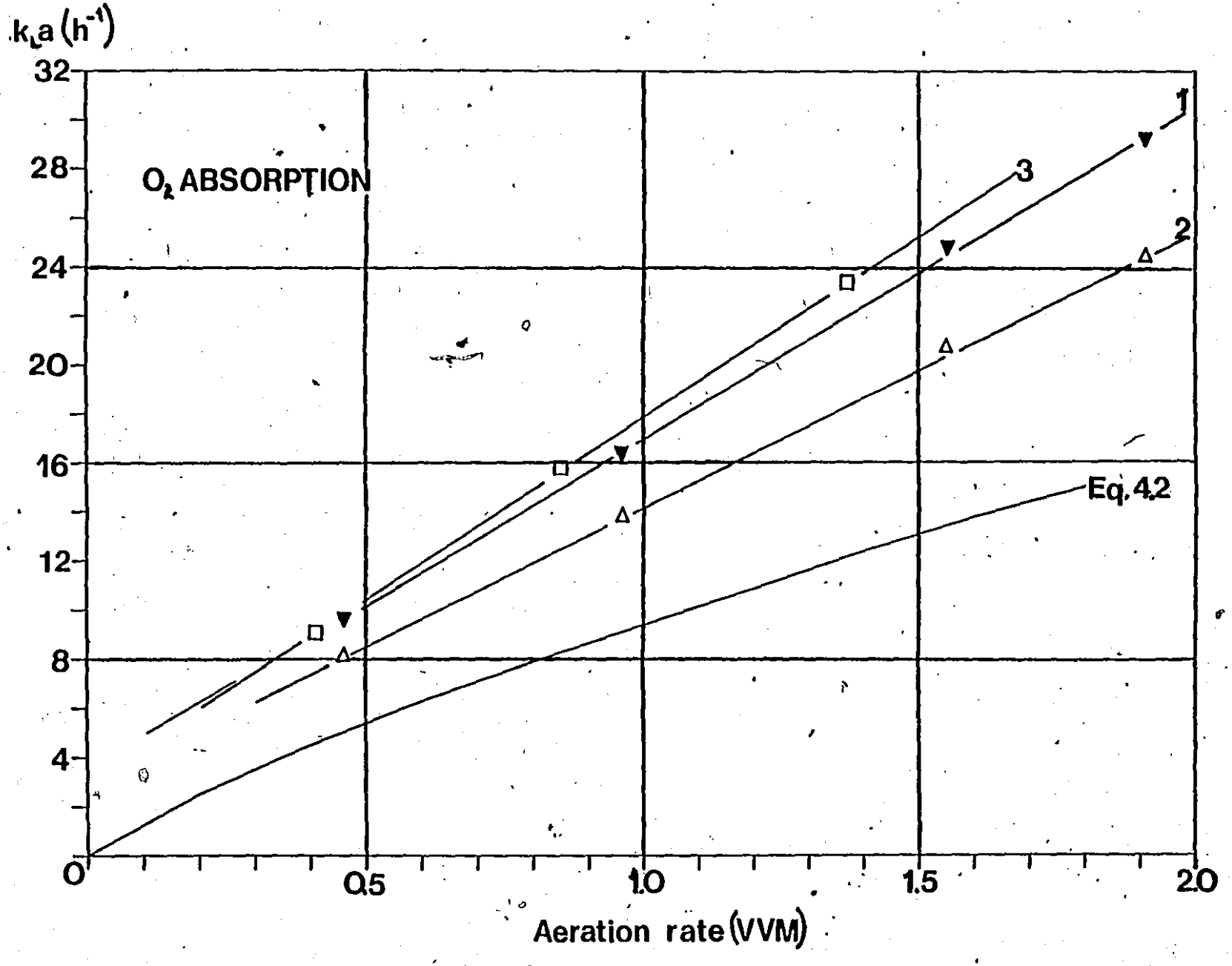

$k_La\ (h^{-1})$
32
28
24
20
16
12
8
4
0
O_2 ABSORPTION
1
2
3
Eq. 4.2
0.5
1.0
1.5
2.0
Aeration rate (VVM)

TABLE 3.9

OXYGEN TRANSFER CAPACITY OF BIOREACTOR

IA AT AN AERATION RATE OF 5 L/MIN.

	Liquid Volume		Aeration Rate (VVM)	DO Probe Location	Diverting Plate Location (cm)	Presence of Matrix	$k_L a$ (h^{-1})
	Height (cm)	(L)					
1	30.5	5.2	0.96	External	31.0	Yes	16.5
2	30.5	5.2	0.96	In Matrix	31.0	Yes	13.9
3	30.5	5.2	0.96	In Matrix	None	Yes	16.7
4	30.5	5.9	0.85	External	31.0	No	14.9
5	39.4	6.6	0.75	External	31.0	No	14.1
6	39.4	6.5	0.77	Internal	31.0	Yes	15.7
7	39.4	6.5	0.77	Internal	None	Yes	15.8
8	27.9	4.4	1.14	Internal	31.0	Yes	23.9
9	27.9	4.4	1.14	Internal	None	Yes	20.6

Linear correlation coefficient r = 0.99.

Line 2: DO probe in the immobilizing structure.

$$k_La = 11.3(VVM) + 3.0 \quad (3.57)$$

Linear correlation coefficient r = 0.99.

Line 3: No immobilizing structure in the bioreactor.

$$k_La = 14.8(VVM) + 2.8 \quad (3.58)$$

Linear correlation coefficient r = 0.99.

The k_La coefficient is not dramatically affected by the immobilizing structure. It is only reduced by its presence by 9% and 24% in the peripheral space and within the immobilizing structure. As shown in Table 3.9, the flow diverting plate height and high liquid levels have no significant effects on k_La. Whereas k_La is increased by 50% over the expected value obtained from Equation (3.57) when the liquid level is maintained at the immobilizing structure level (4.4 L). Consequently, a best aeration rate of Bioreactor IA is ~ 0.9-1.0 VVM. This condition ensures good mixing and sufficient oxygen transfer to the medium (k_La ~ 15 h^{-1}) for good growth of plant cells as recommended in Section 1.2.1.2.

3.2.4.2 Bioreactor IP.

The relative mixing time (θ_m) and the oxygen transfer capacity of Bioreactor IP within the immobilizing structure are mainly a function of its operating conditions (aeration and mixing rates and liquid level). The main structural features of Bioreactor IP (immobilizing layers spacing and structure height) have been determined for high biomass loading and fluid access to the immobilized plant cells.

The θ_m measured inside the immobilizing structure of Bioreactor IP are presented in Figure 3.22 as a function of the mixing rate. Little improvement in internal mixing occurs above an agitation rate of 200 RPM (Impeller Reynolds Number ~ 19760 or Tip speed ~ 81 cm/s). At this mixing rate, the presence of the immobilizing structure has little effect on θ_m. The lowering of the liquid level by 16% (to the immobilizing structure height) increases θ_m by 80%. This emphasized the positive effect of the liquid level above the immobilizing structure on the mixing efficiency inside it. The mixing times measured in Bioreactor IP compare to the mixing times measured in Bioreactor IA.

The effect of the operating conditions on the oxygen transfer capacity (k_La) of Bioreactor IP are presented in

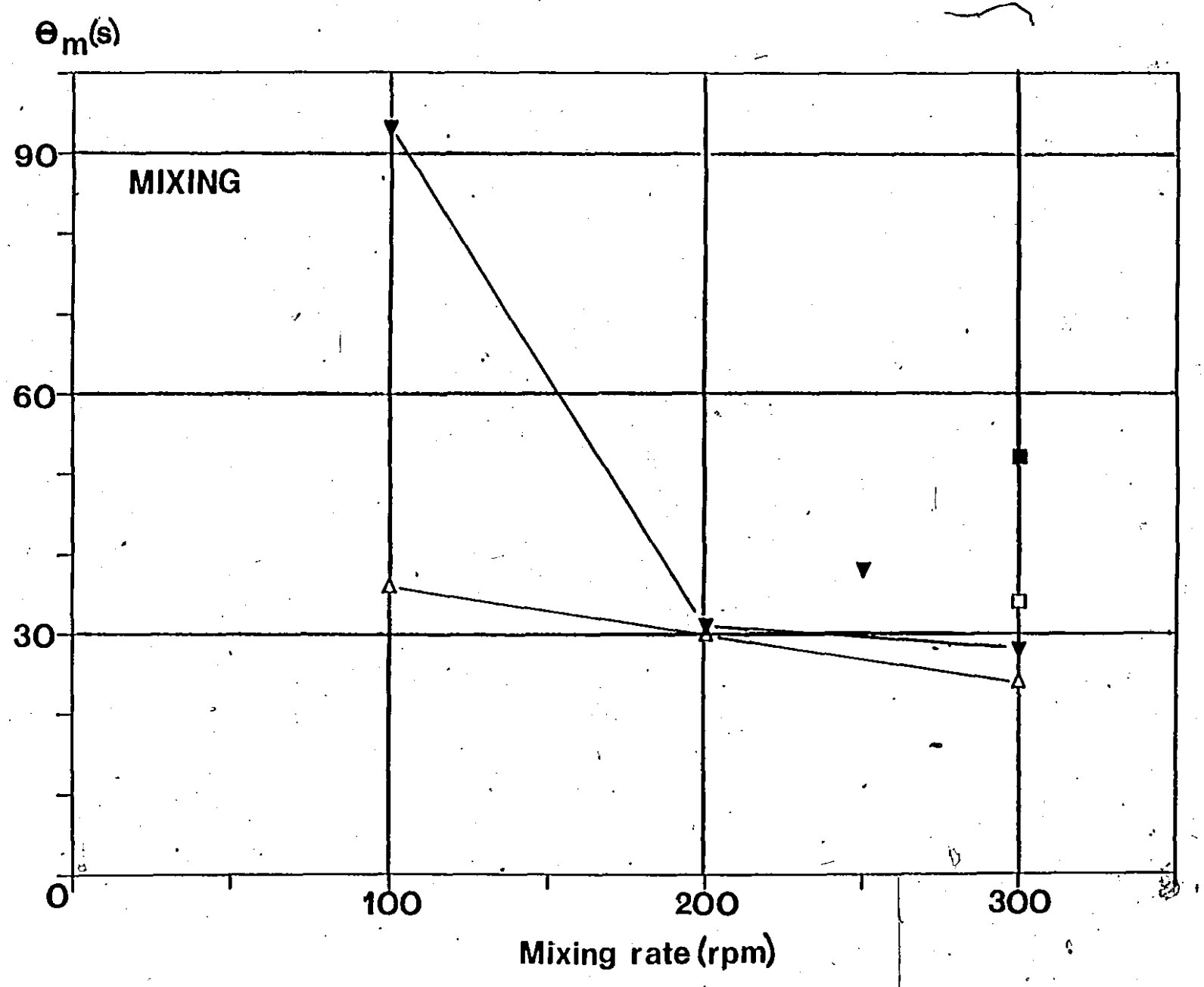
Figure 3.22 - Relative mixing time (θ_m) in Bioreactor IP as a function of the mixing rate.

▼ : 2 L level (16 cm) with the immobilizing structure.

△ : 2 L level (16 cm) without the immobilizing structure.

■ : 1.6 L level (13.5 cm: immobilizing structure level) with the immobilizing structure.

□ : 1.6 L level (13.5 cm: immobilizing structure level) without the immobilizing structure.


Θ_m(s)
90
60
30
0
MIXING
100
200
300
Mixing rate (rpm)

Figures 3.23 and 3.24. Contrary to mixing time, the k_La coefficient is not affected by the liquid level in the bioreactor. However, k_La is significantly reduced (~ 17 to 45%) by the presence of the immobilizing structure with increasing mixing and aeration rates. The effect of the mixing rate on k_La can be correlated linearly, as shown in Figure 3.23, by the following equations.

Line 1: with the immobilizing structure present.

$$k_La = 1.06 \times 10^{-4}(Re_i) + 6.7 \qquad (3.59)$$

Linear correlation coefficient r = 0.99

Line 2: without the immobilizing structure.

$$k_La = 4.45 \times 10^{-4}(Re_i) + 4.3 \qquad (3.60)$$

Linear correlation coefficient r = 0.96

The immobilizing structure damps the effect of the mixing rate on k_La by limiting the air dispersion action of the impeller. This results in a marginal improvement of 27% in k_La upon increasing the mixing speed by 200% when the structure is present. Whereas a similar increase in the mixing rate improves k_La by 94% when the immobilizing structure is not present in the bioreactor.

Figure 3.23 – Oxygen transfer capacity (k_La) of Bioreactor IP as a function of the mixing rate at a 0.2 VVM aeration rate.

▼ : 2 L level (16 cm) with the immobilizing structure.

△ : 2 L level (16 cm) without the immobilizing structure.

■ : 1.6 L level (13.5 cm) with the immobilizing structure.

□ : 1.6 L level (13.5 cm) without the immobilizing structure.

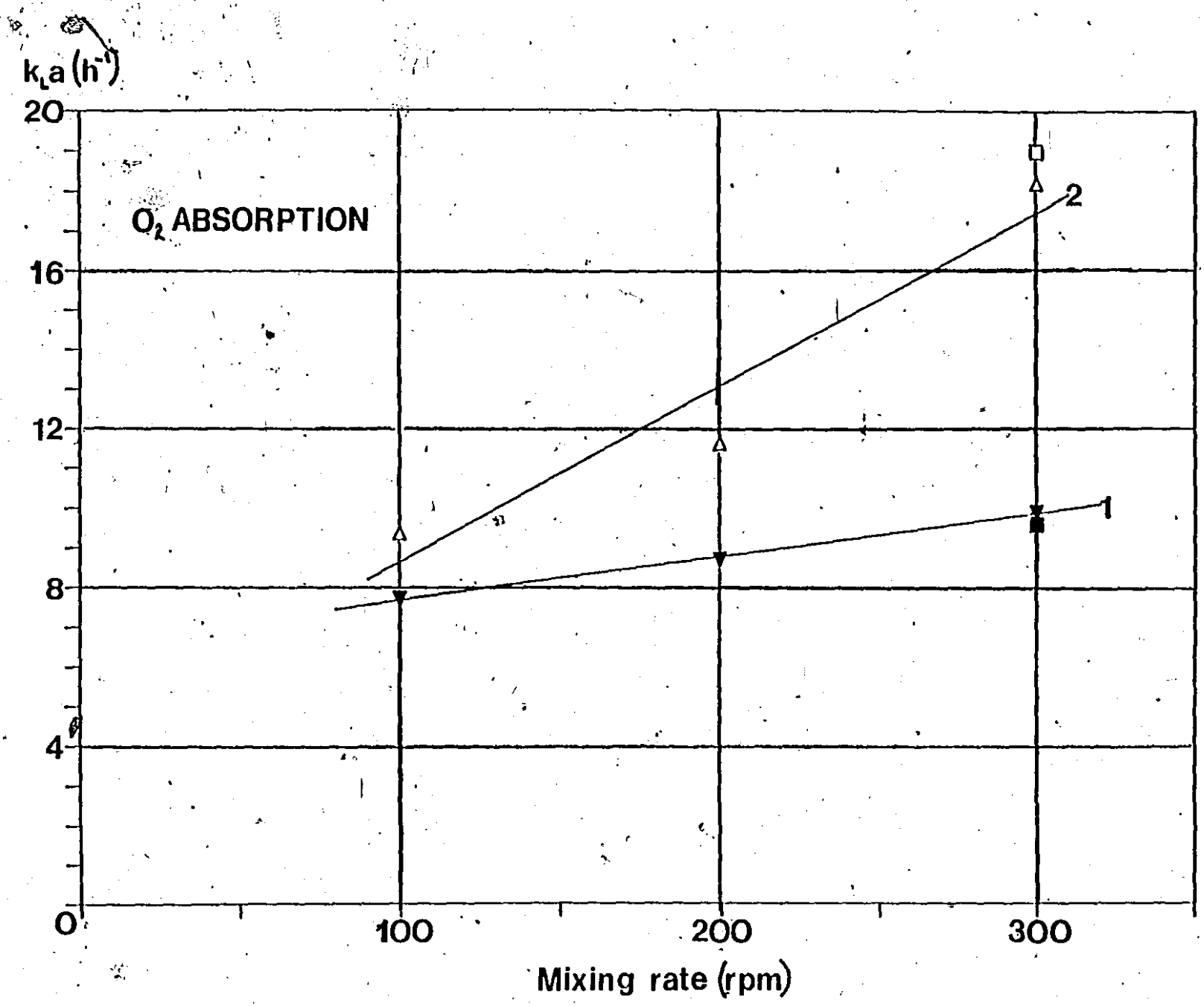

$k_La\ (h^{-1})$
20
16
12
8
4
0
O_2 ABSORPTION
2
1
100
200
300
Mixing rate (rpm)

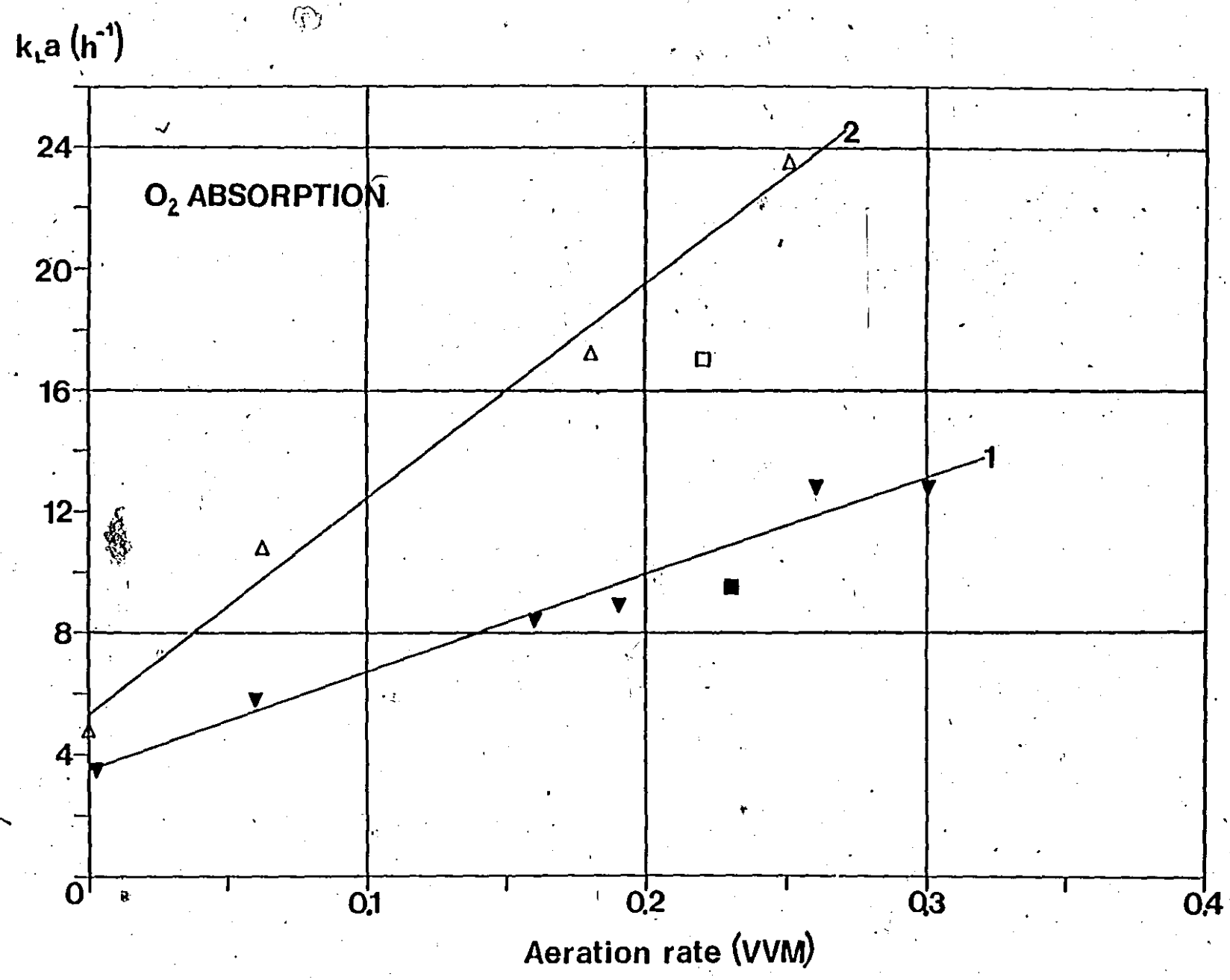
Figure 3.24 - Oxygen transfer capacity (k_La) of Bioreactor IP as a function of the aeration rate at the mixing rate of 300 RPM ($Re_i \sim 29645$).

▼: 2 L level (16 cm) with the immobilizing structure.

△: 2 L level (16 cm) without the immobilizing structure.

■: 1.6 L level (13.5 cm) with the immobilizing structure.

□: 1.6 L level (13.5 cm) without the immobilizing structure.


$k_La\ (h^{-1})$
O_2 ABSORPTION
24
20
16
12
8
4
0
0.1
0.2
0.3
0.4
Aeration rate (VVM)
1
2

The aeration rate represents a better control variable over k_La in this system as shown in Figure 3.24. The k_La coefficient can be linearly correlated to the aeration rate (VVM) by the following equations.

Line 1: with the immobilizing structure present.

$$k_La = 32.4(VVM) + 3.6 \qquad (3.61)$$

Linear correlation coefficient r = 0.99.

Line 2: without the immobilizing structure.

$$k_La = 71.9(VVM) + 5.5 \qquad (3.62)$$

Linear correlation coefficient r = 0.99.

The better response of the k_La coefficient to the aeration rate is indicated by an improvement of ~ 120% in k_La upon a ~ 320% increase in VVM with or without the immobilizing structure. It is interesting to note that surface aeration contributes significantly (3.5 h^{-1} and 4.9 h^{-1} with or without the immobilizing structure) to this small, low H/D (~ 1.5) bioreactor total k_La (~ 6-24 h^{-1}).

The conditions for a best operation of Bioreactor IP were selected as per the above results and to meet the

physical limits of the system. The aeration rate of 0.2 VVM was chosen for sufficient oxygen supply ($k_La \sim 10\ h^{-1}$) and to limit the expansion of the foam layer produced by the culture in the restricted space above the liquid surface (~ 5 cm). While a mixing rate of 300 RPM ($Re_i \sim 29645$ or Tip speed ~ 121 cm/s), which was the systems limit ensuring the rotational stability of the magnetic stirring bar, provided for sufficient agitation and aeration of the culture.

3.3 Performance of the SIPC Bioreactors.

The performance of the SIPC bioreactors was evaluated for biomass immobilization and growth. Suitable culture conditions were determined for each bioreactor. The growth of surface immobilized Catharanthus roseus (line MCR17) cells cultured in these systems was characterized.

3.3.1 Bioreactor IP.

3.3.1.1 Operating Procedure.

The immobilization matrix was formed in the vertical square spiral configuration and placed in the bioreactor described in Section 3.2.2.1. The bioreactor was filled with 1.2 L of the growth medium 185 and was steam sterilized with accessories according to the stardard practice (60 min, 120°C, 15 psi). The sterile bioreactor was filled with additional sterile medium to the operational volume (1.6-1.9. L) and with a suitable plant cell suspension inoculum (8 to 15% of the initial total culture volume). This inoculum was a shake flask grown culture prepared as per Section 2.1. It was generally a 8 to 10. day old culture (See Figure 2.1) as recommended in Section 3.1.2.3.

The bioreactor was fitted with necessary sterile accessories (condenser, feed reservoir(s) etc) and installed on the magnetic stirrer. The culture temperature was maintained at 28 ± 1°C while sterile aeration and mixing rates were adjusted to desire levels (0.2 VVM and 300 RPM).

During the culture period, the medium was regularly sampled (one sample per day or 2 days) for pH and carbohydrate measurement. This sterile sampling was performed with 125 ml sterile filtering Erlenmeyer flasks fitted to the bioreactor sampling port. The sterile air flow to the bioreactor was temporarily increased and the medium was transferred in the sampling flask (~ 25 ml) by vacuum. Each sample was preceded by a line flushing of the sampling port tube. Larger purging of the bioreactor (100 ml to 1500 ml) was performed by the same procedure using larger flasks as required.

At the end of the culture, the bioreactor was rapidly dismantled. The biomass produced was collected and treated as per Section 2.6.3 and Appendix 3. The temperature, pH and dissolved oxygen concentration of the medium were measured. The medium was filtered, weighed and sampled.

3.3.1.2 General Operation Comments.

The inoculated plant cell biomass attached readily and uniformly to the immobilizing matrix. At the standard inoculation ratio used (8% of the total culture volume or 10% of the initial medium volume) all the plant cells were immobilized during the first 24 hours of the culture. The medium was completely free of suspended biomass until the end of the culture period. The final immobilization efficiency of the system (quantity of SIPC/quantity of produced biomass) was 96.4% (average of 49 cultures, s = 1.9%). The use of larger inoculum volumes (up to 15% of the culture volume) required lower initial mixing (125 RPM) and aeration rates (0.1 VVM) to prevent excessive plant cell grinding and foaming. Complete immobilization of the inoculated biomass occured within the first 48 hours of the culture at this higher inoculation ratio.

The medium was remarkably clear and free of cell debris and secreted macromolecular species during the whole culture period. A small and controlled foam layer formed above the medium surface. This foam consisted of a non permanent section (~ 1 cm) immediately adjacent to the liquid surface and a biomass froth (1-2 cm) above the non permanent section. This froth mainly (50 to 100%) was made of a whiteish material and unattached plant cells.

This froth was the sole unattached biomass present in the bioreactor. The formation of this foam layer in SIPC cultures was found to depend on the medium initial sucrose concentration and on the inoculum age. SIPC cultures initiated at a sucrose concentration of 10 g/L produced less than half of the biomass froth observed in cultures started at higher sucrose concentrations (20 or 40 g/L). The use of a 5 day old plant cell suspension inoculum (~ exponential phase, see Figure 2.1) instead of the standard 8-10 day old inoculum resulted also in SIPC cultures with less (~ 30-50%) biomass froth.

3.3.1.3 The Growth of SIPC Cultured in Biorector IP.

The biomass formation pattern of SIPC cultured in Bioreactor IP is illustrated in Figure 3.25. These results were normalized to a 1900 cm^2 immobilizing area. The two most significant features of these growth curves are their linearity during the first 6-7 days of the culture and the occurence of a biomass stable stationary phase thereafter for batch cultures using an initial sucrose concentration (S) of 20 g/L. This stationary phase occured generally (17 out of 19 cultures) without complete carbohydrate consumption from the medium as shown in Figures 3.26 and 3.30.

Figure 3.25 - Normalized growth curves of SIPC cultured in Bioreactor IP.

▼ : S = 20 g/L.

\+ : S = 10 g/L.

□ : S = 40 g/L.

◨ : S = 20 g/L, SB1.

◧ : S = 20 g/L , Su582.

▬ : S = 20 g/L (5d) + APM.

▬ : S = 40 g/L + N_2/PO_4 (day 4 to 8).

▨ : S = 30 g/L, 5d old inoculum.

▨ : S = 40 g/L, 15% inoculum.

■ : S = 20 g/L + N_2/PO_4 (day 7 to 12).

⊞ : S = 10 g/L, medium change S = 20 g/L.

⊡ : S = 20 g/L, 15% inoculum.

◪ : S = 20 g/L, semicontinuous, 15% inoculum.

◪ : S = 20 g/L, semicontinuous.

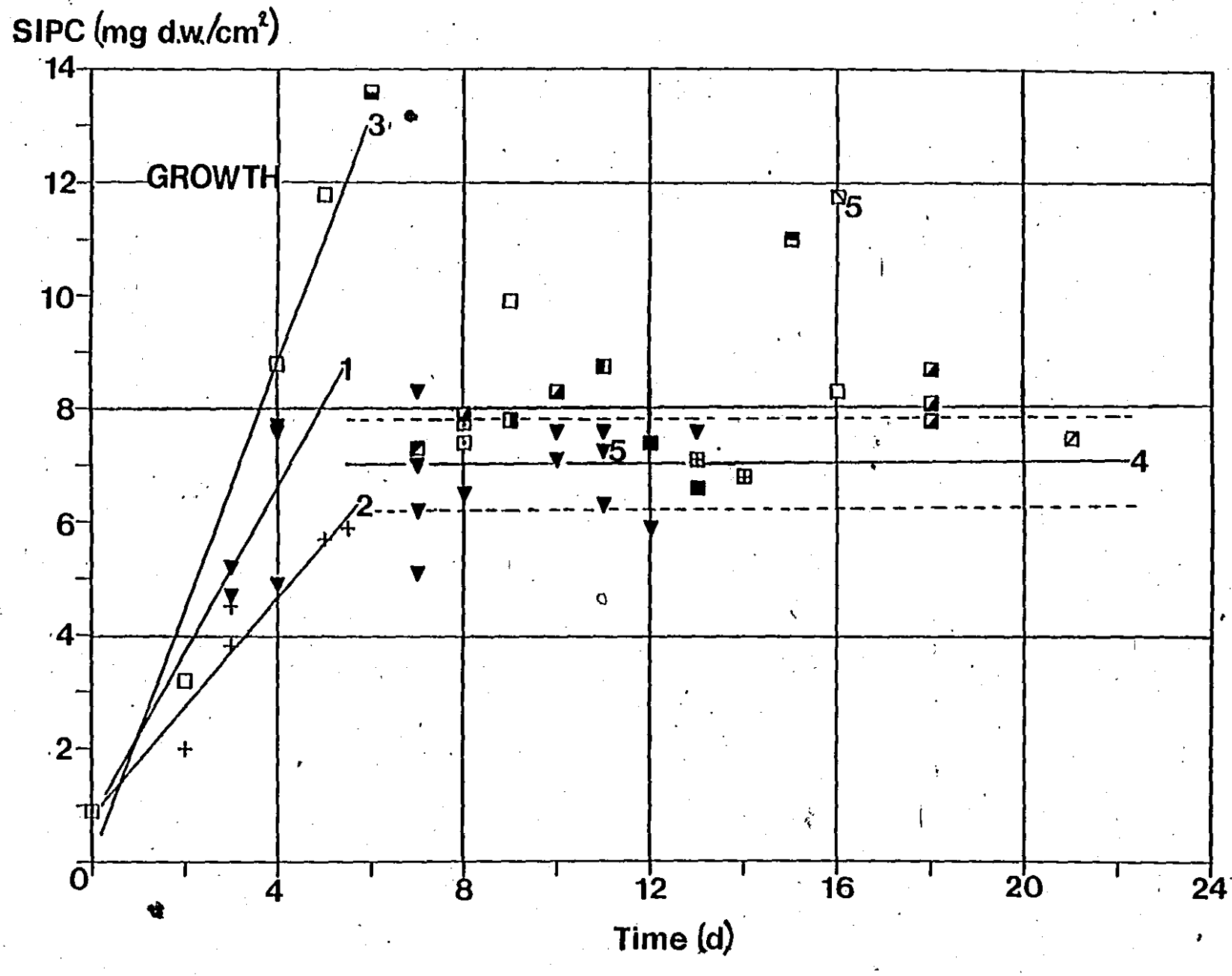

SIPC (mg d.w./cm²)
14
12
10
8
6
4
2
0
GROWTH
3
1
2
5
5
4
0
4
8
12
16
20
24
Time (d)

Figure 3.26 – Final carbohydrate concentration in the medium of SIPC cultured in Bioreactor IP. Symbols are as in Figure 3.25.

SC: Suspension culture.

Line 1: S = 20 g/L CHO= 19.0 – 1.9t (3.66)

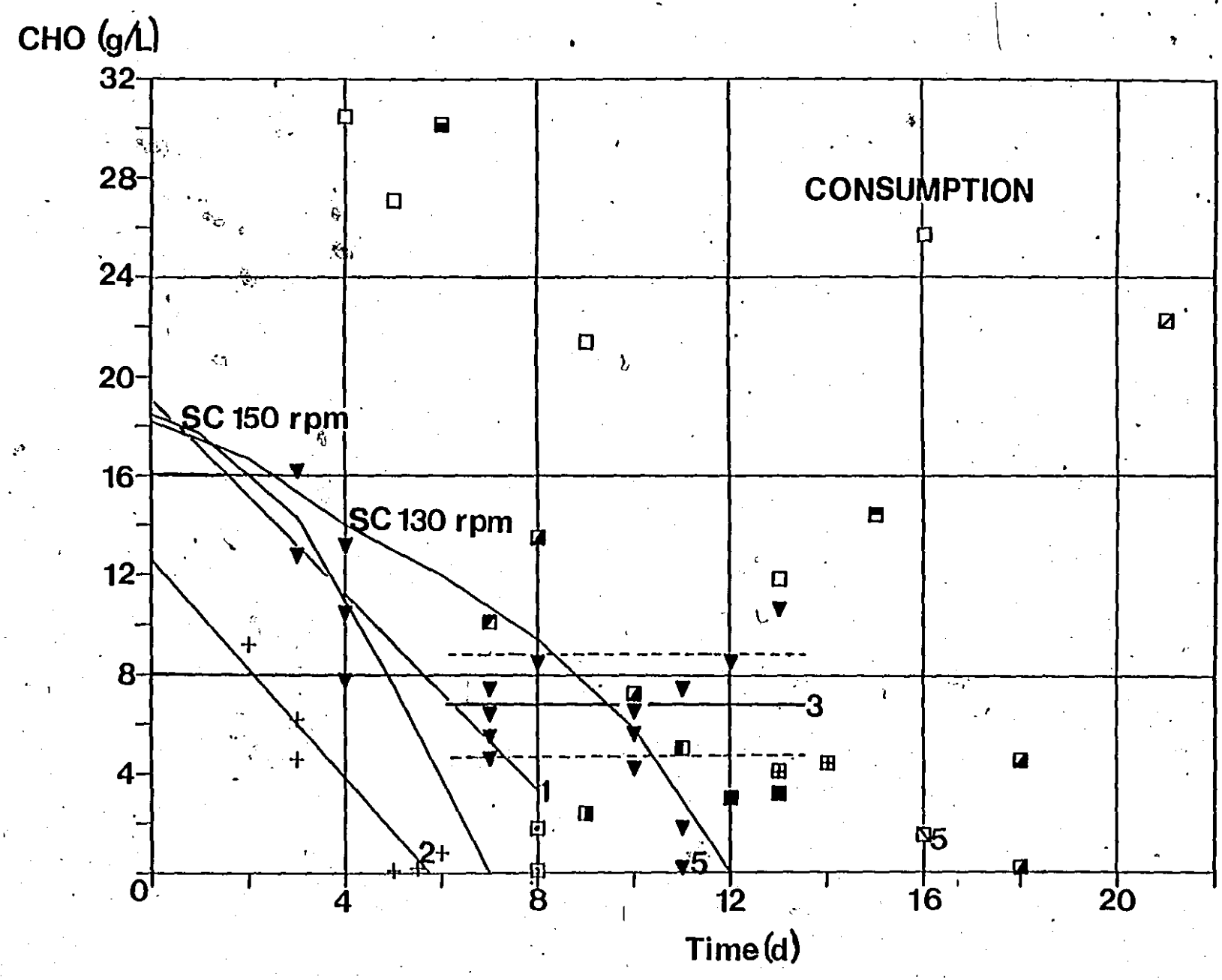
N_o = 9 r = 0.87

Line 3: S = 20 g/L CHO= 6.7 s = 2.0

N_o = 10

Line 2: S = 10 g/L CHO= 12.5 – 2.2t (3.67)

N_o = 6 r = 0.94


CHO (g/L)
32
28
24
20
16
12
8
4
0
4
8
12
16
20
Time (d)
CONSUMPTION
SC 150 rpm
SC 130 rpm
1
2+
3
5
5

The results of Figure 3.26 are correlated in Table 3.10. These growth curves, before the stationary phase, are significantly linear ($r \geq 0.9$). They fit well the empirical SIPC growth model represented by Equation (3.18). The constant growth rates (b [mg d.w./cm^2.d] can be further correlated with the medium initial sucrose concentration (S [g/L] by the following equation.

$$b = 0.041S + 0.60 \qquad (3.68)$$

Linear correlation coefficient $r = 0.99$

The growth curves of Figure 3.26 can be represented in terms of dried biomass concentration ([g d.w./L of broth]) in the bioreactor at harvesting. This corresponds to an equivalent plant cell suspension concentration. These growth curves are illustrated in Figure 3.27 and are correlated in Table 3.11. These growth curves are also fairly linear ($r \geq 0.9$) before the stationary phase. The immobilized biomass growth rates (b'[g d.w./L.d]) and specific growth rates of S = 20 g/L cultures are identical to rates obtained for plant cell suspensions cultured in shake flasks agitated at 150 RPM. The constant SIPC growth rates can be further correlated to the medium initial sucrose concentration (as Equation (3.68)) by the following equation.

TABLE 3.10

GROWTH CORRELATIONS OF SIPC CULTURED IN BIOREACTOR LP

Initial Sucrose Concentration (g/L)	Culture Period (d)	Number of Cultures	Growth Correlations (mg d.w./cm^2)	r(1)	Figure 3.25 Line	Specific Growth Rate μ (d^{-1})	r(1)
20	2 to 6	6(2)	SIPC = 1.47t + 0.78 (3.63)	0.91	1	0.50	0.97
	7 to 14	18(3)	$\overline{SIPC}$ = 7.00 s(4) = 0.75	-	4	-	-
10	2 to 6	6(2)	SIPC = 0.97t + 0.82 (3.64)	0.97	2	0.34	0.94
40	2 to 5	4(2)	SIPC = 2.21t + 0.08 (3.65)	0.98	3	0.52	0.99

(1) r: Linear correlation coefficient.
(2) An origin point was included corresponding to full inoculum immobilization at t = 0.
(3) All S = 20 g/L data were included except
- Fedbatch experiments, (6)
- Su582 and SB1 cultures.
(4) s: Statistical standard deviation shown by dotted lines in Figure 3.25.

Figure 3.27 - Growth curves of SIPC cultured in Bioreactor IP expressed in suspended biomass concentration.

SC: Suspension culture.

Symbols are as in Figure 3.25.

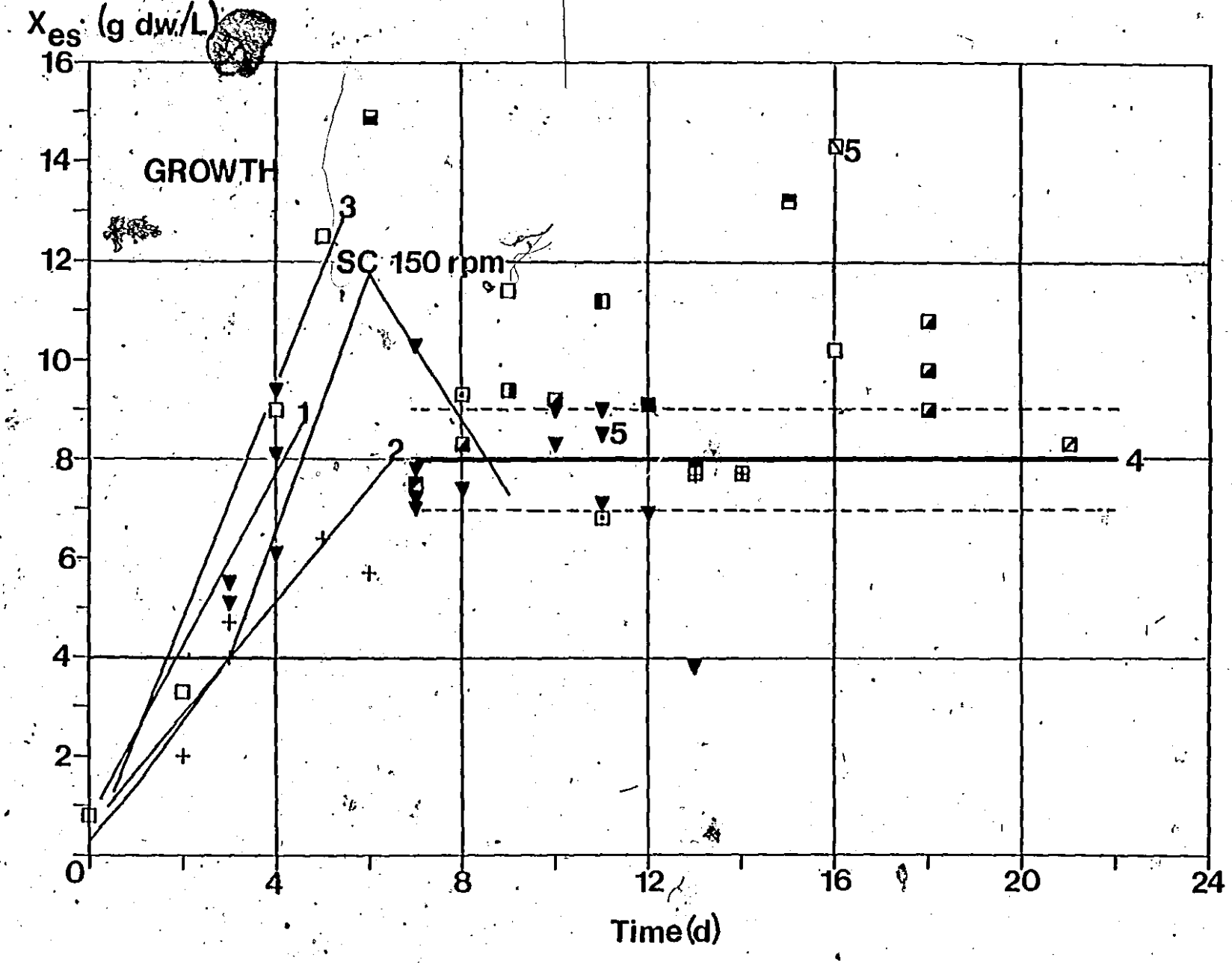

X_{es} (g dw/L)
16
14
12
10
8
6
4
2
0
GROWTH
SC 150 rpm
1
2
3
4
5
5
4
8
12
16
20
24
Time (d)

TABLE 3.11

GROWTH CORRELATIONS OF PLANT CELLS CULTURED IN BIOREACTOR IP

Initial Sucrose Concentration (g/L)	Culture Period (d)	Number of Culture	Growth Correlations (g d.w./L)	r(1)	Figure 3.27 Line	Specific Growth Rate μ (d^{-1})	r(1)
20	2 to 6	6(2)	$X_{es} = 1.77t + 0.53$ (3.69)	0.92	1	0.56	0.98
	7 to 14	18(3)	$\overline{X_{es}} = 8.0$ $s^{(4)} = 1.0$	-	4	-	-
10	2 to 6	6(2)	$X_{es} = 1.12t + 0.63$ (3.70)	0.97	2	0.38	0.95
40	2 to 6	4(2)	$X_{es} = 2.35t - 0.069$ (3.71)	0.99	3	0.55	0.99
Suspension Control (Figure 2.1) 20	0 to 6	Number of points 8	$X = 1.92t - 0.40$ (3.72)	0.98	SC	0.56	0.95

(1) r: Linear correlation coefficient.
(2) The inoculation point was included (0.8 g d.w./L at t = 0).
(3) All S = 20 g/L results were included except:
- Fedbatch experiments (6).
- Su582 and 6B1 cultures.
(4) s: Statistical standard deviation shown by dotted lines in Figure 3.27.

$$b' = 0.039S + 0.83 \qquad (3.73)$$

Linear correlation coefficient r = 0.97

The biomass stationary phase observed for SIPC cultures performed according to the standard protocol used limits the total biomass content of Bioreactor IP as shown in Figure 3.27. Except for the SIPC cultures carried out at S = 40 g/L, most cultures performed for more than 6 days (22 out of 26 cultures) were limited to biomass concentration of 8 ± 1 g d.w./L. The biomass concentration of the SIPC cultures performed at S = 40 g/L was most probably overestimated as indicated by the higher yields observed (Figure 3.30) because of the high carbohydrate concentrations (> 20-30 g/L) present in the medium which may have partly interfered with dry biomass measurements.

The following methods were investigated to increase this biomass concentration in Bioreactor IP.

1- Use of a 15% (of total culture volume) inoculum.
2- Periodical addition (10 cc/d from day 7 to day 12) of a concentrated nitrogen/phosphate solution to a 20 g/L SIPC culture. This solution was made in nutrients proportions equivalent to the standard B5 formulation (Appendix 2) and to a quantity of 75% of a regular 1 litre B5 medium.

3- Initiation of a regular S = 20g/L SIPC culture containing only 50% of the nutrients except for sucrose. The other 50% of the nutrients was added after 2 days of culture.

4- Growth of S = 10 g/L SIPC cultures for 3, 4 or 5 days, then replacement of 80% of the liquid phase with fresh medium of a 20 g/L sucrose concentration.

5- Growth of S = 20 g/L SIPC cultures for 4 days, then daily replacement of part of the medium (~ semi-continuous operation) with the same fresh medium at rates of 480, 330 or 175 g/d for 2, 3 or 6 days respectively.

6- Periodical addition (20 cc/d from day 4 to day 8) of a same nitrogen/phosphate solution as in Method 2 above to a S = 40 g/L SIPC culture.

As shown in Figures 3.25 and 3.26, Methods 1 to 4 were unsuccessful. The biomass concentration at the stationary phase was not affected by these changes. The use of a 15% inoculum did not improve the biomass concentration. Methods 1 to 3 resulted only in some increase in carbohydrate consumption (Figure 3.26). Method 4, even though it allowed a 80% increase in total nutrient availability to growing SIPC, resulted only in a biomass formation equivalent to a normal batch culture (at

S = 20 g/L, Figure 3.25). In this case, the carbohydrate consumption rate declined from 2.1-2.4 g/L.d to 0.3 g/L.d within 4 days after the medium change.

The semi-continuous Method 5 resulted in some slow (~ 0.25 g of dried biomass/L.d) improvement in total biomass formation (13 to 35%), especially at the slower addition rate. The carbohydrate consumption was increased mainly at the higher addition rates (Figure 3.26).

The daily addition of the nitrogen/phosphate concentrated solution from day 4 to a S = 40 g/L SIPC culture (Method 6) resulted in a significant increase (44%) in carbohydrate consumption (Figure 3.26: from a final carbohydrate concentration of 26 g/L for an untreated culture to 14.5 g/L). A similar increase (~ 30%) was noted in the biomass formation of this culture (Figures 3.25 and 3.27), even though this result may have been partly masked by the high carbohydrate concentration present in the medium. This was the most significant improvement in SIPC biomass formation obtained using the methods listed above.

It is important to observe that the SIPC biofilm was more responsive to some of these changes when they occured before the 5th or 6th day of the culture. This is illustrated by the results obtained when using Method 4 as

compared to Method 2. A similar behaviour was observed upon changing the medium of a 3 or 4 day old S = 10 g/L SIPC culture as compared to a 5 day old culture (Method 4). Higher carbohydrate consumptions were observed for the younger cultures after the medium change.

One of the last C. roseus SIPC cultures performed in Bioreactor IP involved assessing the immobilization efficiency of a 5 day old plant cell suspension inoculum (~ exponential phase, see Figure 2.1) at the standard SIPC culture conditions. This particular SIPC culture performed better than SIPC cultures inoculated with stationary phase inoculum. Its immobilization efficiency (97.1%) was comparable to other SIPC cultures. The biomass produced was equivalent to other SIPC cultures. However, less foam was formed on top of the liquid phase as noted in Section 3.3.1.2 and, more important, all carbohydrates present in the medium were consumed at the 11th day of the culture (data points marked "5" in Figures 3.25 to 3.27 and Table 3.12). This last result was rather surprising.

A second similar SIPC culture was performed to verify this result and simultaneously to assess its possibility for high biomass formation using a S = 30 g/L 185 medium. A constant high carbohydrate consumption rate was observed during the whole 16 day period of this SIPC culture (1.78

g/L.d, 8 samples, linear correlation coefficient of 0.97). The biomass concentration attained was the highest of all SIPC cultures performed in Bioreactor IP (see point ⊠ in Figures 3.25 to 3.27). This concentration was 14.3 g of dried biomass per litre of final medium plus wet biomass present in the reactor.

This is an important result for the application of the surface immobilization technique to the culture of plant cells. It stresses the importance of the plant cell suspension inoculum quality (age), rather than quantity, to the biomass formation and control of SIPC cultures.

A few additional experiments were carried out to verify the application of the surface immobilization technique and its scale up in Bioreactor IP to the culture of other types of plant cells. The selected plant cell types were Soybean (cell line SB1) and Tobacco (cell line Su582). These cell lines were maintained in shake flask suspensions as described in Section 2.1. The SIPC culture of these two plant cell types were carried out according to the standard protocol presented in Section 3.3.1.1 with medium 1B5 (S = 20 g/L).

The results of these SIPC cultures are summarized in Figure 3.28 and Table 3.12. They are compared to their

Figure 3.28 – Growth curves of Tobacco cell line Su582 and Soybean cell line SB1 cultured in shake flask suspensions and in Bioreactor IP under standard conditions.

Cell line Su582: suspension: △ : CHO

▼ : biomass

SIPC: + : CHO

○ : biomass

Cell line SB1: suspension: □ : CHO

◆ : biomass

SIPC: × : CHO

● : biomass

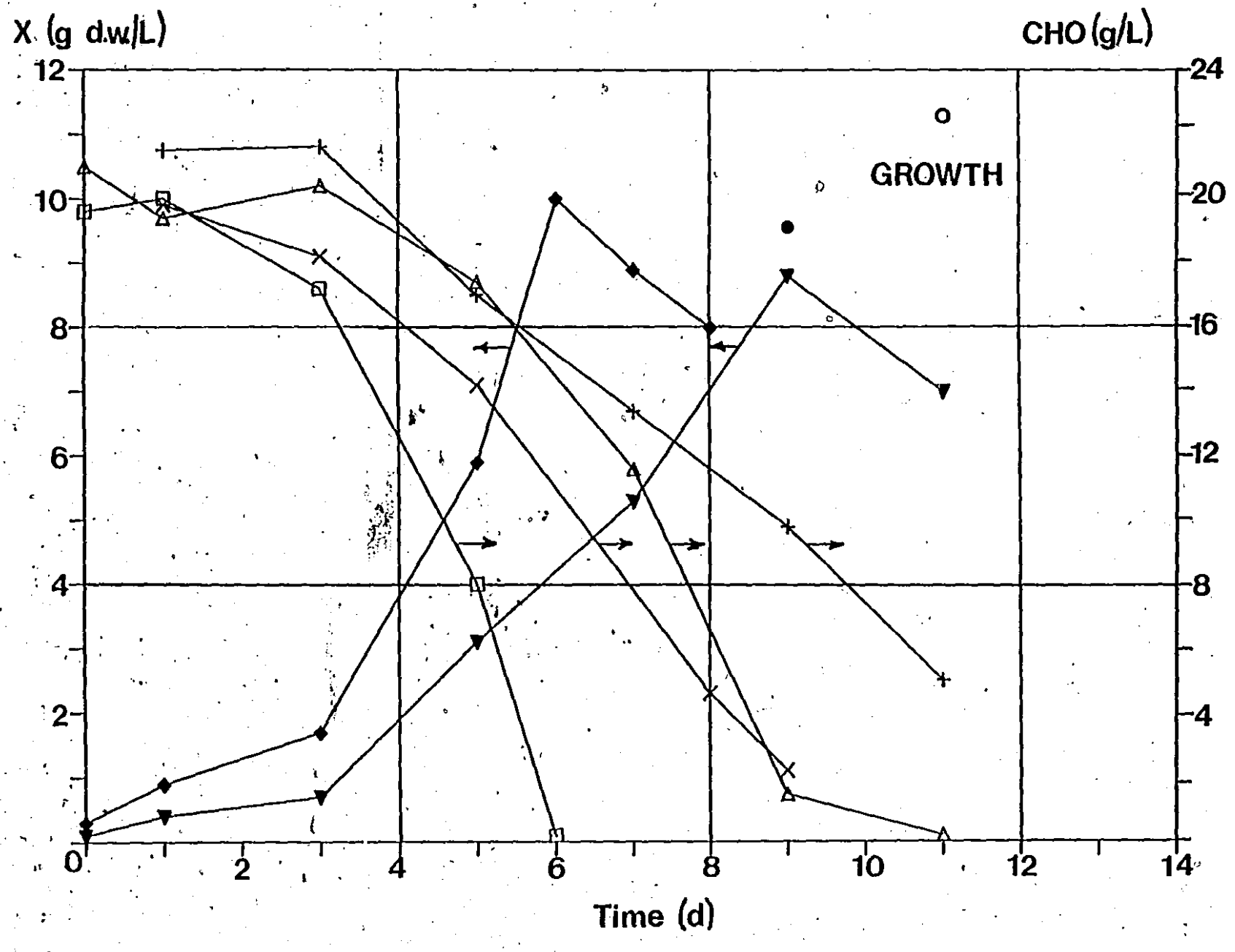

X (g d.w/L)
CHO (g/L)
GROWTH
12
10
8
6
4
2
0
24
20
16
12
8
4
2
4
6
8
10
12
14
Time (d)

TABLE 3.12

COMPARISON OF VARIOUS PLANT CELL LINES GROWTH.

	Catharanthus roseus Cell Line MCR17		*Tobacco* Cell Line Su582	*Soybean* Cell Line SB1
Suspension Culture	(Figure 2.1)		(Figure 3.28)	(Figure 3.28)
Inoculum (age, %)	7 d 10% (I/V_{om})		8d 10%	7 d 10%
Time to Max Biomass	6		9	6
CHO Consumption	CHO =22.1-3.37 t (g/L) (3.74) no=8 r=0.96		CHO =23.7-2.1t (g/L) (3.78) no=7 r=0.94	CHO =22.6-3.2t (g/L) (3.81) no=5 r=0.93
X formation	X = 1.92t-0.40 (g d.w./L) (3.75) no=8 r=0.98		X=0.95t-0.92 (g d.w./L) (3.79) no=6 r=0.96	X= 1.50t-0.77 (g d.w./L) (3.82) no=5 r=0.92
Specific g.r. (μ)(d^{-1})	$0.56 d^{-1}$ no=8 r=0.95		$0.59 d^{-1}$ no=6 r=0.92	$0.55 d^{-1}$ no=6 r=0.99
Biomass Yield (%)	56.2%		42%	49%
SIPC Culture (IP)	(Figure 3.30-IP:10)		(Figure 3.28)	(Figure 3.28)
Inoculum (age, %)	9d 10%	5d 10%	8d 10%	9d 9%
Culture Time (d)	10	11	11	9
CHO Consumption	CHO =21.2-2.1t(g/L) (3.76) no=4(7d) r=0.98	CHO =21.4-2.2t(g/L) (3.77) no=6 r=0.98	CHO =25.2-1.7t (g/L) (3.80) no=6 r=0.98	CHO = 24.0-2.33t (g/L) (3.83) no=5 r=0.98
Av. Specific g.r.(μ)(d^{-1})	0.22_{10d} 0.31_{7d}	0.22_{11d} 0.31_{7d}	0.21_{11d}	0.28_{9d}
Av. Norm. Biofilm g.r.(b_1)($\frac{mg\ d.w.}{cm^2 \cdot d}$)	0.67_{10d} 0.96_{7d}	0.61_{11d} 0.96_{7d}	0.75_{11d}	0.80_{9d}
Biomass Yield (%)	46.2%	35.6%	55.2%	43.9%
Normalized SIPC($\frac{mg\ d.w.}{cm^2}$)	7.6	7.3	.8.8	7.8
Imm. Efficiency (%)	96.2%	97.1%	94.2	98.5
W/D Ratio	33.0	36.1	55.5	31.1

suspension culture and to the culture of C. roseus cells. The growth of these three cell lines in shake flask suspensions is comparable, with line Su582 and SB1 somewhat slower (50% and 22%) and lower in yield (42% and 49% as compared to 56%) than line MCR17. Their SIPC cultures show lower carbohydrate consumption rates as compared to corresponding suspension cultures (36%, 19% and 27% respectively for lines MCR17, Su582 and SB1). These rates (for SIPC) are comparable for the three cell lines, with the rate of cell line Su582 lower by ~ 20% as compared to the two other cell lines. The maximum biomass concentration attained in both culture systems compares well for line SB1 (8.8 as compared to 9.4 g d.w./L in the SIPC system), but it is lower in the suspension culture system for line Su582 (10.0 as compared to 11.2 g d.w./L) (Figure 3.28). The suspension culture of line MCR17 exhibited a higher maximum biomass concentration than SIPC cultures (11.5-12.0 g d.w./L as compared to 8.5-9.0 g d.w./L.). These results are reflected in the yields and lower growth rates of the SIPC cultures presented in Table 3.12.

The carbohydrate consumption results presented in Figure 3.28 indicate that the SIPC cultures of lines SB1 and Su582, with the protocol used, do not lead to the early biomass stationary phase observed when culturing C. roseus

plant cells in this system using a 8-9 day old inoculum. This result was expected for cell line Su582 since a 8 day old suspension inoculum is in its exponential phase of growth (Figure 3.28). This is equivalent to a 5 day old inoculum of cell line MCR17 for which the early SIPC biomass stationary phase was observed not to occur. The high carbohydrate consumption rate (2.33 g/L.d) of the Soybean SIPC culture during the whole culture period was not expected since the 9 day old inoculum used was a stationary phase suspension culture (Figure 3.28).

The other investigated characteristics of SIPC cultures performed in Bioreactor IP were the pH of the medium, the biomass yield, the carbohydrate consumption rate, the wet-to-dry weight ratio and the oxygen consumption rate of the SIPC biomass.

The pH of a SIPC culture medium (line MCR17, normal protocol) was self-maintained at ~ 5.1 (average of 15 cultures, s = 0.3) during the growth phase (first 6-7 days of the culture). This was observed for cultures terminated at or before this time and for cultures carried out for longer periods but sampled regularly. Thereafter the pH of the medium increased within 24 hours to a value of ~ 5.5 (average of 31 cultures, s = 0.3). No real pattern was noted other than this sudden change (not truly overlapping)

at the 6-7th day of the culture. The pH value of shake flask suspension cultures increased progressively to ~ 5.6-5.7 at the complete disappearance of carbohydrates from the medium (~ 6th day of cultures agitated at 150 RPM). Thereafter the pH declined to ~ 4.8-5.1 with a decrease in the biomass concentration (see Figure 2.1).

SIPC cultures using a 5 day old suspension inoculum of plant cells of line MCR17 showed higher pH of the medium (5.5-6.0) during the whole culture period. The culture of Tobacco plant cells (Su582) resulted in a similar pH pattern respectively in shake flask suspension and in Bioreactor IP as observed for the culture of cell line MCR17 using the standard protocol. The culture of cell line SB1 resulted in a similar pH pattern for both systems, a progressive increase to ~ 6.1 at the end of the culture.

The final biomass yields of the various SIPC cultures performed in Bioreactor IP are presented in Figure 3.29. Some of these results have to be assessed with care because of the possible interference of high carbohydrate concentrations in the medium (> 20 g/L) on the measurement of dried biomass quantities. This is particularly important for SIPC cultures carried out with an initial sucrose concentration of 40 g/L. A declining biomass yield was observed during the growth phase as anticipated in

Figure 3.29 – Biomass yield of SIPC cultured in Bioreactor IP.

Symbols are as in Figure 3.25.

Line 1: S = 20 g/L $y = 92.2 - 6.7t$ (3.84)

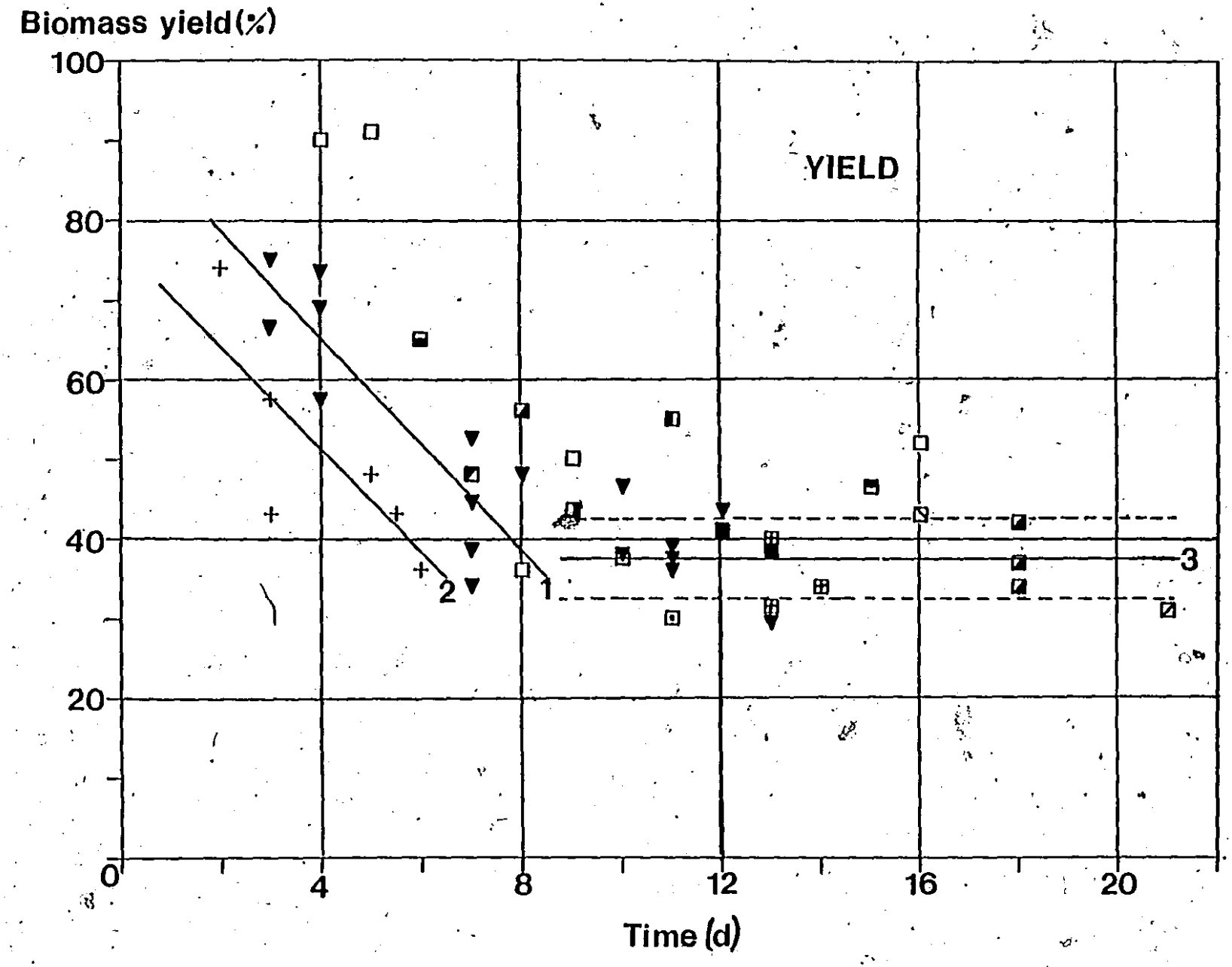
$N_o = 11$ $r = 0.88$

Line 3: S = 20 g/L y = 37.6% S = 5.0%

$N_o = 13$

Line 2: S = 10 g/L $y = 76.6 - 6.5t$ (3.85)

$N_o = 6$ $r = 0.81$


Biomass yield(%)
100
80
60
40
20
0
4
8
12
16
20
Time (d)
YIELD
1
2
3

Section 3.2.3.2. A constant yield of ~ 37.6% was observed thereafter.

The carbohydrate consumption pattern of all batch SIPC cultures of cell line MCR17 carried out according to the standard protocol showed a constant high rate (> 1.5 g/L.d) during the growth phase. Thereafter, this rate was significantly reduced (≤ 0.6 g/L.d). This was illustrated by the final carbohydrate concentration of all SIPC cultures performed in Bioreactor IP presented in Figure 3.26. These results were compared to the carbohydrate consumption pattern of shake flask cultured plant cell suspension (SC). A similar consumption pattern was observed for each SIPC culture. A few of these results are presented in Figure 3.30 and Table 3.13. The carbohydrate consumption rate of growing SIPC cultures (~ 2 g/L.d) are corresponding to rates observed for shake flask suspension cultures agitated at 130 and 150 RPM (1.4 g/L.d and 3.37 g/L.d.).

The wet-to-dry weight ratios (W/D) of the SIPC biomass cultured in Bioreactor IP are presented in Figure 3.31. The data points of the experiments are scattered.

Figure 3.30 - Carbohydrate consumption of SIPC cultures IP9/11 (200 RPM, 0.2 VVM) and IP 24/26 (300 RPM, 0.2 VVM) in Bioreactor IP.

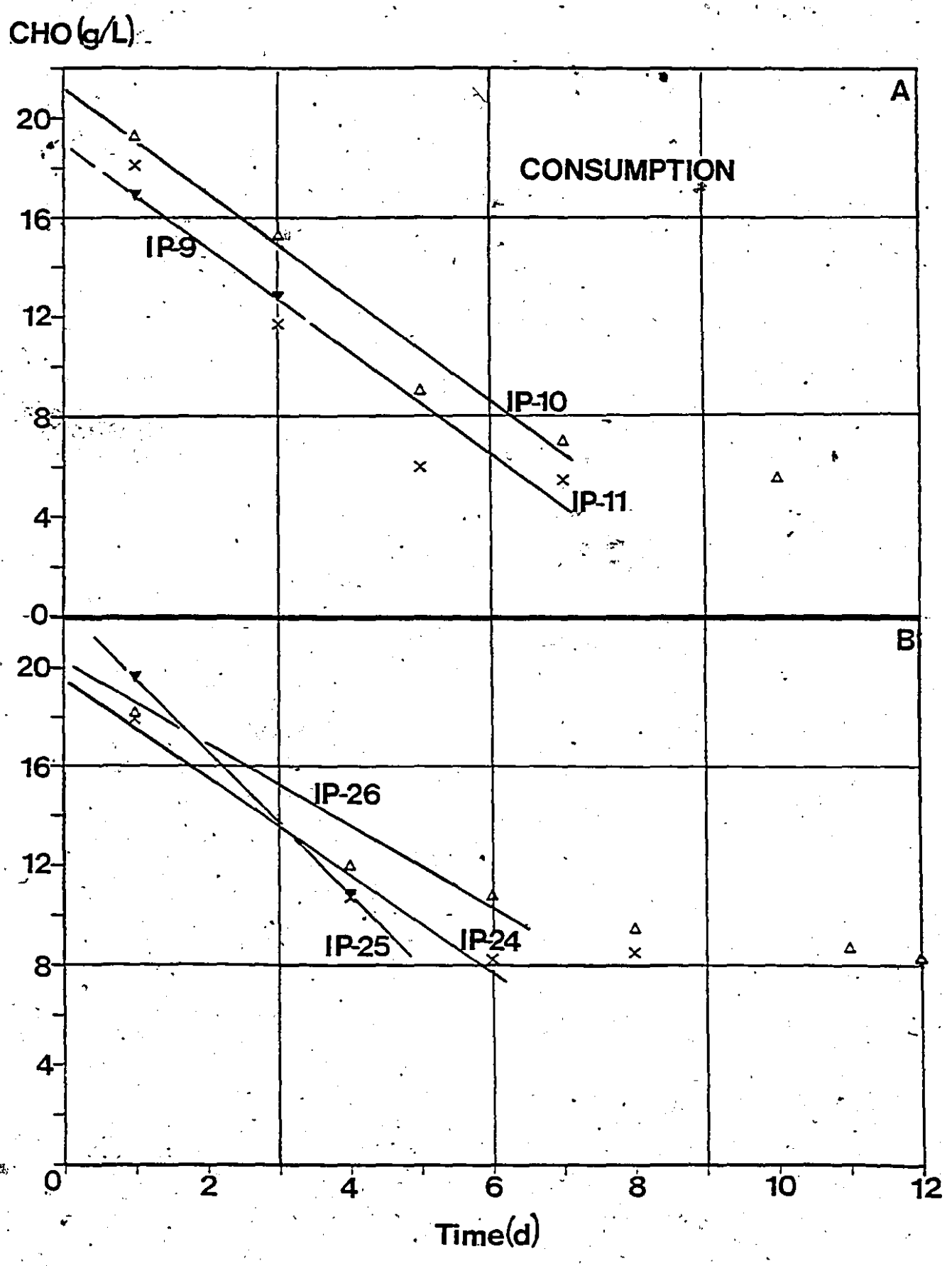

CHO (g/L)
A
CONSUMPTION
IP-9
IP-10
IP-11
B
IP-26
IP-25
IP-24
20
16
12
8
4
0
0
2
4
6
8
10
12
Time(d)

TABLE 3.13

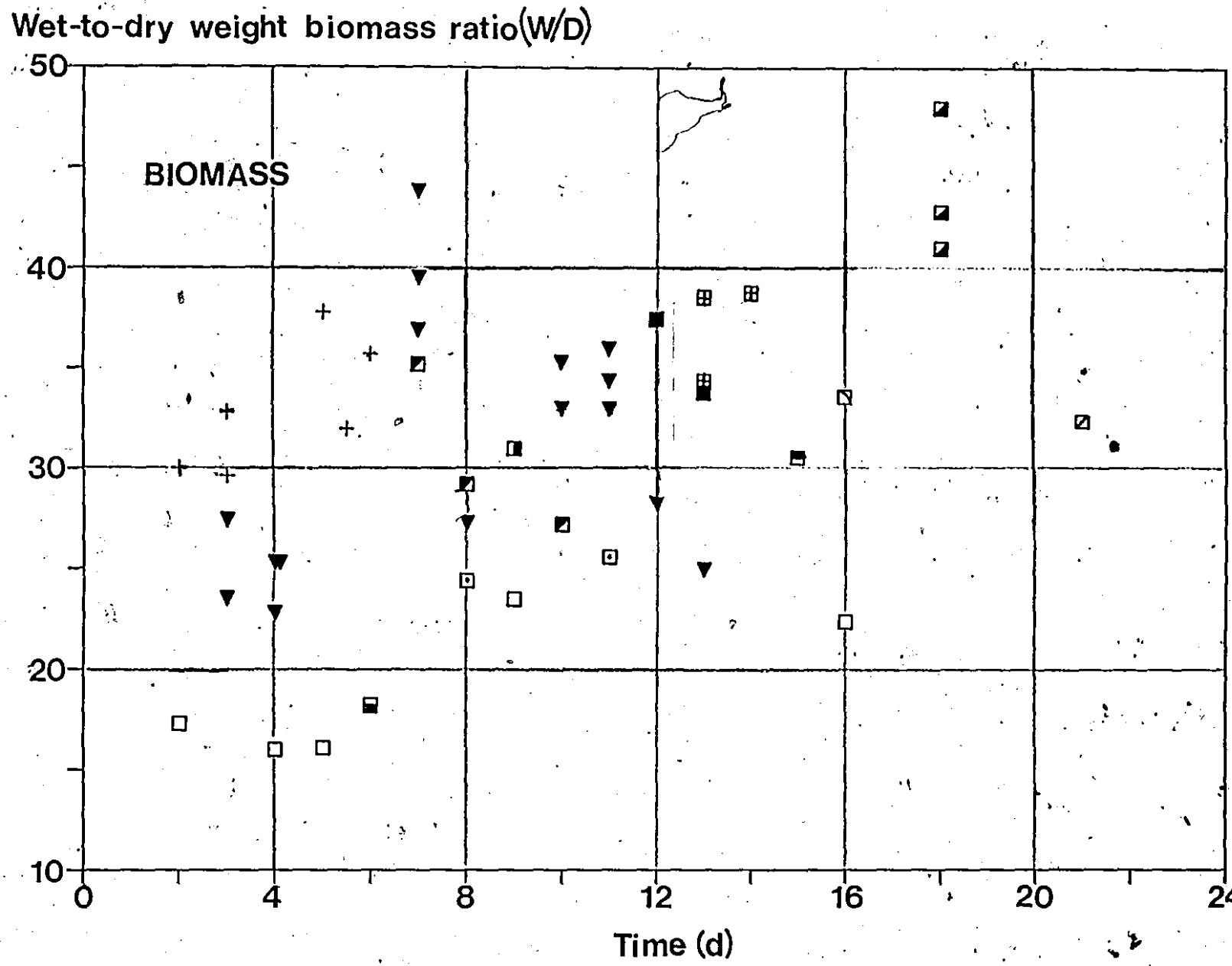
CARBOHYDRATE CONSUMPTION RATES OF SIPC CULTURES IP9/11 AND IP24/26 IN BIOREACTOR IP

IP	Total Culture Period (d)	Time to Stationary Phase (d)	Carbohydrate Consumption during Growth			CHO Consumption during S. Phase (g/L·d)
			No of points	(g/L)	r	
9	3	–	2	$\frac{\Delta CHO}{\Delta t}$ = 2.1 g/L·d	–	–
10	7	7	4	CHO = 21.2–2.1 t (3.86)	0.98	–
11	10	7	4	CHO = 19.0–2.2 t (3.87)	0.95	0.48
24	8	6	3	CHO = 19.5–2.0 t (3.88)	0.98	–
25	4	–	2	$\frac{\Delta CHO}{\Delta t}$ = 3.0 g/L·d	–	–
26	12	6	3	CHO = 20.4–1.7t (3.89)	0.96	0.44

See Figure 3.30.

Figure 3.31 - Wet-to-dry biomass ratio of SIPC cultured in Bioreactor IP.

Symbols are as in Figure 3.25.


Wet-to-dry weight biomass ratio(W/D)
BIOMASS
50
40
30
20
10
0
4
8
12
16
20
24
Time (d)

A good proportion of these W/D results (33 out of 41 results) are higher (average of 32.9 for 37 cultures, s = 6.2) than the theoritical ratio of 25 mentioned in Section 3.1.2.3.

An average wet biofilm thickness (T [mm]) can be calculated from these experimental results by the following equation.

$$T = (\frac{W}{D})SIPC/\rho_b \qquad (3.94)$$

with SIPC: average SIPC dried biomass surface concentration (mg d.w./cm²),

ρ_b: SIPC wet biofilm density (~ 1.01 g/cc).

This average thickness is presented in Figure 3.32 in relation to SIPC surface concentrations. This average T is

Figure 3.32 – Relationship between the wet SIPC biofilm thickness and the dried biomass concentration of cultures performed in Bioreactor IP. Symbols are as in Figure 3.25.

Line 1: S = 20 g/L $T = 0.42SIPC - 0.58$ (3.95)

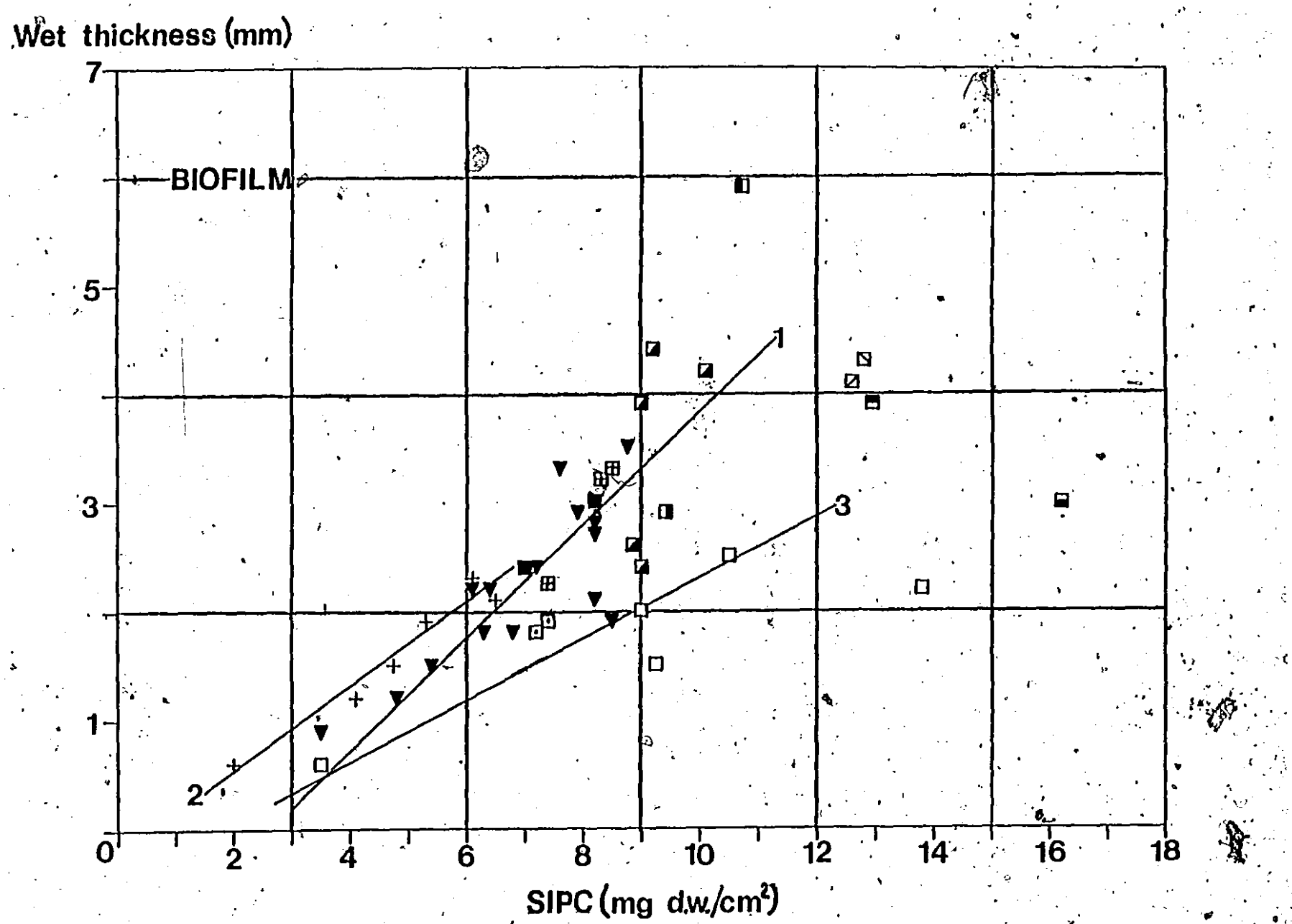
$N_o = 30$ $r = 0.86$

Line 2: S = 10 g/L $T = 0.35SIPC - 0.09$ (3.96)

$N_o = 7$ $r = 0.98$

Line 3: S = 40 g/L $T = 0.26SIPC - 0.19$ (3.97)

$N_o = 8$ $r = 0.87$


Wet thickness (mm)
7
5
3
1
0
2
4
6
8
10
12
14
16
18
BIOFILM
1
2
3
SIPC (mg d.w./cm²)

linearly correlated by Equations (3.95) to (3.97). The first two equations give an average SIPC biomass W/D ratio of ~ 40-50. Equation (3.95) indicates that a 3 mm wet SIPC biofilm (main SIPC bioreactor design criterion - see Section 3.2.1) corresponds to a biomass surface concentration of 8.5 mg d.w./cm². This corresponds to a maximum allowed plant cell biomass concentration of 8.5 to 9.8 g d.w./litre of reactor volume at the maximum and immobilizing structure liquid levels. This range of concentrations was predicted in Section 3.2.2.1 and was attained for S = 20 g/L SIPC cultures with or without biomass formation stimulation (Figure 3.27).

The final characteristic of SIPC cultures evaluated is the oxygen consumption rate of the immobilized biomass. This rate can be estimated either from the oxygen mass transfer rate (O_2CR_m) or by the carbohydrate consumption rate (O_2CR_c) according to the following equations.

$$O_2CR_m = \frac{(k_La)_s(DO^*-DO_s)*V_{L_s}}{32*X_T} \qquad (3.98)$$

$$O_2CR_c = \left(\left(\frac{dCHO}{dt}\right)_s \frac{(1-y_x)}{180}\right)\frac{6}{24}*\left(\frac{V_L}{X_T}\right) \qquad (3.99)$$

with O_2CR: oxygen consumption rate of the immobilized biomass (μMO_2/mg d.w.h),

$(k_La)_f$: final oxygen transfer coefficient of the bioreactor (h^{-1}),

DO^*: dissolved oxygen concentration at saturation (and equilibrium) of the medium at the operating conditions (ppm),

DO_f: measured dissolved oxygen concentration in the medium at the end of the culture (ppm),

V_{Lf}: medium volume at the end of the culture (L),

X_T: total dried immobilized biomass in the bioreactor at the end of the culture (mg d.w.),

$(dCHO/dt)_f$: final average carbohydrate consumption rate (g/L.d),

Y_x: final biomass yield.

The $(k_La)_f$ coefficient was estimated from the water measurements as per Equation (3.61). The measured DO^* concentration of medium 1B5 at the normal operating conditions of the system was 7.3 ppm.

The comparison of the O_2CR_m and O_2CR_c calculated results showed both to be in the same range. However, the latter data were more scattered and, on the average, lower than the O_2CR_m results (average of 28 results: -11%

relative to O_2CR_m data, with s = 31%). This difference originated probably from an overestimation of $(k_La)_+$, the limited accuracy of the experimental results and the impossibility of determining a true differential biomass yield. Consequently, the O_2CR_m results obtained from Equation (3.98) were used and are presented in Figure 3.33.

These results show a decline in the oxygen consumption rate of the SIPC biomass during the growth phase and a constant rate of ~ 0.10 μMO_2/mg d.w..h thereafter. These results are consistent with the O_2CR range of 0.2-3.4 μMO_2/mg d.w..h mentioned in Section 1.2.1.1 for cultured plant cells, even though they are in the low end of this range. These results are also consistent with the calculated O_2CR_c results (Equation (3.99)) of growing plant cell suspensions (line MCR17) cultured in shake flasks presented in Figure 3.33.

3.3.2 Bioreactor IA.

3.3.2.1 Operating Procedure.

The matrix was formed in the vertical square spiral configuration and placed in the vessel described in Section 3.2.2.2. The bioreactor was either filled with 4.0 L of the growth medium 1B5, or not, and steam sterilized with

Figure 3.33:- Oxygen consumption rate of SIPC biomass cultured in Bioreactor IP.

Symbols are as in Figure 3.25.

Flask suspension cultures O_2CR_c (150 RPM):

◆ : S = 40 g/L.

◇ : S = 20 g/L.

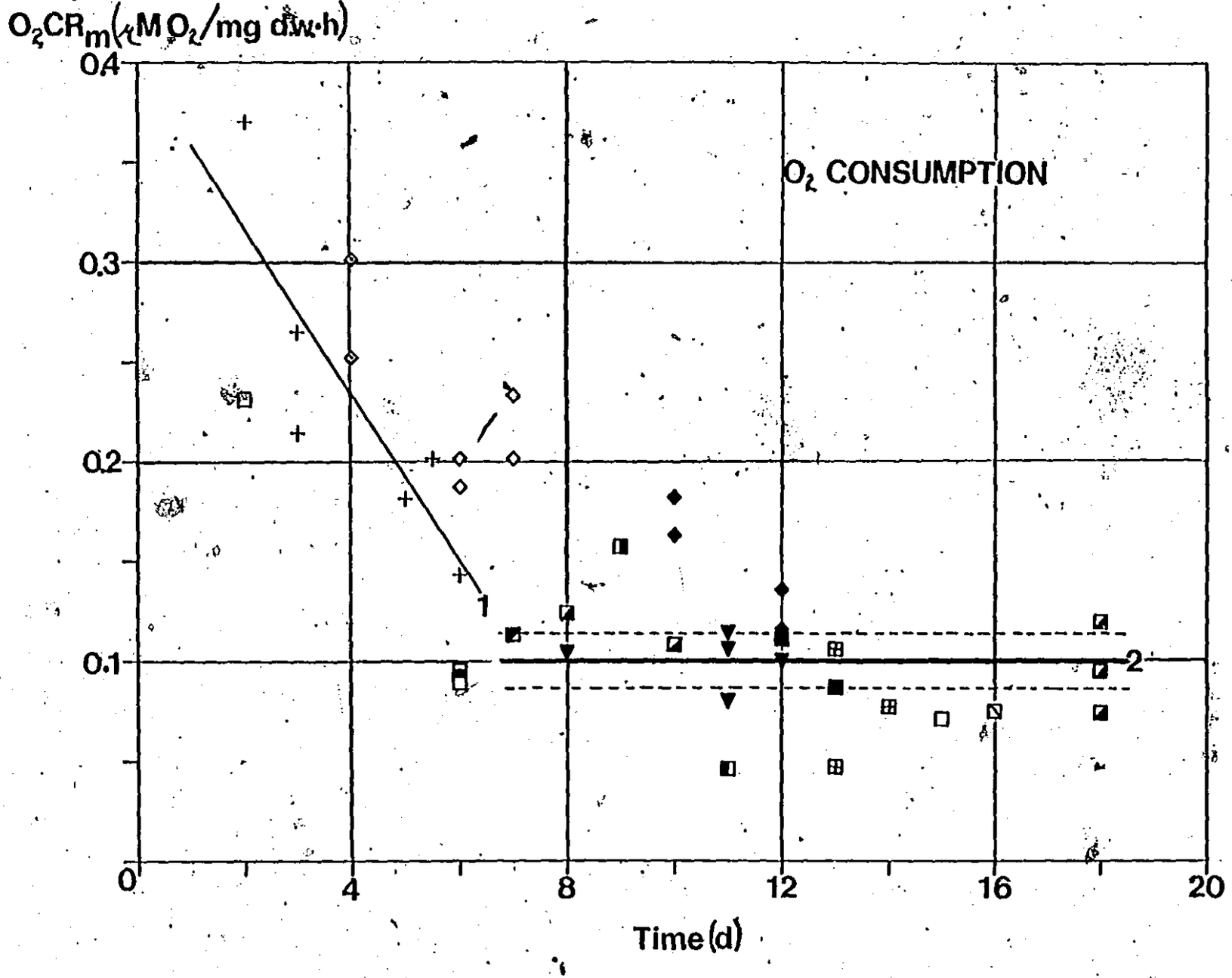
◈ : S = 10 g/L.

Line 1: S = 10 g/L $O_2CR = 0.40 - 0.04t$ (3.100)

$N_o = 6$ r = 0.86

Line 2: S = 20 g/L $O_2CR = 0.10 \mu MO_2/mg$ d.w.h

$N_o = 16$ s = 0.015


O_2CR_m (μMO_2/mg d.w.h)
0.4
0.3
0.2
0.1
0
4
8
12
16
20
Time (d)
O_2 CONSUMPTION
1
2

accessories according to the standard practice (60 min., 121°C, 15 psi). The sterile reactor was subsequently filled to the operational level (6.5, 5.2 or 4.4 L) with sterile medium and with a suitable suspension plant cell inoculum (8 to 16% of the initial total culture volume) prepared as per Section 2.1 and similar to Bioreactor IP (see Section 3.3.1.1).

The bioreactor was fitted with necessary sterile accessories (condenser, feed reservoir etc) and installed in its holding structure. The culture temperature was maintained at 27±1°C. The culture liquid level and the aeration rate were adjusted to the desired values.

During the culture period, the medium was regularly sampled by drainage as for Bioreactor IP. At harvesting, the culture was treated as for Bioreactor IP (see Section 3.3.1.1).

3.3.2.2 General Operation Comments.

The immobilization efficiency of Bioreactor IA was the same as for Bioreactor IP. All the inoculated plant cells were attached to the matrix within the first 24 hours of the culture. The medium was completely free of suspended biomass until the end of the culture. The final

immobilization efficiency of the system, as defined in Section 3.3.1.2, was 96.0% (average of 30 cultures, S = 2.2%).

The use of a large inoculum (16% as compared to the standard 8-10% volume ratio used) did not improve the growth of SIPC in Bioreactor IA as for Bioreactor IP. The use of this larger inoculum resulted in a slight increase of the amount of plant cells trapped in the foam layer of the culture (~ +1% of the total biomass present in the bioreactor). The biomass froth formed on top of SIPC cultures performed in Bioreactor IA was of a similar nature as for Bioreactor IP (see Section 3.3.1.2). However, it was better contained (larger head space) and controlled (flow diverting plate effect) than in the smaller system.

3.3.2.3/Operational Hydrodynamics of Bioreactor IA.

The hydrodynamics of Bioreactor IA was evaluated with water in Section 3.2.4.1. Subsequently, the actual operating conditions of this system were determined for a best culture of surface immobilized plant cells. These culture conditions are presented in this Section.

The good biomass handling performance of Bioreactor IA mentioned in the previous Section required a close control

of two operating conditions, particularly during the immobilization period of the culture (first 24 hours, until complete attachment of the inoculated plant cells). The aeration rate was limited to 2.6 L/min (~ 0.4-0.6 VVM) during this period. This low aeration rate was intended to minimize the entrapment of the inoculated plant cells in the foam of the culture (see Section 1.2.1.2). Thereafter, the aeration rate was increased to the desired level.

The second important operating condition was the culture liquid level. As mentioned in Section 3.2.4.1, high liquid levels resulted in excessive fluid by-pass around the immobilizing structure. Lower liquid levels (< 5.2 litres), especially to the top of the immobilizing structure, favored better mixing within the vertical spiral wound configuration. This behaviour was confirmed during the experimentation of Bioreactor IA. The liquid level could influence drastically the distribution of the inoculated biomass in the immobilizing structure and its uniformity of attachment. High liquid levels (> 5.2 litres or above the flow diverting plate) led to the formation of a thicker wet biofilm on the external matrix layer of the immobilizing structure as compared to internal layers coverage. Lower initial (first 24 hours of the culture) liquid levels, particularly those reaching only to the top of the immobilizing structure, resulted in an improved

uniform distribution and attachment of the inoculated plant cells as well as in a more uniform grown SIPC biofilm throughout the vertical spiral wound configuration. This result was not quantified because of the difficulties involved. After this immobilization period, the medium level was raised to the flow diverting plate height (~ 5.2 litres) for uniform liquid mixing and growth.

The biomass formation phase was affected by three operating variables as shown in Table 3.14. Low aeration/mixing rates (Experiments 14 and 17: 0.46-0.60 VVM_o (relative to the total initial culture volume)) resulted in low yields (~ 30%) and in lower final biomass concentrations as compared to other cultures (~ 17 to 35%). The biomass formation was not affected by aeration/mixing rates above 0.8-0.9 VVM. A 0.9 VVM aeration rate was selected to ensure sufficient oxygen transfer to the liquid phase (k_La ~ 13.2 h^{-1} - Equation (3.57)) for the culture of the SIPC.

With the culture protocol used (stationary phase inoculum of cell line MCR17), the final concentration of the biomass in the reactor (X_{max}) depended on the initial medium volume of the culture. This is shown in Table 3.14 for SIPC cultures performed with 6 litres of medium (except for Experiments 7 (low immobilizing area) and 14 (low

TABLE 3.14

PERFORMANCE OF BIOREACTOR IA.

IA #	I. Area (cm^2)	V_{om} (L)	VVM.	t (d)	CHO (g/L)	X_{es} ($\frac{g\ d.w.}{L}$)	SIPC(1) ($\frac{mg\ d.w.}{cm^2}$)	Max biofilm growth r $\frac{mg\ d.w.}{cm^2 \cdot d}$	b_1(1)	t (d)	y_1 (%)	Notes
3	7884	6.0	1.43	11	0.6	10.7	9.3	0.84	1.10	8	46.9	
4	7132	6.2	1.38	12	0.9	13.3	9.7	0.63	0.75	12	40.3	
8	6108	6.1	1.15	11	11.6	10.8	8.0	0.87	0.88	8	49.2	
9	6346	6.1	0.78	11	10.9	10.7	7.6	0.78	0.83	8	52.9	
12	6264	6.1	0.94	13	4.5	11.8	7.8	1.13	1.18	8	39.5	
7	4544	6.1	0.93	12	7.1	8.6	5.7	1.08	0.82	6	32.3	
14	6302	6.1	0.60	13	20.5	7.6	5.7	0.76	0.80	6	28.7	S = 30 g/L
15	6226	5.2	0.91	12	6.5	6.3	5.2	0.72	0.75	6	35.4	
16	6386	5.3	1.41	12	6.7	6.7	5.4	0.72	0.77	6	34.6	
17	6415	5.1	0.46	12	8.5	5.5	4.4	0.58	0.62	6	31.6	
19	6241	5.1	0.91	6	8.8	7.3	5.9	0.83	0.86	6	45.0	
20	6278	5.1	0.92	9	6.0	8.1	6.5	0.90	0.94	6	44.6	
5	5137	5.8	1.45	9	12.0	7.1	5.6	0.92	0.79	6	33.2	
25	5934	5.0	0.93	14	14.4	8.3	8.0	0.74	0.73	8	40.9	Med. replaced at day 6 4.0L
26	6266	5.1	0.95	10	15.7	8.5	7.1	0.76	0.79	8	45.2	"
30	5744	4.6	1.01	15	0	12.4	9.9	0.65	0.62	15	43.7	Semicontinuous, day 4→10 890 cc/d

(1): Normalized to 6000 cm^2.

aeration rate) as compared to 5 litre cultures. However, this high liquid level introduced serious mixing disparities within the immobilizing structure (see Section 3.2.4.1 and Figure 3.20). This can be resolved by a fedbatch or semicontinuous operation of the system (Experiment 30).

The third important variable was the available immobilizing area relative to the culture volume. This influenced directly the thickness of the wet SIPC biofilm produced from a given volume of medium. This is shown in Table 3.14 for Experiment 7 as compared to Experiments 8, 9 and 12. Lowering by ~ 25% the immobilizing area relative to a same initial medium volume (~ 6 litres) resulted in decreases respectively of 22% and 31% in the final biomass concentration and in the yield. A same increase of the immobilizing area relative to the same medium volume (Experiments 3 and 4 as compared to Experiments 8, 9 and 12) did not lead to a significant improvement in the biomass produced at the same operating conditions.

From these results, a set of best operating conditions of Bioreactor IA was determined and is summarized in Table 3.15.

TABLE 3.15

BEST OPERATING CONDITIONS OF BIOREACTOR IA WITH THE STANDARD PROTOCOL USED.

Operating Conditions	Selected Level
Inoculation Ratio	8-10% of total culture volume
Immobilizing Area	Structure B (Table 3.7) ~6500 cm^2
Immobilization Period (1° day)	
Culture Liquid Level	Top of matrix structure (4.4 litres)
Aeration/Mixing Rate	0.6 VVM
Growth Period (>1° day)	
Culture Liquid Level	Flow diverting plate level (5.2 litres)
Aeration/Mixing Rate	0.9 VVM
High Biomass formation	Semi-continuous operation before 5° day or using a 5 day old inoculum and higher initial sucrose concentrations (S~30-50 g/L).

3.3.2.4 The Growth of SIPC Cultured in Bioreactor IA.

The biomass formation pattern of SIPC cultured in Bioreactor IA is illustrated in Figure 3.34. The results were normalized to a 6000 cm^2 immobilizing area. As observed for Bioreactor IP, this pattern is characterized by a linear growth phase up to the 6th day of the culture followed by a stable biomass stationary phase without complete consumption of carbohydrates. The occurrence of this last phase was also confirmed by measurements of carbohydrate concentrations as shown in Figures 3.35 and 3.38. The results of Figure 3.34 are correlated in Table 3.16.

The growth curves of Figure 3.34 can also be represented in terms of the dried biomass concentration per litre of wet biomass plus medium present in the bioreactor. This corresponds to an equivalent plant cell suspension concentration. Figure 3.36 shows these growth curves which are correlated in Table 3.17. From the results of Tables 3.16 and 3.17, it can be seen that the growth rates of $S = 10$ g/L and $S = 20$ g/L SIPC cultures performed in Bioreactor IA are lower (~ 30-40%) than the growth rate of a shake flask suspension culture agitated at 150 RPM.

Figure 3.34 - Normalized growth curves of SIPC cultured in Bioreactor IA at the best operating conditions.

▲ : S = 20 g/L.

◭ : S = 10 g/L.

△ : S = 40 g/L.

□ : S = 40 g/L + N_2/PO_4.

⊞ : S = 20 g/L, medium changed after 6 days.

■ : S = 20 g/L + N_2/PO_4.

◪ : S = 20 g/L, semicontinuous.

⊡ : S = 20 g/L, 16% inoculum.

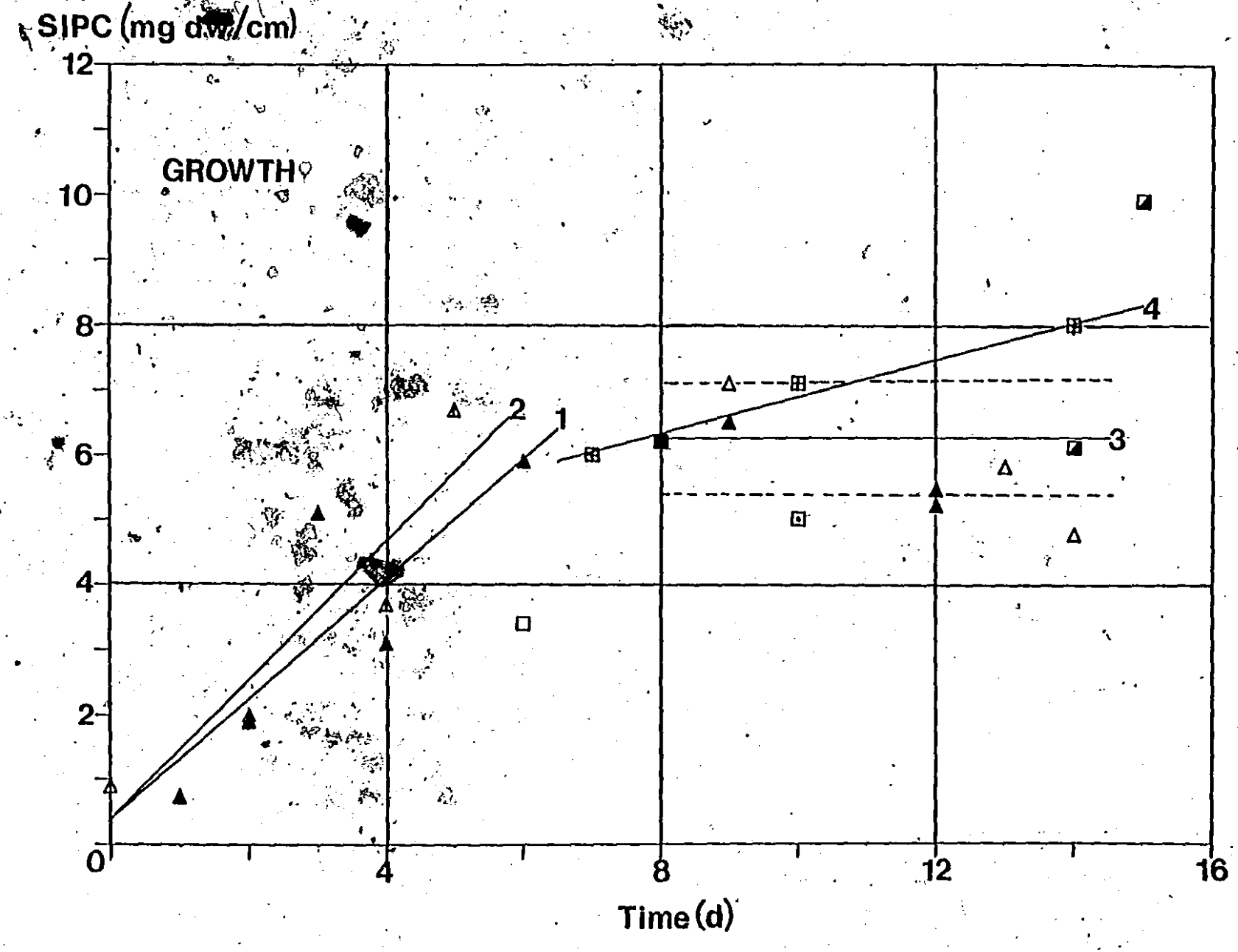

SIPC (mg d.w./cm)
GROWTH
12
10
8
6
4
2
0
4
8
12
16
Time (d)
1
2
3
4

Figure 3.35 - Final carbohydrate concentration in the medium of SIFC cultured in Bioreactor IA. Symbols are as in Figure 3.34.

Line 1: S = 20 g/L CHO= 23.2 - 2.2t (3.104)

N_o = 5 r = 0.97

Line 2: S = 10 g/L CHO= 13.1 - 2.5t (3.105)

N_o = 3 r = 1.0

Line 3: S = 20 g/L CHO= 8.3 s = 2.4

N_o = 12

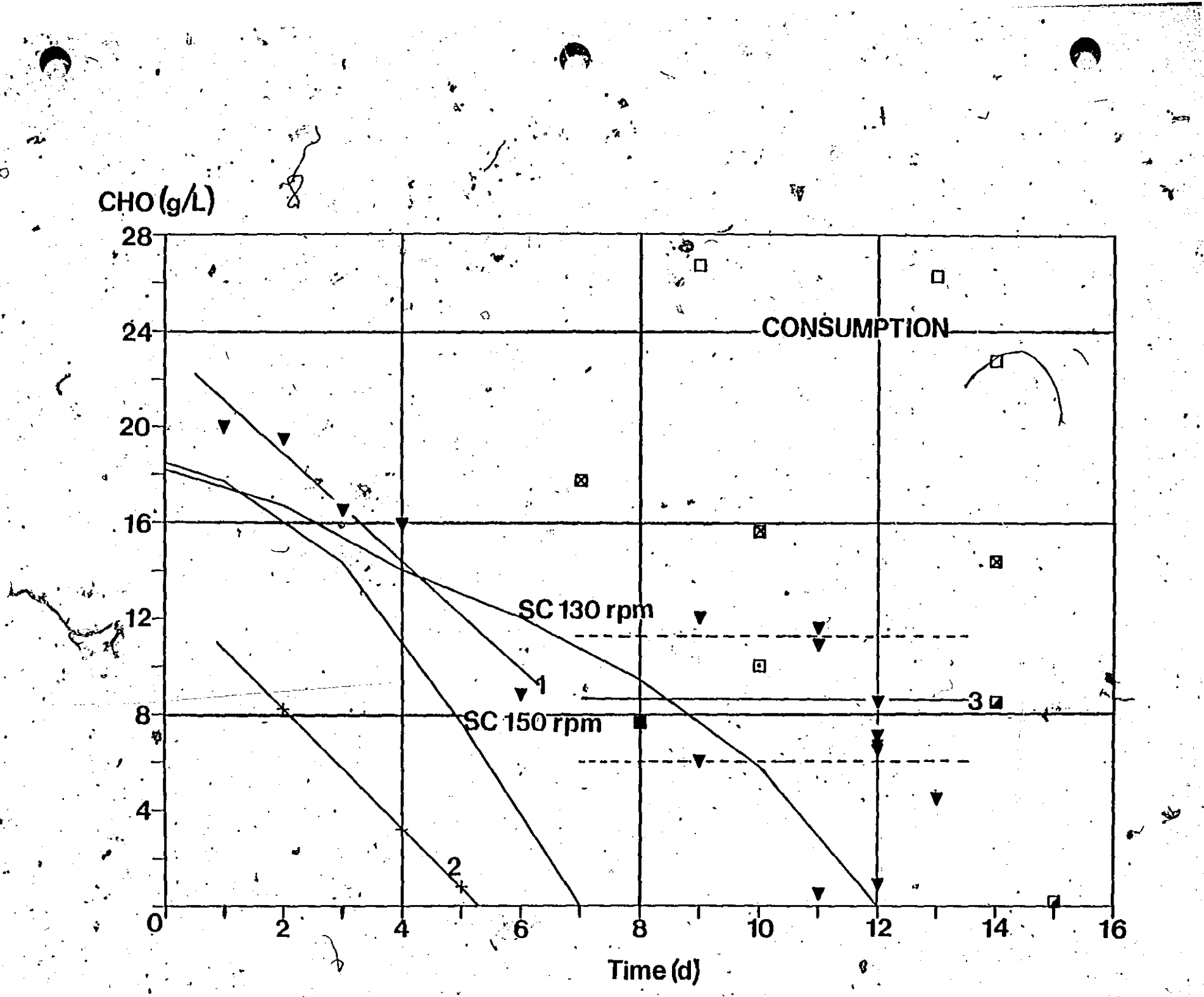

CHO (g/L)
28
24
20
16
12
8
4
0
2
4
6
8
10
12
14
16
Time (d)
CONSUMPTION
SC 130 rpm
SC 150 rpm
1
2
3

TABLE 3.16

GROWTH CORRELATIONS OF SIPC CULTURED IN BIOREACTOR IA

Initial Sucrose Concentration (g/L)	Culture Period (d)	Number of Cultures	Growth Correlations (mg d.w./cm^2)	r(1)	Figure 3.34 Line	Specific Growth Rate μ (d^{-1})	r(1)
10	2 to 5	4(2)	SIPC = 1.07t + 0.39 (3.101)	0.94	2	0.38	0.99
20	2 to 6	5(2)	SIPC = 0.93t + 0.41 (3.102)	0.85	1	0.30	0.89
20 & 40	8 to 14	11(3)	SIPC = 6.2 s(4) = 0.9	-	3	-	-
20(5)	7 to 14	3	SIPC = 0.28t + 4.11 (3.103)	0.99	4	0.04	0.98

(1) r: Linear correlation coefficient.
(2) An origin point was included, corresponding to full inoculum immobilization at t=0.
(3) All S=20 g/L and S=40 g/L data included except the final semi-continuous experiment.
(4) s: Statistical standard deviation shown by dotted lines in Figure 3.34.
(5) Cultures at S=20 g/L with complete medium replacement after 6 days of culture.

Figure 3.36 – Total biomass concentration of SIPC cultured in Bioreactor IA.

Symbols are as in Figure 3.34.

SC: Suspension culture.

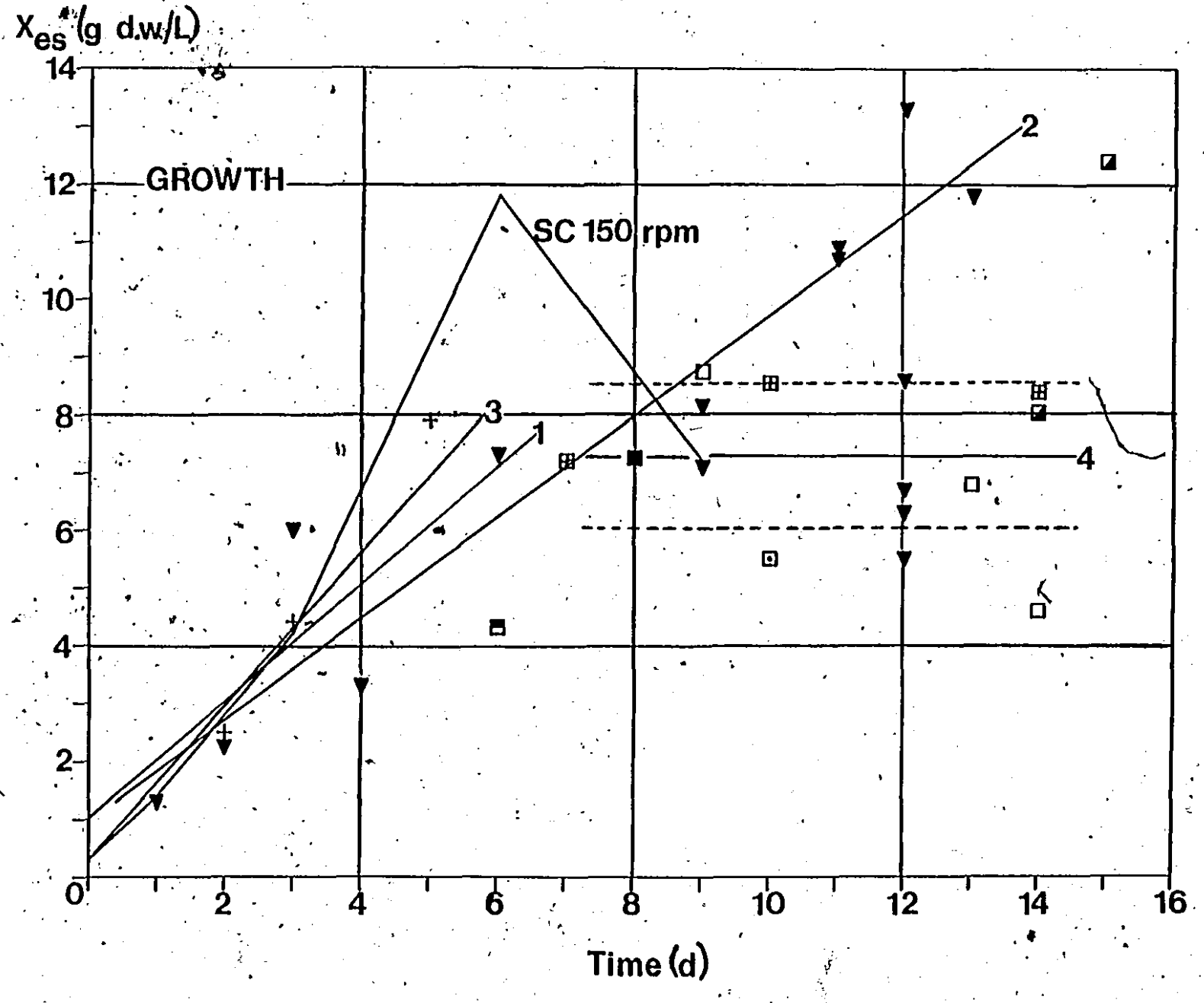

X_{es} (g d.w/L)
GROWTH
SC 150 rpm
1
2
3
4
Time (d)
0
2
4
6
8
10
12
14
16

TABLE 3.17

GROWTH CORRELATIONS OF PLANT CELLS CULTURED IN BIOREACTOR IA

Initial Sucrose Concentration (g/L)	Culture Period (d)	Number of Cultures	Growth Correlations (g d.w./L)	r(1)	Figure 3.36 Line	Specific Growth Rate μ (d^{-1})	r(1)
20	1 to 6	6(2)	X_{eS}= 1.07t + 0.63 (3.106)	0.88	1	0.38	0.87
20	1 to 13	(2)12(3)	X_{eS}= 0.90t + 0.97 (3.107)	0.97	2	0.18	0.92
20	8 to 13	16(4)	X_{eS}= 7.2 s(5)= 1.2	-	4	-	-
10	2 to 6	4(2)	X_{eS}= 1.30t + 0.32 (3.108)	0.95	3	0.43	0.99
Suspension Control (Figure 2.1) 20	0 to 6	No of pts 8	X = 1.92t-0.40 (3.109)	0.98	SC	0.56	0.95

(1) r: Linear correlation coefficient.
(2) The inoculation point was included (0.8 g d.w./L at t = 0).
(3) All points of Equation (3.102) plus Experiments 3,4,8,9,12 and 20 (Table 3.14).
(4) All S = 20 g/L and S = 40 g/L experiments except Experiments 3,4,8,9,12 and 30 (Table 3.14).
(5) s: Statistical standard deviation shown by dotted lines in Figure 3.36).

High biomass concentrations in Bioreactor IA were attained with high initial medium volumes (~ 6 litres), with an immobilizing area ~ 6000 cm^2 and by semi-continuous operation (see Table 3.14).

The other characteristics of SIPC cultures performed in Bioreactor IA which were evaluated, were the pH of the medium, the biomass yield, the carbohydrate consumption rate, the wet-to-dry weight ratio and the oxygen consumption rate of the SIPC biomass.

The pH of the SIPC culture media was as noted for Bioreactor IF:

Culture time $\leq$ 6 days: pH = 5.1 (average of 56 readings, s = 0.2).

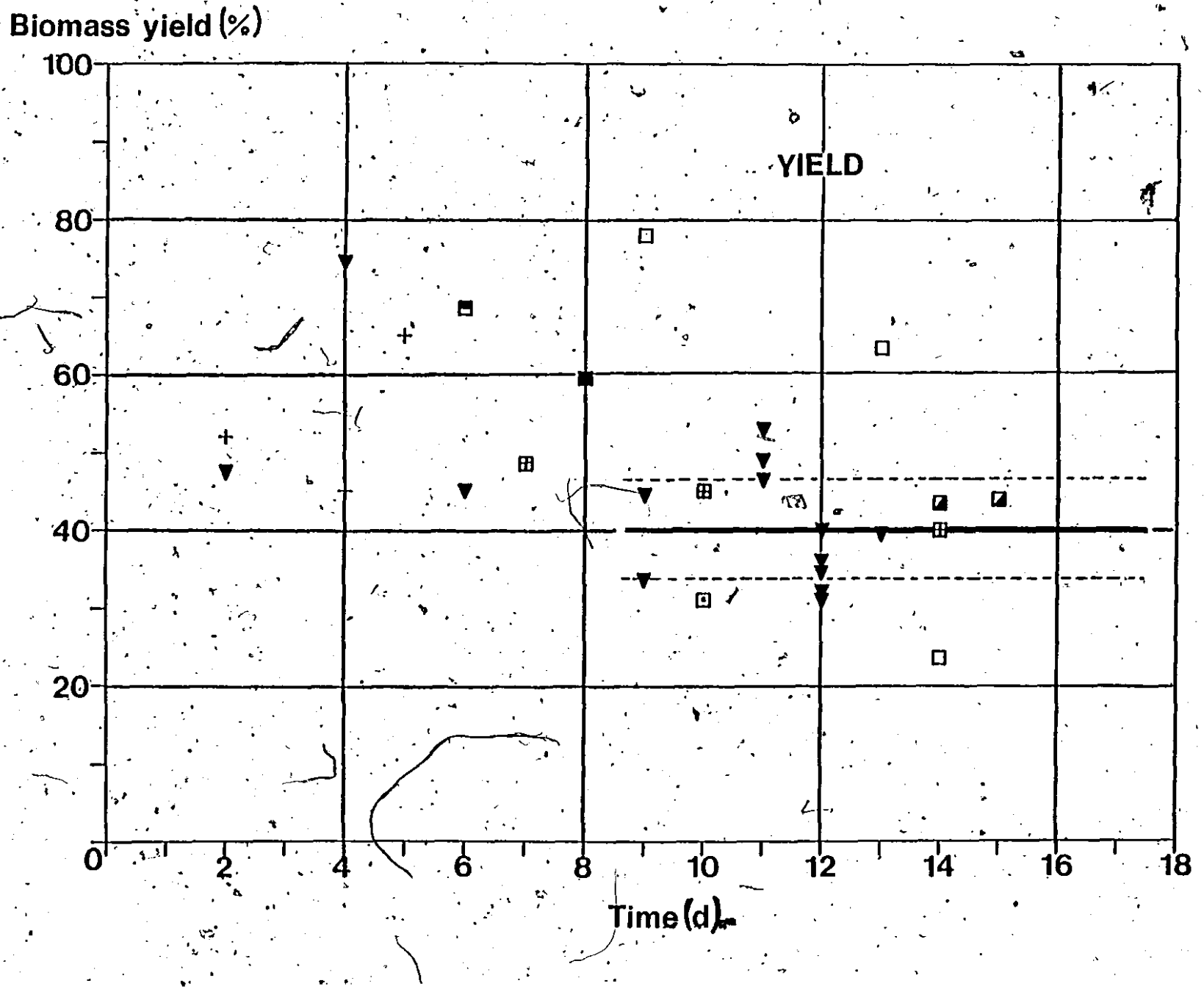
Culture time $>$ 6 days: pH = 5.5 (average of 50 readings, s = 0.2).

This was observed for sampled and harvested cultures. Again, no real pattern was noted other than this "sudden" change from ~ 5.1 to ~ 5.5 within 24 hours from 6th day of the culture.

The final biomass yield of the various SIPC cultures performed in Bioreactor IA are shown in Figure 3.37. A declining trend is observed before the 8th day of the

Figure 3.37 – Biomass yield of SIPC cultured in Bioreactor IA.

Symbols are as in Figure 3.34.


Biomass yield (%)
100
80
60
40
20
0
YIELD
2
4
6
8
10
12
14
16
18
Time (d)

culture and a constant average yield of ~ 40% (average of 16 cultures, s = 6.6%) is noted thereafter for S = 20 g/L SIPC culture. A similar trend was observed for Bioreactor IP cultures (Figure 3.29).

As observed for other SIPC culture systems, the carbohydrate consumption rate of batch SIPC cultures performed in Bioreactor IA was constant during the growth phase (~ 2.2-2.5 g/L.d - Figure 3.35 and 1.2-2.8 g/L.d - Figure 3.38 and Table 3.18). This rate was reduced during the stationary phase (0.1-1.2 g/L.d). The carbohydrate consumption rates of growing SIPC cultures are comparable to those observed for shake flask suspension cultures agitated at 130 and 150 RPM (1.4 g/L.d and 3.37 g/L.d).

The wet-to-dry biomass ratio of the SIPC cultures performed in Bioreactor IA are presented in Figure 3.39. These results appear to have less scatter than the results presented for Bioreactor IP (Figure 3.31). An increasing trend of W/D with time also be observed. The W/D ratios can correlated with the following equation for S = 20 g/L and S = 20 g/L cultures.

$$W/D = 1.25t+14.1 \qquad (3.116)$$

for 21 cultures, linear correlation coefficient of 0.73.

Figure 3.38 – Carbohydrate consumption of SIPC cultures IA3,4,15/17 and IA18/20 in Biorector IA.

IA		I. area (cm^2)	Vo_m(L)	VVM_o	IA		I. area (cm^2)	Vo_m(L)	VVM_o
3	▼	7884	6.0	1.43	18	▼	6408	5.1	0.91
4	△	7132	6.2	1.38	19	△	6241	5.1	0.92
15	+	6226	5.2	0.91	20	+	6278	5.1	0.92
16	□	6386	5.3	1.41					
17	◆	6415	5.1	0.46					

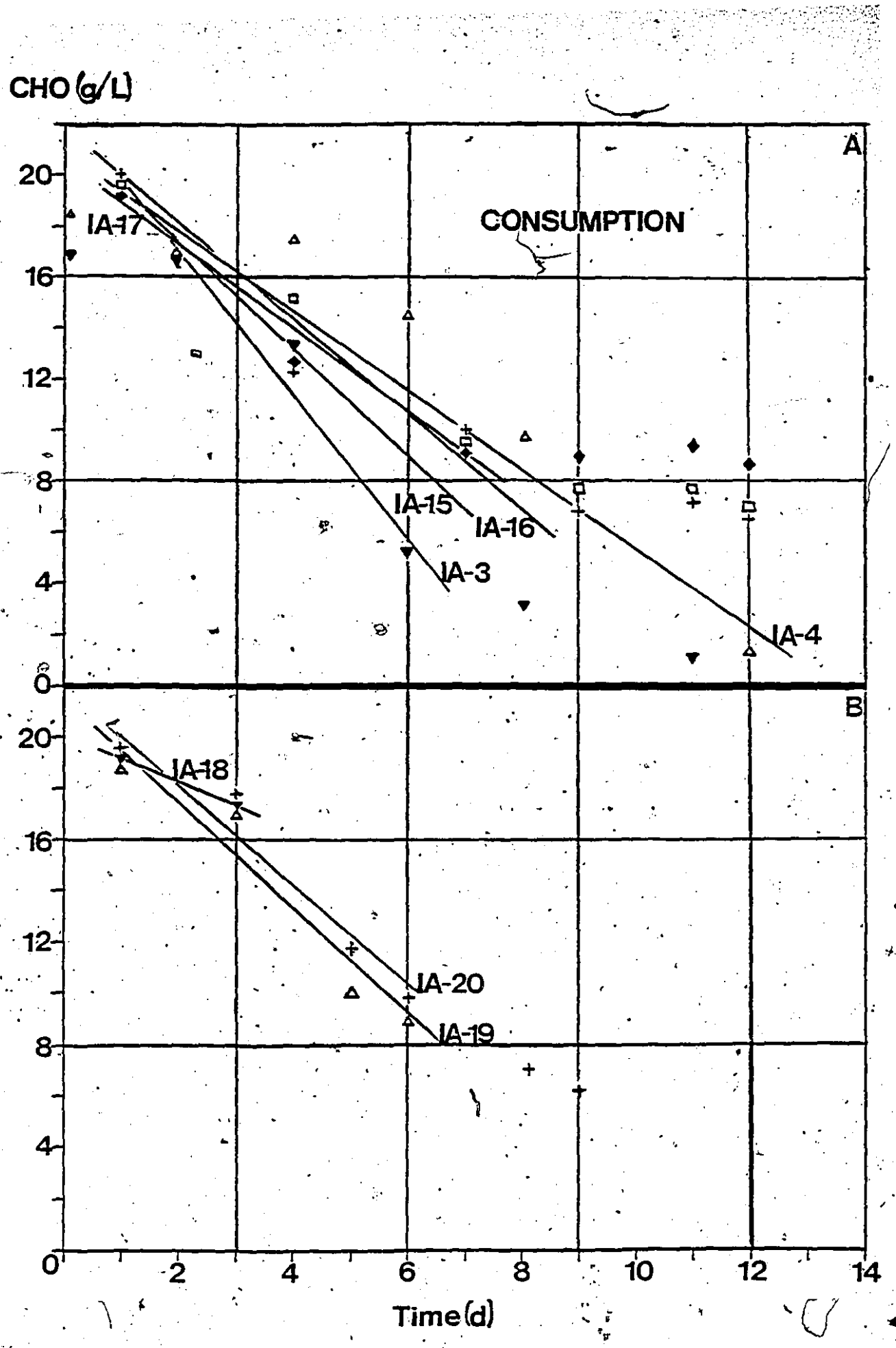

CHO (g/L)
A
CONSUMPTION
IA-17
IA-15
IA-16
IA-3
IA-4
B
IA-18
IA-20
IA-19
20
16
12
8
4
0
2
6
10
14
Time (d)

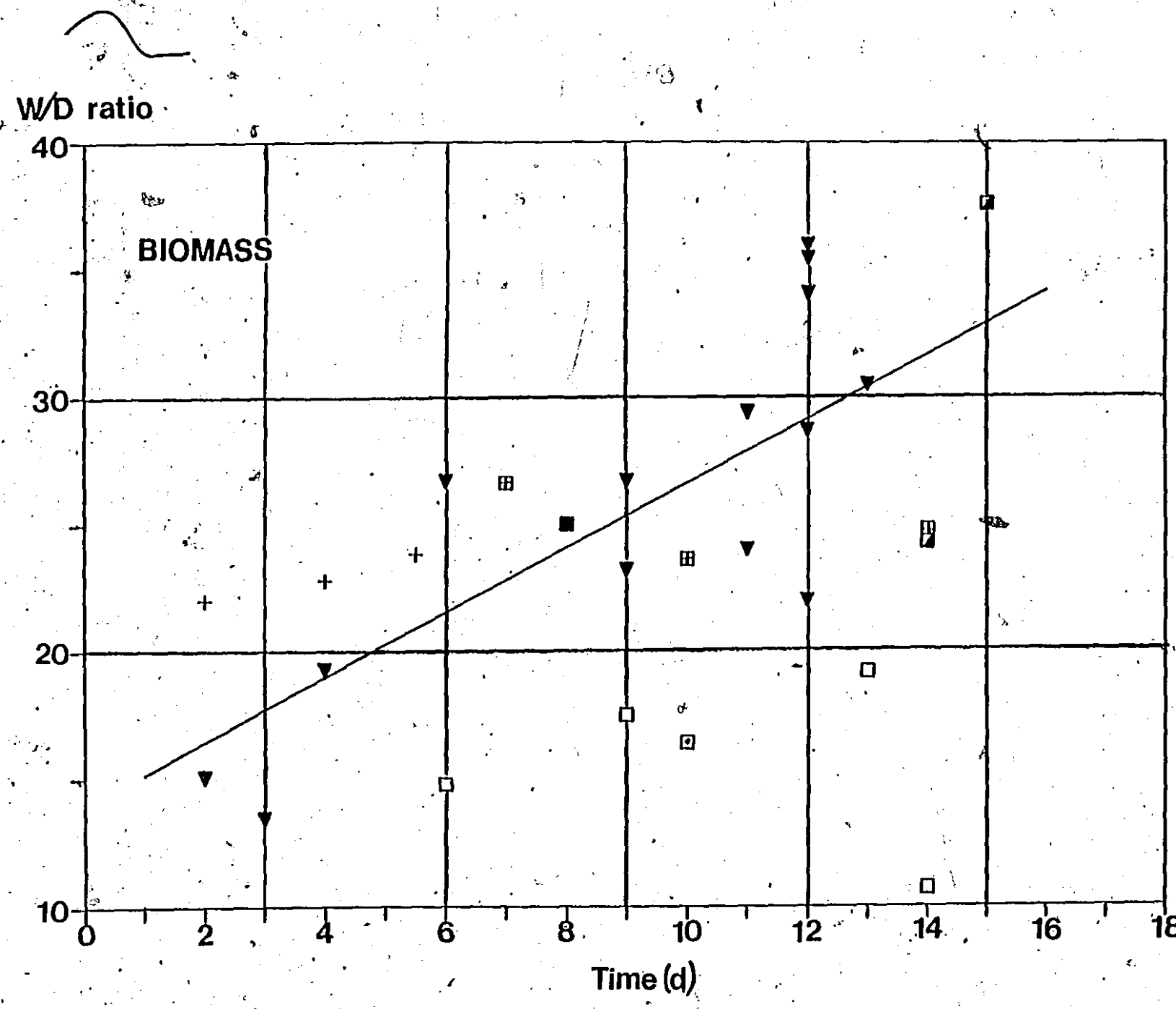
TABLE 3.18

CARBOHYDRATES CONSUMPTION RATES OF SIPC CULTURES IA3,4, 15/17 and 18/20.

IA	Total Culture Period (d)	Time to Stationary Phase (d)	Carbohydrate Consumption at Growth			CHO Consumption during S. Phase (g/L·d)
			No of points	(g/L)	r	
3	11	6	3	CHO = 23.1-2.9t (3.109)	0.96	0.88
4	12	–	6	CHO = 20.8-1.5t (3.110)	0.95	–
15	12	7	3	CHO = 21.4-2.0t (3.111)	0.99	0.62
16	12	7	3	CHO = 21.7-1.8t (3.112)	0.99	0.50
17	12	7	3	CHO = 20.5-1.6t (3.113)	0.99	0.10
18	3	–	2	$\Delta CHO/\Delta t$ = 1.2 g/L·d	–	–
19	6	6	4	CHO = 21.6-2.1t (3.114)	0.98	–
20	9	~6	4	CHO =22.2-2.0t (3.115)	0.97	1.23

See Figure 3.38.

Figure 3.39 - Wet-to-dry SIPC biomass ratio of SIPC cultures performed in Bioreactor IA. Symbols are as in Figure 3.34.


W/D ratio
40
30
20
10
BIOMASS
0
2
4
6
8
10
12
14
16
18
Time (d)

This correlation is remarkably close to Equation (3.93) of Figure 3.31 for growth stimulated SIPC cultures in Bioreactor IP. The lower scatter of the experimental results presented in Figure 3.39 can be partially explained by the lower average wet biofilm thickness (Equation (3.94)) shown in Figure 3.40 for SIPC cultures performed in Bioreactor IA (0.2-2.6 mm) as compared to similar results presented in Figure 3.32 for Bioreactor IP (0.2-4.5 mm).

The average wet biofilm thicknesses of Figure 3.40 can be linearly correlated to the surface concentrations SIPC by Equations 3.117-3.119. The first two equations give an average W/D ratio of ~ 24-33. A 3 mm wet biofilm thickness corresponds to a SIPC biomass surface concentration of ~ 9.2 mg d.w./cm^2. This gives an allowable biomass concentration of 11.5 to 13.6 g d.w. per litre of reactor volume, with an immobilizing area of 6500 cm^2 (Structure B - Table 3.7) and at the flow diverting plate (5.2 L) and immobilizing structure (4.4 L) heights, which can be cultured in Bioreactor IA. This SIPC surface concentration compares to the value determined for Bioreactor IP in Section 3.3.1.3 (8.5 mg d.w./cm^2). The higher allowable biomass concentration of Bioreactor IA as compared to Bioreactor IP (8.5-9.8 g d.w./L) comes from the lower A/v ratio of the smaller system (1.0 cm^{-1} as compared to 1.25 cm^{-1}). These concentrations for Bioreactor IA are

Figure 3.40 – Relationship between the wet SIPC biofilm thickness and the dried biomass concentration of SIPC cultures performed in Bioreactor IA. Symbols are as in Figure 3.34.

Line 1:S = 20 g/L $T = 0.31SIPC - 0.24$ (3.117)

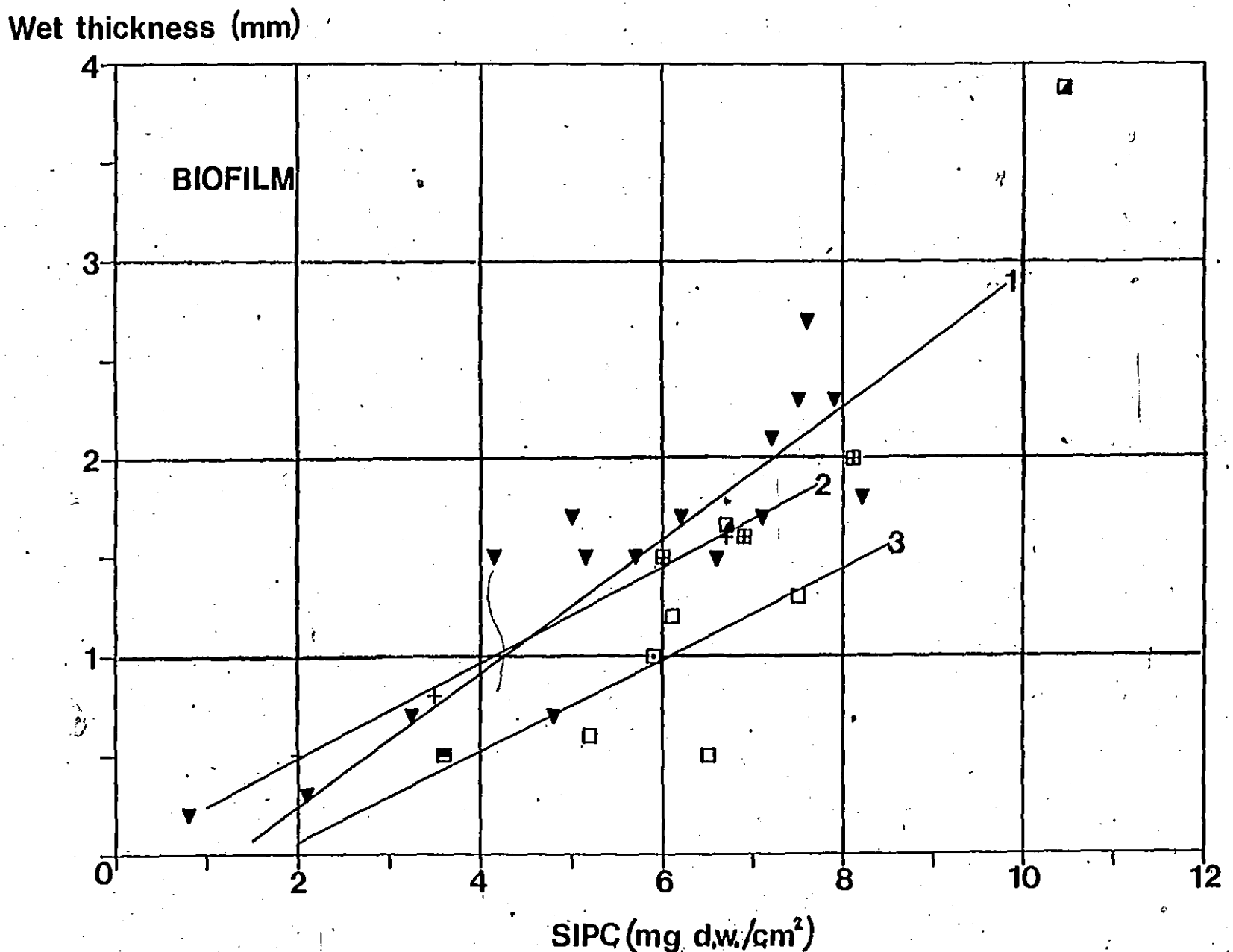
$N_o = 24$ $r = 0.91$

Line 2:S = 10 g/L $T = 0.24SIPC - 0.02$ (3.118)

$N_o = 4$ $r = 0.99$

Line 3:S = 40 g/L $T = 0.17SIPC - 0.07$ (3.119)

$N_o = 5$ $r = 0.94$


Wet thickness (mm)
BIOFILM
4
3
2
1
0
2
4
6
8
10
12
1
2
3
SIPC (mg d.w./cm²)

consistent with the values reported in Table 3.8 for the immobilizing Structure B (7.6-15.1 g d.w./L).

This range of allowed biomass concentrations was attained in Bioreactor IA for batch cultures 3, 4, 8, 9 and 12 (Table 3.14) performed with an initial medium volume of ~ 6 litres. The semicontinuous Experiment 30 produced a 12.4 g d.w./L culture and a 10.4 mg d.w./cm^2 biofilm (immobilizing area of 5744 cm^2). The average wet biofilm thickness (W/D=37.6) of this culture was 3.9 mm. This resulted in an unacceptable biomass packing of the immobilizing structure.

The final characteristic of SIPC cultures evaluated is the oxygen consumption rate of the immobilized biomass. As mentioned in Section 3.3.1.3, this rate can be estimated from Equations 3.98 (O_2CR_m) and 3.99 (O_2CR_c). The comparison of the O_2CR_m and O_2CR_c calculated results for cultures performed in Bioreactor IA showed both to be in the same range. The latter data were on the average 10% higher than the O_2CR_m results (average of 12 cultures, s = 27%). The reasons of this difference were discussed in Section 3.3.1.3, but include the higher evaporation rate observed in Bioreactor IA which lowered the measured carbohydrate consumption rate. The O_2CR_m results of the

SIPC cultures performed in Bioreactor IA are presented in Figure 3.41.

These results show some declining trend during the growth phase and a constant rate of ~ 0.069 μMO_2/mg d.w.h. thereafter. These O_2CR_m are lower than all previously mentioned figures (see Section 3.3.1.3). The difference in the overlapping O_2CR_m ranges of the SIPC biomass cultured in Bioreactor IP (0.085-0.115 μMO_2/mg d.w.h) and in Bioreactor IA (0.040-0.098 μMO_2/mg d.w.h) was already indicated in their respective deviation from the O_2CR_c calculated results (-11% and +10%). The higher O_2CR_m values obtained in Bioreactor IP cultures can be explained by the experimental method used to measure the k_La and the DO_* values. The DO probe could not be installed in the immobilizing structure of Bioreactor IP because of the small size of the vessel (see Section 2.8.2). The k_La measured for IP (Equation (3.61)) and used for the O_2CR_m calculations is most probably overestimated by about 20% relative to a k_La which would have been determined inside the immobilizing structure. This 20% figure was obtained by analogy with Bioreactor IA when comparing k_La measured inside (Equation (3.57)) and outside (Equation (3.56)) the immobilizing structure. The second cause of the higher O_2CR_m of Bioreactor IP cultures comes from the impossibility of measuring the final culture dissolved

Figure 3.41 - Oxygen consumption rate of SIPC cultured in Bioreactor IA.

Suspension cultures O_2CR_c (150 RPM).

◆ : S = 40 g/L.

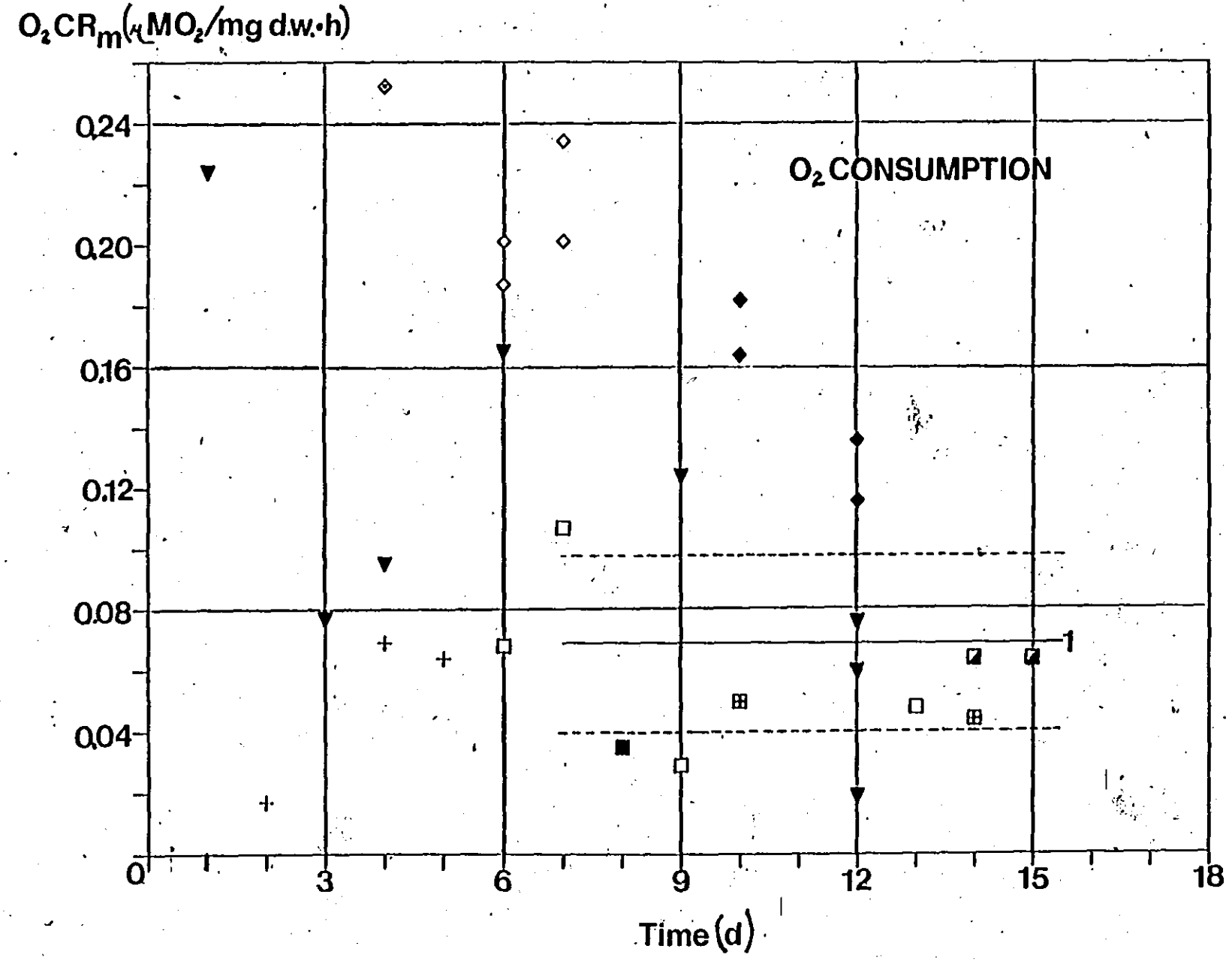
◇ : S = 20 g/L.

◈ : S = 10 g/L.

Symbols are as in Figure 3.34.

Line 1: S = 20 g/L O_2CR=0.069 μMO_2/mg d.w.h

N_o = 9 s = 0.029 μMO_2/mg d.w.h


$O_2 CR_m (\mu MO_2/mg\ d.w.\cdot h)$
0,24
0,20
0,16
0,12
0,08
0,04
0
3
6
9
12
15
18
Time (d)
O_2 CONSUMPTION
1

oxygen concentration (DO_s) rapidly. A period of ~ 5 minutes was required to access the medium after stopping aeration and mixing. This introduced an error in determining DO_s. Consequently the O_2CR_m measured for cultures performed in Bioreactor IA are more representative of the oxygen consumption rate of a SIPC biomass.

In summary, the estimated average oxygen consumption rate of a surface immobilized plant cell biomass decreases during the growth phase to a constant level of ~ 0.07 μMO_2/mg d.w.h during the stationary phase. These rates are lower than for growing suspension cultures by at least 20% during the growth phase of SIPC, and by at least 65% during the stationary phase of SIPC (Figure 3.41).

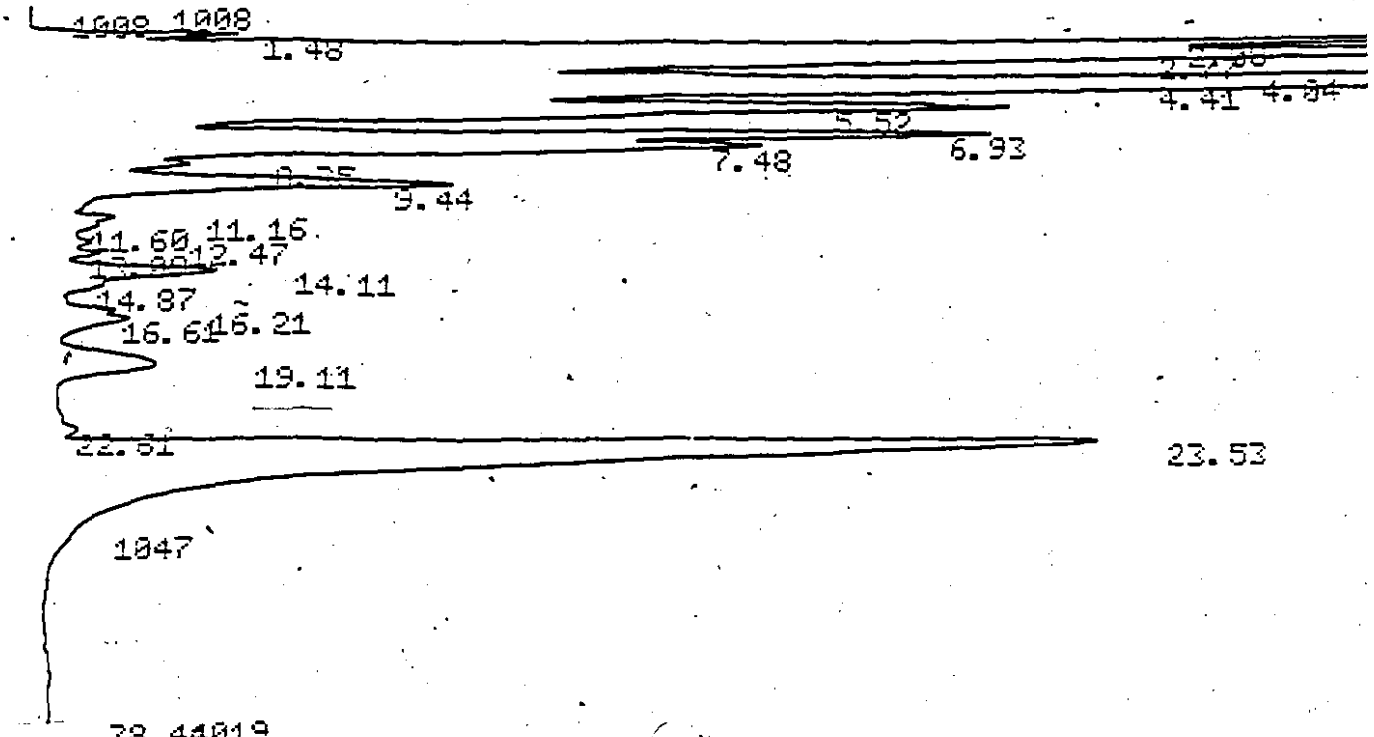
3.4 Production of Secondary Metabolites from SIPC.

The objective of this research project was to develop a suitable immobilization culture system for the production of secondary metabolites from cultivated plant cells. It was important to evaluate if the cells cultured by the surface immobilization technique retained this type of biosynthetic capacity. This was verified for the production of indole alkaloids from C. roseus cells (line MCR17) using the APM medium. This production mode results in the biosynthesis of numerous compounds from productive plant cell lines (see Section 1.2.2 and Figure 3.42). These products are retained in the biomass. The results presented in the following Tables and Figures were for the most easily identified and significant products.

The first experiment was performed to compare the production of alkaloids of suspension and SIPC shake flask cultures of C. roseus cells in the APM medium. This experiment was carried out at McGill University. The extraction of the products and their analysis was performed at NRC-PBI in Saskatoon. The results of this experiment are summarized in Table 3.19. The presence of most SM compounds in the SIPC biomass was confirmed analytically. The two immobilizing materials performed well. The lower SM concentrations of the immobilized plant cells may

Figure 3.42 – HPLC analysis of extracted alkaloids from the SIPC biomass of culture IA6 (25µl, 2 cc/min, 3000 psi, R=0.16).

CHANNEL A INJECT 07/09/86 10:20:32



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FILE 1. METHOD 0. RUN 347

PEAK#	AREA%	RT	AREA	
1	0.56	1.48	81109	
2	14.36	2.08	2080620	
3	16.016	2.77	2320588	Strictosidine lactam
4	14.253	4.04	2065130	
5	15.633	4.41	2265079	
6	7.667	5.52	1110903	
7	4.895	6.93	709235	Ajmalicine
8	3.938	7.48	570627	
9	0.664	8.35	96281	
10	3.121	9.44	452180	Catharanthine
11	0.275	11.16	39884	
12	0.243	11.6	35142	
13	0.266	12.47	38539	
14	0.225	13.09	32643	
15	1.056	14.11	152962	Tabersonine
16	0.256	14.87	37052	
17	0.306	16.21	44282	
18	0.507	16.61	73526	
19	1.128	19.11	163510	
20	0.071	22.61	10337	
21	14.561	23.53	2109866	Serpentine

TABLE 319

PRODUCTION OF INDOLE ALKALOIDS FROM C. ROSEUS CELLS (LINE MCR17) CULTURED IN SHAKE FLASKS

Main Culture Parameters(1)			Strictosidine Lactam (µg/g d.w.)	Ajmalicine (µg/g d.w.)	Tabersonine (µg/g d.w.)	Serpentine (µg/g d.w.)	TLC Analysis		Catharanthine
Technique	I. Material	Biomass Produced					Vindoline	19 Epivindo.	
Suspension	-	9.6 g d.w./L	927.2	4.8	212.7	1543.2	P(2)	P	F(3)
Suspension(7)	-	13.0 g d.w./L	1261.1	94.4	330.7	1261.5	F	F	F
SIPC	Q8	17 mg d.w./cm²	257.4	25.4	32.3	406.8	VF(4)	VF	-
			108.3	10.3	16.6	205.5	VF	VF	-
SIPC	Q8	7.0 g d.w./L(5)	648.0	60.0	109.7	3224.2	F	F	-
SIPC	7612	21 mg d.w./cm²	129.7	17.3	13.7	121.2	F	F	-
SIPC	7612	6.6 g d.w./L(5)	374.6	12.4	98.9	1288.1	P	P	F
Suspension(6)	-	11 g d.w./L	866.5	114.8	240.8	1033.9	F	F	F
Suspension(7)(6)	-	13.9 g d.w./L	594.9	157.6	208.7	908.9	F	F	F
SIPC(6)	Q8	21 mg d.w./cm²	165.9	121.7	71.9	486.8	VF	VF	-
			277.5	128.4	42.6	463.6	F	F	-
SIPC(6)	Q8	5.7 g d.w./L(5)	852.4	161.5	92.5	1054.0	F	F	VF
SIPC(6)	7612	20 mg d.w./cm²	606.2	49.8	36.4	545.8	F	F	-
SIPC(6)	7612	8.3 g d.w./L(5)	869.0	68.1	99.4	1044.7	F	F	F

(1) 27°C, Culture Time: 32 days, APM medium with sucrose, 130 rpm
(2) P: Present
(3) F: Faint
(4) VF: Very Faint
(5) Residual suspended biomass
(6) APM medium with glucose instead of sucrose
(7) 150 rpm

originate from an overestimation of the dried biomass (unwashed freeze dried samples) and/or different biosynthesis kinetics.

Subsequently, this production was assessed in the SIPC bioreactors. The first experiment was performed in Bioreactor IA (IA6). It involved an APM culture carried out according to standard conditions as shown in Table 3.20. Four shake flask suspension cultures (APM, 130 RPM, same inoculum) were carried out simultaneously for comparison. This number of flask cultures was required to ensure the availability of a sufficient volume of plant cell suspension for sampling during the whole experiment.

The biosynthesis patterns of the of the most significant alkaloids produced in these suspension cultures are illustrated in Figure 3.43. The highest concentration of these products in the biomass occured between the 12-14th day and the 20-21th day of the cultures, depending on the compound. Thereafter, these particular products were catabolized. No product was detected in the medium of these cultures.

The concentrations of these products in the suspended biomass at the 26th day of culture (Figure 3.43: ~ 200-400 µg/g d.w.) compare to the concentrations of the

TABLE 3.20

PRODUCTION OF INDOLE ALKALOIDS FROM C. ROSEUS CELLS (LINE MCR17) CULTURED IN THE SIPC BIOREACTOR IA (IA6)

Culture Conditions	Harvesting	Ajmalicine (µg/g d.w.)	Serpentine (µg/g d.w.)	Strictosidine lactam (µg/g d.w.)	Tabersonine (µg/g d.w.)	TLC Analysis			
						Tryp.	H. icin	Loch	Valles.
I. Area: 6479 cm^2	SIPC=4.6mg d.w./cm^2 ((W/D) =27.5) E	242.6	365.0	620.8	-	P(1)	P	P	P
Medium: APM, 6.1 L.		291.5	262.4	614.7	-	-	-	-	-
Inoculum: 562 g of 9d old suspension		290.6	260.1	625.6	-	-	-	-	-
Aeration rate: 0.9 VVM		291.5	553.8	783.2	254.8	P	P	P	P
Temperature: 27° C	Medium: 4.04L								
Time: 26 days	(µg/L med)+	159.0	112.0	-	-	-	-	-	-

(1) P: Present
Tryp.: Tryptamine.
H. icin: Horhammericine.
Loch: Lochnericine.
Valles: Vallesiachotamine.

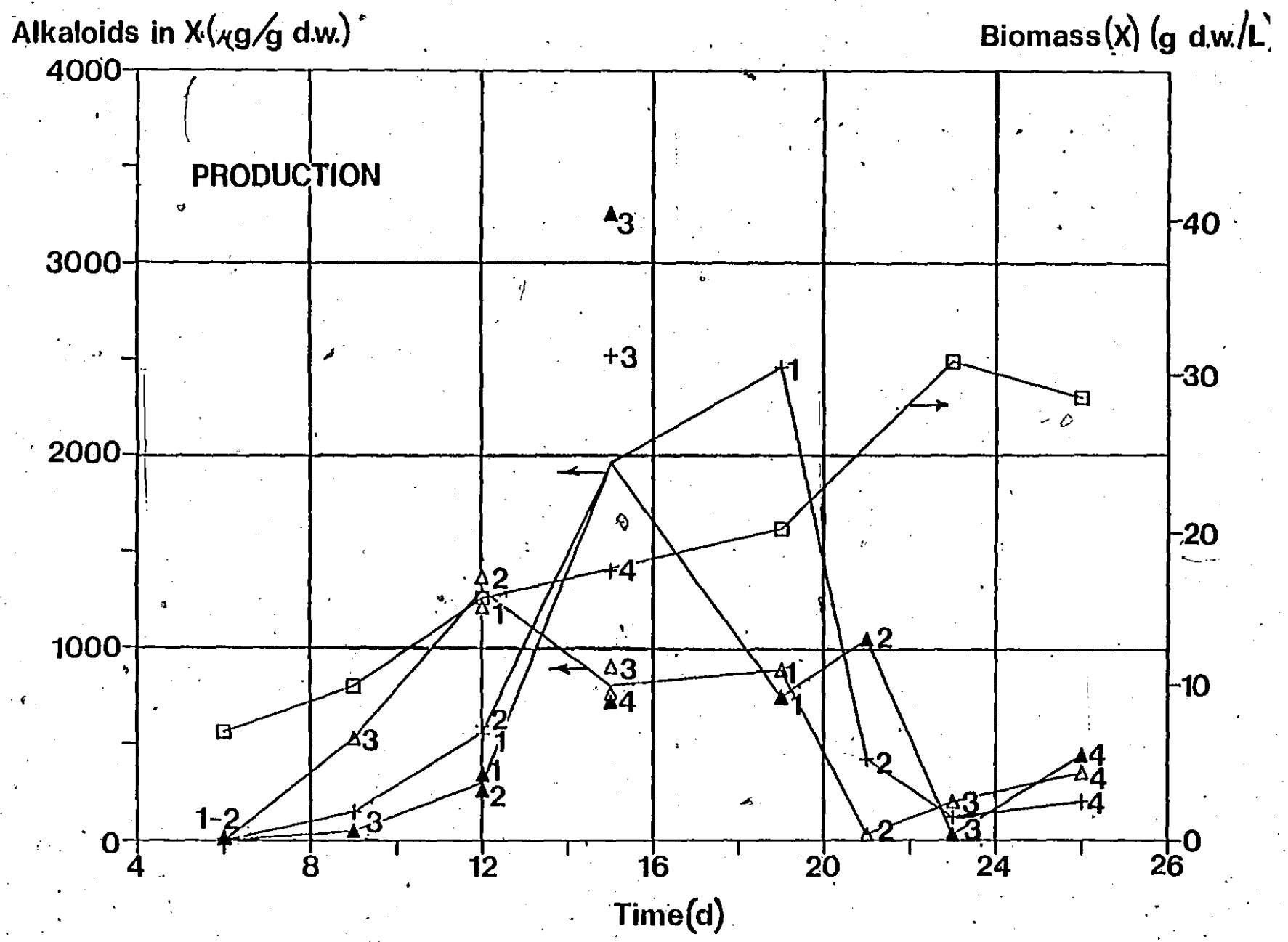
Figure 3.43 – Production of indole alkaloids from C. roseus cells (line MCR17) cultured in shake flasks with the APM medium.

△ i: Ajmalicine in the biomass of flask suspension culture i (fSci).

+ i: Tabersonine in the biomass of fSCi.

▲ i: Serpentine in the biomass of fSCi.

□ : Biomass concentration.


Alkaloids in X (μg/g d.w.)
Biomass (X) (g d.w./L)
PRODUCTION
4000
3000
2000
1000
0
40
30
20
10
0
4
8
12
16
20
24
26
Time(d)

same compounds measured in the immobilized plant cell biomass of the SIPC culture IA6 at harvesting (day 26) presented in Table 3.20. It was not possible to determine the products biosynthesis patterns in this SIPC culture since the biomass was not accessible for analysis without dismantling the bioreactor.

However, some alkaloids were detected in the medium of the SIPC culture IA6. The secretion patterns of the few compounds detected are illustrated in Figure 3.44. These trends resemble the biosynthesis patterns observed for the same compounds produced by the suspended plant cells in Figure 3.43. The secretion mechanism was rapidly stimulated (within 110 minutes) by the addition of 1.2 litres (~ 30% of the culture final liquid volume) of sterile distilled water (pH = 8.6 after sterilization adjusted with KOH before sterilization). This last result presented in Figure 3.43 was not corrected for the dilution effect of the water addition.

Few other production schemes were examined to improve the productive capacity of the SIPC bioreactors. They are summarized in Table 3.21. All schemes were marginally successful. The biomass content of the bioreactors was increased. The SIPC biomass was successfully elicited with the Pythium aphanidermatum sterile fungal extract (Section

Figure 3.44 – Concentration of indole alkaloids detected in the medium of the SIPC culture IA6.

▼: Serpentine.

△: Ajmalicine.

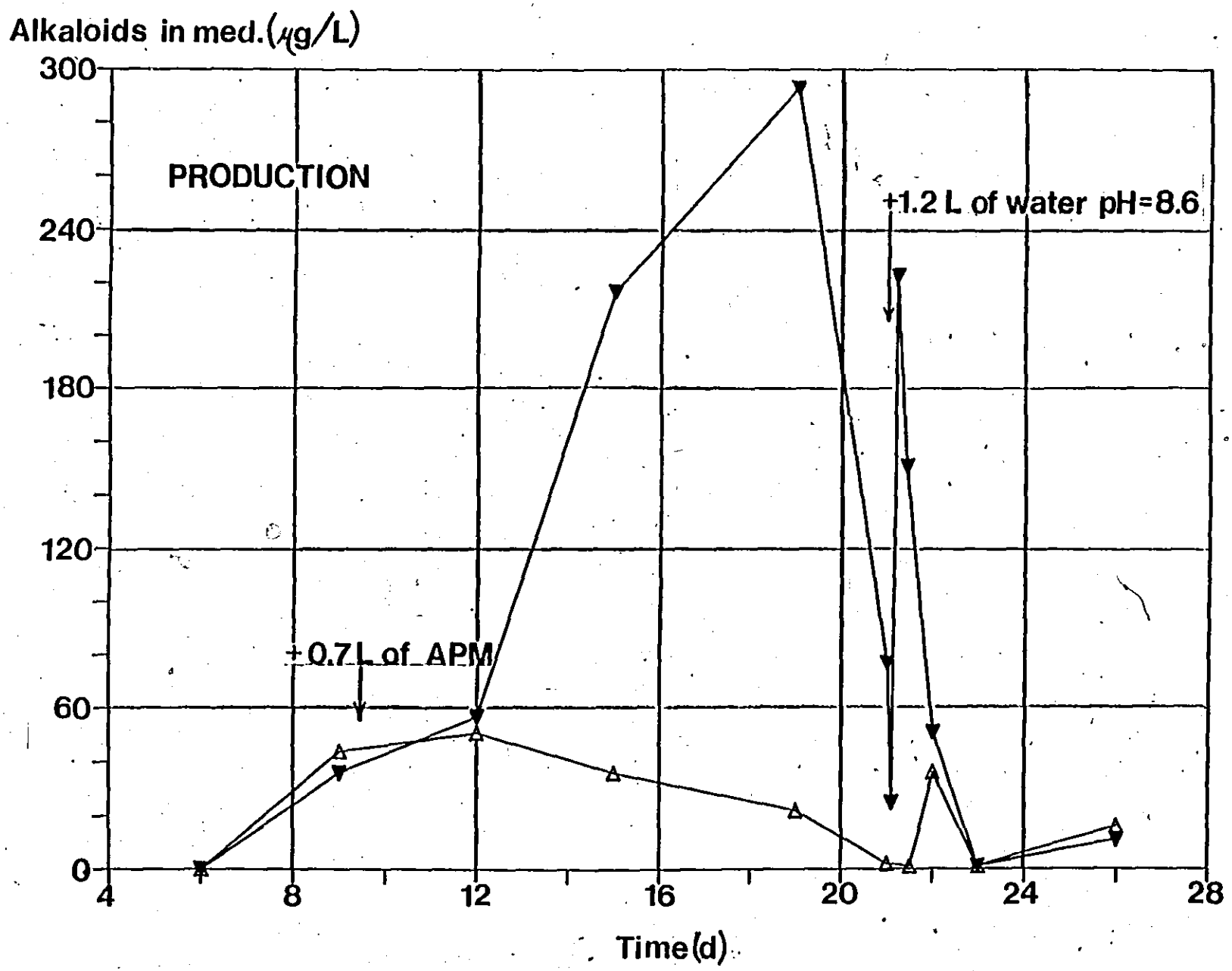

Alkaloids in med.(µg/L)
300
240
180
120
60
0
PRODUCTION
+1.2 L of water pH=8.6
+0.7 L of APM
4
8
12
16
20
24
28
Time(d)

TABLE 3.21

SECONDARY METABOLITES PRODUCTION SCHEMES FOR SIPC BIOREACTORS.

Exp.	I. Area (cm^2)	Growth Phase				Production Phase	Results	Notes
		1B5 $V_{(L)}$	% Inoc.	Aeration rate	Time (d)			
IA10	6400	5.9	8.4	0.9 VVM	13	Purged: 1.65 L of 1B5. Addition: 3.45 L of APM. 10 days.	10.2 mg d.w./cm^2. Small quantities of - Serpentine - Tryptamine - S. lactam & other SM produced.	0.5 L of 1B5 left in bioreactor inhibited biosynthesis.
IA11	6260	6.1	8.5	0.9 VVM	13	Addition of 280 g of Elicitor.	Dead culture after 30-50 hours from Elicitor addition.	Dead biomass completely detaching from the matrix.
IA13	6460	6.1	8.5	0.9 VVM	13	Addition of 280 g of Elicitor. Medium dumped after 24h, Refil: 2.95 L. Batch: 5 hours Continuous: 92 hours 1.7 cc/min 0-44 hrs Batch: 2 days	5.2 mg d.w./cm^2 Small quantities of - Ajmalicine, - Tryptamine & other SM produced.	Non-optimized protocol. Fully viable cells.
IP47	1540	1.5	10%	300 RPM 0.2 VVM	6	Addition of 92g of Elicitor. Medium changed after 24h. Batch for 2 days.	10.4 mg/cm^2. Small quantities of Tryptamine and other SM produced	Non optimized protocol. Fully viable cells.

1.2.2). However, the alkaloids biosynthesized were produced in low quantities. The growth and production phases of this type of plant cell culture need to be further optimized for maximum SM production. This work was not part of the scope of this research project.

4.0 DISCUSSION.

4.1 Characteristics of the Surface Immobilization Technique.

The main factor that contributed to the success of this technique was obviously the selection of Material A07 as the immobilizing matrix. This material is a non-woven geotextile. Its highly irregular fibrous surface, its inertness and a probable biocompatibility between the polyester based fibres and the plant cells favored the excellent biomass attachment observed.

The mechanism of attachment of the cells to the matrix was described in Section 3.1.2.1. It involved the adaptation of the plant cell's shape to the microconfiguration of the support material and its secretion of some compound accumulating between the cell wall and the immobilizing material. These compounds are suspected to be polysaccharides and/or proteins of the types that have been reported to be secreted by plant cells cultured in suspension (11,27,34-37) and/or similar to the middle lemma extracellular glueing pectin substances occuring in plants (32). This was emphasized by the use of stationary phase suspension inocula of sticky and polysaccharide secreting plant cells (2). The results

presented in Figure 3.1 to 3.7 support an hypothesis of the cell wall plasticizing during growth on the immobilization surface, shape adaptation to the support microconfiguration and subsequent rigidifying in place.

The immobilized plant cells were not just viable but were required to participate actively in the adhesion process described above. In fact, the death of the plant cells was shown to result in the complete detachment of the biomass from the immobilizing matrix (see Table 3.21, Experiment IA11). The limited thickness of the biofilm (~ 3 mm), its good diffusive properties (see section 3.2.3.2 - Assumption A2 (48,186,201-203)), the slowness of the plant cell metabolism (t_d ~ 1-2 days), the presence of accumulated intracellular nutrients, the full bathing of the biomass by the medium and the more mass transfer efficient hydrodynamics of the culture system were all likely contributing to ensure good viability retention of surface immobilized plant cells.

The adhesion process resulted in a biomass-support bond strong enough to resist the culturing conditions of the bioreactors without biomass leakage from the immobilizing structure. However, the application of the surface immobilization technique required the formation of Material A07 into a space efficient and easily accessible fixed

structure, such as the vertical spiral wound configuration used in this work, to prevent biomass attrition and abrasion. A similar result was observed for the entrapment of plant cells in polyurethane foam (206). However, the scale up of this last technique was done using a free floating particulate form of the immobilizing matrix in a complex culture system of questionable (sterile) reliability and biomass retention (171).

The other factors which have likely contributed to the successful scale up of the surface immobilization technique were the availability of a large immobilizing area relative to the culture volume (~ 0.8-1.2 cm^{-1}) and the fluid mixing pattern within the immobilizing structure. These last factors appear to have overridden the requirement for a stationary phase inoculum noted in Section 3.1.2.3 for a good attachment and growth of SIPC cultured in shake flasks. This was shown by the successful C. roseus SIPC cultures performed in Bioreactor IP using an exponential phase (5 day old) plant cell suspension inoculum.

4.2 Scale up of the Surface Immobilization Technique.

4.2.1 SIPC Bioreactors Mass Transfer Characteristics.

The operating conditions of both bioreactors were determined relative to their respective unique configuration. This was to ensure comparable uniform and efficient mixing and sufficient oxygen transfer to the medium to meet the needs of the culture within the immobilizing structure. An exponential correlation of the form of Equation (4.1) is suggested to assess the efficiency of the oxygen transfer from the sparged air to the liquid phase in aerated vessels (207,208).

$$k_La \propto (VVM)^{n_1} \qquad (4.1)$$

Values of $n_1 \sim 0.7-1.2$, with $n_1 = 0.8$ for distilled water are reported. This correlation applied to the results of Figures 3.21 and 3.24 gives n_1 values of ~ 0.77 and 0.52 respectively for Bioreactors IA and IP for all conditions evaluated. This shows an oxygen transfer efficiency in relation to the air flow rate in Biorector IA comparable to other airlift configurations. Whereas the lower efficiency observed for Bioreactor IP comes from the hindering effect of the immobilizing matrix on the air dispersion action of the mixing bar and/or the less efficient air-sparger used.

The low A_R/A_D ratio of Bioreactor IA (~ 0.03 to 0.09 at full design biomass loading) was selected to minimize space losses for immobilization. This structural parameter was shown to influence significantly the mass transfer characteristics of internal loop airlift vessels according to the following equations applicable for $1.5 \leq A_R/A_D \leq 7.7$ (188,207).

$$k_La = 0.75(1+A_D/A_R)^{-1.2}(u_g)_r^{0.8} \quad (4.2)$$

$$\frac{\theta_m}{\theta_c} = 3.5(A_D/A_R)^{0.5} \quad (4.3)$$

$$\theta_c = 8.3(1 + \frac{A_D}{A_R})^{-1/3} (\frac{(v_L)^2_r}{D_c g})^{1/3} \quad (4.4)$$

$$(v_L)_r = 0.66 (A_D/A_R)^{0.78} (u_g)_r^{1/3} \quad (4.5)$$

with $(u_g)_r$: superficial gas velocity in the riser tube (m/s),

$(u_L)_r$: superficial liquid velocity in the riser tube (m/s),

θ_c: circulation time (s),

D_c: internal diameter of the vessel (m).

The 0.8 exponent of Equation (4.2) originates from Equation (4.1). The low A_R/A_D feature of Bioreactor IA limited its k_La to the recommended range of 10-15 h^{-1} for plant cell culture (52) as anticipated according to Equation (4.2). This compares to the high k_La observed in airlift bioreactors ($\geq$ 100-400 h^{-1}) (143). This low A_R/A_D did not affect significantly the mixing efficiency of the system. Equation (4.2) is compared to the k_La values obtained for Bioreactor IA in Figure 3.21. The higher (~ 45%) experimental values of Bioreactor IA are not caused by the presence of the immobilizing structure (Line 3: no structure) or of the flow diverting plate (see Table 3.9). This difference can be explained by the k_La measurement technique used to determine Equation (4.2) (CO_2) and/or its A_R/A_D range of application as compared to the low A_R/A_D ratio of Bioreactor IA.

The application of the surface immobilization technique to other more oxygen demanding cell cultures may require some operational and/or structural changes of these bioreactors at least to improve their oxygen transfer capacity to the liquid phase. These changes include a better air sparger in Bioreactor IP and a higher aeration rate or an increase of the A_R/A_D ratio of Bioreactor IA.

The uniform mixing of the culture broth within the low

H/D (~ 0.77) immobilizing structure of Bioreactor IP was achieved efficiently by the upward swirling motion imparted by the mixing bar to the liquid phase. The direction of the liquid flow relative to the winding orientation of the vertical spiral structure of the immobilizing matrix did not influence the plant cells attachment and growth processes.

Achieving mixing uniformity within Bioreactor IA immobilizing structure (H/D ~ 1.6) required an optimum location of the flow diverting plate above the riser tube and close level control. The reason of this particular hydrodynamics behaviour of Bioreactor IA can be found in the results presented in Figure 3.20.

It was mentioned in Section 3.2.4.1 that the measured mixing times (θ_m) compared surprisingly well to the average circulation times (θ_c) predicted by the hydrodynamics model of Equation (3.12) in two situations. This occured when the immobilizing structure was removed from the bioreactor and when the pH electrode was placed in its peripheral spacing for liquid level above the flow diverting plate (H_L $\geq$ 5.2 L). All θ_m measurements were as shown in Figure 3.4, with no real indication of a circulation time (for example, a cyclic response before final homogeneity) other than the initial lag time observed. This lag time was ~ 27% of θ_m

(average of 84 results, s = 6.7%). This would give a θ_m/θ_c ~ 3.7. This value is larger than the 0.8-1.8 ratios observed in Figure 3.20 for θ_m measured in the peripheral spacing for all VVM and in the immobilizing structure for aeration rates $\geq$ 0.9 VVM ($H_L \leq 5.2$ L) relative to the θ_c values predicted by Equation (3.12).

Equation (4.3) and a second correlation (188), which involves the dispersion of the tracer in the liquid phase, could be used to estimate this ratio. Both correlations give either too high (Equation (4.3) ~ 20) or too low (~ 10^{-4}) values of θ_m/θ_c as compared to the results of Figure 3.20. The main reason of these differences is most probably associated to the unique configuration of Bioreactor IA.

The surprising compatibility observed between some measured θ_m of Figure 3.20 and the predicted θ_c may be coincidental, more or less associated with the particular measurement technique used. An alternative explanation involves the structure of Bioreactor IA, the flow pattern generated and the type of tracer used. This mixing vessel is composed of a central section, the immobilizing structure, which occupies ~ 80% of the liquid volume of the reactor, where laminar flow occurs, limiting dispersion. This section is surrounded by a liquid volume occupying the

residual 20% of the vessel (top and bottom sections of the reactor, riser tube and peripheral spacing around the immobilizing structure), which is intensively mixed. It is interesting to note that this 20% figure compares to the relative duration of the lag time observed in measuring θ_m (~ 27% of θ_m). In addition, Bioreactor IA is small (8.3 L) and its slenderness ratio (H/D ~ 3) is low for an airlift vessel and the HCL used diffuses rapidly. These facts explain to some extent the shape of the response curve obtained (Figure 2.4), especially upon aeration rates higher than 0.5 VVM at the optimum flow diverting plate height and liquid level.

The results presented in Figures 3.19 and 3.20 show a large and constant difference (by a factor of ~ 10) between the θ_m measured at the vessel periphery and within the immobilizing structure at high liquid levels (Line 1 in Figure 3.20). This indicates excessive fluid by-pass in the peripheral spacing relative to flow in the structure as mentioned previously. It is then reasonable to conclude that the time parameter measured at the vessel periphery is more associated to a circulation time. Whereas the parameter measured inside the structure is more of a mixing time. This explains the results observed in Figure 3.20 and the good compatibility of some of them to Equation (3.12). These results confirm the validity of the

hydrodynamics model represented by Equation (3.12) to estimate the k_L of the bioreactor. The optimization of the flow diverting plate location and of the operating conditions of Bioreactor IA to attain mixing uniformity were aimed at reducing this difference between θ_m (inside the structure) and θ_c (at the vessel periphery).

4.2.2 SIPC Bioreactors Performance.

The inoculated plant cells were rapidly, uniformly and efficiently immobilized and the SIPC as well retained in both bioreactors. This good performance can be attributed to the combined optimized action of the numerous factors, either structure, operation or immobilization technique associated, discussed in the previous Sections. This resulted in limited foam production, which can be further reduced by using an exponential phase suspension inoculum and/or regulating the concentration of carbohydrates in the culture medium. This resulted also in the formation of a uniform biofilm and in the efficient separation of the biomass from the medium.

Three structural features of the bioreactors have significantly influenced their performance. The low H/D ratios of the vessels (IP: 1.5, IA: 3.0) and of the immobilizing structures (IP: 0.77, IA: 1.63) have

contributed to the uniformity of the inoculum distribution within the immobilizing structure and of the grown SIPC biofilm. They allowed for short but frequent passage of the inoculated plant cells over the immobilizing surface which resulted in a more uniform deposition.

A compromise had to be made in selecting the immobilizing layers spacing and the available immobilizing area for a maximum biomass content of the bioreactor. The results of this work have shown that a spacing of 1.0-1.3 cm and a biofilm thickness of ~ 3 mm provided for good viability of the biomass and sufficient flow passage. These factors should be taken into account upon further scale up of this technique.

4.2.3 The Culture of SIPC in Bioreactors.

4.2.3.1 Growth Behaviour of SIPC.

It was difficult to compare the growth rates of SIPC to that of suspension cultured plant cells. Their respective mechanism of growth is different. Plant cells cultured in suspension grow in aggregates by tridimensional expansion and division. Large aggregates are broken by fluid shear (see Section 3.2.3.2). The respective basis of biomass concentration measurement of these two culture

modes are different (area against volume). The concept of the specific growth rate (μ) (growth by binary fission) is not truly applicable to the culture of surface immobilized plant cells. However, μ remains an easy and useful measure of growth which is not associated with a spatial dimension ($\mu \equiv_D \frac{1}{X}\frac{dX}{dt}$), [$t^{-1}$]. Consequently it was used in this work, in conjunction with biomass growth rates, for comparison purposes.

The growth pattern of the SIPC biomass, cultured according to the standard protocol used, was more easily distinguished in the bioreactors than in the flask systems. The high quantity of suspended biomass masked this pattern in shake flasks. The low A/V ratio ($\sim$ 0.3 cm^{-1}) of the magnetically stirred flask system resulted in relatively high amounts of nutrients available to the space limited growing SIPC biofilm. This may have caused the variability of the results presented in Figure 3.13 and 3.14.

This growth pattern of SIPC cultures consisted of a linear growth phase, up to the 6-8th day of culture, followed by a stationary phase of a constant biomass concentration. The characteristics of this growth pattern and of the biomass produced are summarized in Table 4.1 for

TABLE 4.1

COMPARATIVE CULTURING OF *C. ROSEUS* LINE MCR17 PLANT CELLS

Culture Characteristics (s=20 g/L)	SIPC Culture Systems				Shake Flask Suspension Culture	
	Mag Stirred Flask (200 RPM)	Bioreactor IP (300 RPM, 0.2 VVM)		Bioreactor IA (0.9 VVM)	130 RPM	150 RPM
A/V (cm^{-1})	0.3	1.0		0.8-1.2	-	-
Growth Phase						
Time (d)	8	6-7		6-7	12	6
CHO (g/L)	11.3±1.6	6.7±2.0		8.3±2.4	0	0
pH range	5.0±0.3	5.1±0.3		5.1±0.2	5.0 →	5.8
		b_1 ($\frac{mg\ d.w.}{cm^2 \cdot d}$)	X_{es}	b_1 ($\frac{mg\ d.w.}{cm^2 \cdot d}$)	X_{es}	
Growth Rate						
Biomass (g d.w./L·d)	1.08 $\frac{mg\ d.w.}{cm^2 \cdot d}$	1.47	1.77	0.93	1.07	0.81 → 1.92
$\mu(d^{-1})$	0.25	0.50	0.56	0.30	0.38	0.24 → 0.56
d(CHO)/dt (g/L·d)	0.84	2.1		2.2		1.44 → 3.37
O_2CR_C (μMO_2/mg d.w.·h)	0.34	Decreasing		Decreasing		0.08 → 0.19
Stationary Phase						
$SIPC_{max}$ (mg d.w./cm^2)	8.5±2.2 (100 cm^2)	7.0±0.7 (1900 cm^2)		6.2±0.9 (6000 cm^2)	-	-
$X_{es\ max}$ (g d.w./L)	-	8.1±1.0		7.2±1.2	11.0	11.7
pH range	5.0±0.3	5.5±0.3		5.5±0.2	5.6 →	4.8
O_2CR_m (μMO_2/mg d.w.·h)	0.25_C	0.10±.015		0.069±0.029	-	-
d(CHO)/dt (g/L·d)	0.62	0.46		0.67	-	-
Biomass yield	24.5±7%	37.6±5.0%		40.0±6.6%	57.1%	56.2%
W/D ratio	42.9±12.5	32.9±6.2		$\frac{W}{D}$ = 14.1±1.25t (3116)	22.9±7.6	26.0±8.3
Biofilm thickness (mm)	~3.6	T=0.42SIPC-0.58 (3.95)		T=0.31SIPC-0.24 (3114)	-	-
Immobilization Efficiency	75.5±12.2%	96.4±1.9%		96.0±2.2%	-	-

the three SIPC culture systems developed. They are compared to shake flask suspension cultures. The lower A/V ratio of the flask system explains the slower and slightly extended growth phase (8d) and the higher SIPC biomass loadings observed (8.5 ± 2.2 mg d.w./cm^2).

The two SIPC bioreactors performed well, with the IP system showing higher growth rates (~ 60% relative to IA) and final biomass concentration (X_{max}) (~ 13% relative to IA). This comes from the higher A/V ratio of Bioreactor IA and the resulting lower relative quantity of nutrients (other than carbohydrates) available for growth as compared to Bioreactor IP and to the magnetically stirred flask system (A/V ~ 0.3 cm^{-1}). This fact will be discussed further with the characteristics of the unusual stationary phase of SIPC cultures.

The biomass growth and carbohydrate consumption rates of SIPC cultures performed in the bioreactors are intermediary between that of shake flask cultures agitated at 130 RPM and 150 RPM. The SIPC cultures were optimized for mass transfer while higher shake flask mixing speeds may cause shearing of the plant cells. The availability of nutrients, extracellular carbohydrates for suspension cultures (Figure 2.1) (2) and intracellular rapidly assimilated nutrient(s) for SIPC cultures (see following

discussion), and the inherent division rate of plant cells limit the growth in both types of culture.

This makes the biomass formation phase of a SIPC culture bioreaction controlled and its Effectiveness Factor (η_m - Equation (3.42)) ~ 1. This process is not mass transfer limited above the mixing and aeration rates determined for a best operation of the SIPC bioreactors. This can be confirmed by comparing the growth and carbohydrate consumption rates of SIPC cultures performed in the bioreactors (~ 1.1-1.7 g d.w./L.d and 2 g CHO/L.d) to the following equations derived in Section 3.2.3.4 for $\eta_m > 1$.

$$v_m << 57.6C'_B \text{ (g/L.d)} \qquad (3.53)$$

$$v_m << 6.5C'_B \text{ (g/L.d)} \qquad (3.55)$$

where C'_B is the nutrient (carbohydrate) concentration and v_m is the observed reaction rate. This conclusion can be extended to the production of secondary metabolites from cultured plant cells since the highest production rate reported (~ 0.9 g of rosmarinic acid/L.d (7)) is still lower than Equations (3.53) and (3.55) values.

The Efficiency Factor (η_1 - Equation (3.41)) of the growth phase of SIPC cultures performed in Bioreactor IP is ~ 1 relative to a shake flask suspension culture agitated at 150 RPM. Comparing the results of Table 3.20 and Figure 3.43 indicate that η_1 of the SM production phase is at least of the order of 1. This stresses that these processes are bioreaction limited.

The negative effect of high mixing rates on the growth of SIPC cultured in the flask systems observed in Table 3.3 and 3.4 cannot be explained. This may result from some restricting physiological response(s) of the SIPC biofilm to these culture conditions, from some inhibition to the attachment process or some detachment of the biomass (shake flask system) and/or from some inhibitory effect of the high relative availability of some nutrients.

The conclusion that mass transfer does not control the SIPC processes above the aeration and mixing rates determined for the bioreactors suppports the growth model developed in Section 3.2.3.2. The simple empirical Equation (3.18) proposed to describe the growth of a SIPC biofilm (with no mass transfer restrictions) was very useful in correlating the growth phase behaviour of these

cultures performed in the flask (Tables 3.3 and 3.4) and bioreactor systems (Tables 3.10 and 3.16).

$$SIPC = Io/A + bt \qquad (3.18)$$

However, the nature of this immobilization technique precludes any direct measurement of the biomass concentration in the bioreactor. This model was modified to include the medium carbohydrate concentration. This substrate represents the major carbon source for the formation of biomass and it is easily measured. The biofilm growth rate (b' [mg d.w./cm^2.d]) was related to the measured carbohydrate disappearance rate from the medium by Equation (3.32).

$$b \tilde{=} y_1 \frac{V_M}{A} \left(\frac{dC_s}{dt}\right)_M \qquad (3.32)$$

It was found experimentally that the end of the growth phase was marked by a significant decline in the carbohydrate disappearance rate from the medium (Table 4.1: ~ 2 g/L.d to ~ 0.5 g/L.d). The close monitoring of this rate allows assessing b and determining the end of the growth phase. From these results, the productive biomass content of the bioreactor can be estimated from Equation (3.18).

The validity of this approach was verified by comparing the measured biofilm growth rates (b_M) determined as per Equation (3.18) to estimated rates (b_E) calculated from Equation (3.32) for SIPC cultures performed in the bioreactors. The growth phase period (t) was determined as discussed above. The yield factor used in Equation (3.32) was obtained from Figures 3.29 and 3.37. The V_M/A ratio used was the initial culture value.

The results of S = 10 g/L and S = 20 g/L SIPC cultures carried out in Bioreactor IP show

$$b_M = b_E$$

for 32 experiments performed under various conditions (batch, chemicals addition, semicontinuous etc) with a standard deviation of 16%. The results of similar experiments carried out in Bioreactor IA give

$$b_M = 1.18 b_E$$

for 21 cultures with a standard deviation of 24%. The latter higher difference (b_M/b_E) comes mainly from the higher evaporation rate occuring in the airlift Bioreactor IA. This increases artificially the carbohydrate concentration measured and diminishes the disappearance

rate and b_E.

Consequently, the simple and crude model of Equations 3.18 and 3.32 represents reasonably well (± 20%) the biomass formation phase of the SIPC cultures performed in the bioreactors. Its accuracy can be significantly improved upon closer monitoring of $(dC_B/dt)_M$, of the medium volume and of the evaporation rate as well as by using a better yield model and by evaluating the effect of the biofilm volume (last term of Equation (3.29)) on this growth model. This could be achieved only with a better instrumented and controlled SIPC bioreactor system.

The stationary phase of SIPC cultures inoculated with a stationary phase suspension of C. roseus cells is rather unusual but similar for the three culture systems presented in Table 4.1. It occurs without carbohydrate depletion in the medium and with a constant biomass concentration. During this phase, carbohydrates are slowly consumed (~ 0.5 g/L.d). The presence of carbohydrates in the medium during the stationary phase explains, at least partly, the constant SIPC biomass concentration observed (209). This behaviour of the immobilized biomass is different from the growth of a suspended biomass using medium 155 and any viable inoculum of a sufficient volume (5-20%). The normal growth limiting nutrient of this medium is carbohydrate

(sucrose) at initial concentrations lower than 50 g/L (2). Carbohydrate exhaustion from the medium causes the suspension culture to enter a stationary phase which is characterized by some decline in the dried biomass concentration (Figure 2.1). Some other mechanism(s) appear(s) to limit the growth of the SIPC cultures.

This phenomenon was not caused by mass transfer restrictions for the reasons discussed previously nor by the sterilization of the immobilizing material with the growth medium. However it was found to depend on the plant cell type and line cultured and on the inoculum age.

This behaviour can be explained by considering the structure of a SIPC biofilm and the particular growth metabolism of cultured plant cells. The growth of SIPC is mainly unidirectional and is space limited to the immobilizing area. This growth is most probably restricted to the biofilm external layer while subsurface plant cells experience some volumetric pressure inhibiting cell expansion and division.

Plant cells, and in particular C. roseus cells, used to inoculate a volume of medium for suspension culturing are known to accumulate readily (within 2 days after inoculation) and to store large quantities ($\geq$ 60%) of some

nutrients from the fresh medium (210-213). A good example is phosphate which is less damaging to the culture if absent from the medium. These stored nutrients are subsequently redistributed to the cell progeny upon division. Growth and the amount of biomass produced are proportional to the total intracellular concentration of these nutrients in the culture and to their availability at division (210-213).

The inherent structure of a growing SIPC biofilm consists of younger (nutrients demanding) cells exposed to a depleted medium and growing on top of older (nutrients poorer) cells. This growth pattern restricts seriously the availability of essential intracellular nutrients for continued growth.

This behaviour was found to be cell type and inoculum age dependent. Different nutrient(s) uptake rates, suspension growth patterns and culture histories can explain the observed variability per plant cell type. This implies that some protocol optimization may be required for each type of plant cell to attain self-growth control in SIPC cultures.

Knobloch et al (212) offered an interesting

explanation for the inoculum age factor. The stored intracellular phosphate (and/or other nutrient(s)) of inoculated stationary phase plant cells would be in a form making it unavailable for growth-connected metabolism. In this case, the growth of the SIPC biofilm depends only on the phosphate present in the medium which is accumulated rapidly by the nutrient starved cells. A part of the intracellular phosphate (and/or other the nutrient(s)) of inoculated growing plant cells is readily available for growth. The phosphate present in the medium is probably accumulated less rapidly by these immobilizing plant cells. Consequently, a larger quantity of phosphate is available for the growth of the culture and more remains available in the medium over a longer period for the growing external layer of the SIPC biofilm.

This mechanism explains the needlessness of using large inoculum volumes (16%). It was tested by the various chemicals addition and medium change schemes experimented in Bioreactor IP (Section 3.3.1.2). Most schemes resulted in marginal improvements in biomass formation (0 to ~ 10% for semicontinuous operation, see Figure 3.25) or in some increase in carbohydrate consumption (~ 40-100% relative to other cultures, see Figure 3.26). However, the periodical addition of a concentrated nitrogen/phosphate solution from day 4 (growth phase) of a S = 40 g/L SIPC culture did

respond by a significant increase in biomass formation (~ 30%) and in carbohydrate consumption (~ 40%). This result supports the growth limiting mechanism discussed above.

In summary, both these space and intracellular nutrients availability restrictions imposed by this culture mode on (surface immobilized) plant cells resulted in the limited growth observed. This discussion suggests a (batch cultured) SIPC biofilm to be heterogeneous in depth (formed of cells progressively poorer in intracellular nutrients near its surface) and homogeneous in surface. The biofilm grows at its periphery, probably with some degree of synchrony, at a constant rate as long as essential nutrients are extracellularly and intracellularly available. The plant cells underneath this dividing and expanding layer undergo stationary phase differentiation which should promote secondary metabolism. This biofilm structure, growth behavior and metabolism suggest a different (complex) physiology of SIPC as compared to a suspension cultured plant cell biomass, which seems not to have been studied for secondary metabolites production elsewhere than in this work.

The occurrence of the particular stationary phase of a SIPC biofilm cultured according to the standard protocol used represents an important advantage of this culture

technique. It offers a natural, easy and non-damaging means of controlling the growtn of the biomass for the efficient long term operation of this immobilizing system.

4.2.3.2 Characteristics of the SIPC Biomass.

The two SIPC bioreactors produced a viable plant cell biofilm of comparable characteristics (yield, oxygen consumption rate, wet-to-dry weight ratio and medium pH control; see Table 4.1). As compared to shake flask suspension cultures, the SIPC biomass is more stable at stationary phase since carbohydrates are still present in the medium (209) and consequently it may be less subject to cell lysis as indicated by the stability of the pH of the medium.

The estimated O_2CR of the SIPC biomass follows a normal declining trend during the growth phase and attains a constant low level during the stationary phase (~ 0.07 μMO_2/g d.w..h). The O_2CR (~ 0.07 - 0.3 μMO_2/mg d.w..h) presented in Figures 3.33 and 3.41 are lower than the range of 0.2 - 3.4 μMO_2/mg d.w..h) reported for growing suspensions of plant cells mentioned in Section 2.2.1.. The SIPC biomass O_2CR are also lower (~ 25 - 50%) than the average estimated O_2CR_c of growing C. roseus (line MCR 17) cell suspensions cultured in shake flasks agitated at 150

RPM presented in these Figures. Simultaneously the yield of the SIPC biomass declines during the growth phase (Figures 3.29 and 3.37) and is lower than for a suspension cultured biomass (40% as compared to 56%).

This behaviour results from the structure of the growing biofilm discussed in the previous Section. Growth is restricted to the biofilm external layer while the subsurface plant cells enter rapidly a stationary phase metabolism. This metabolism is characterized by a low oxygen consumption rate and a low carbohydrate utilization rate strictly for maintenance purposes. This explains the lower estimated average O_2CR and biomass yields observed for SIPC cultures as the proportion of stationary to growing biomass increases when compared to a suspension culture.

The wet-to-dry biomass weight ratio of SIPC increases with time (Figures 3.31 and 3.39) as the proportion of stationary to growing biomass in the biofilm increases. The possible declining trend noted in Figure 3.31 for S = 20 g/L batch SIPC cultures in stationary phase may indicate a possible contraction of the biofilm which would not affect the dried biomass concentration. These ratios are higher than for a suspension cultured plant cell biomass (30 to 50 as compared to ~ 25, see Table 4.1). A

similar result was reported for plant cells entrapped in foam particles (W/D ~ 33.5) (206). This higher W/D of the immobilized biomass is partly explained by the fact that it was not vacuum filtered after its removal from the bioreactor as compared to a suspended biomass. This W/D ratio of SIPC represents an actual wet-to-dry biofilm measurement.

This high actual W/D ratio of SIPC is the factor which limits the amount of active plant cell biomass that can be cultured in a given (immobilization as well as suspension) bioreactor volume. The practical biomass capacity of both SIPC bioreactors were determined accordingly for their efficient operation. These capacities are ~ 10 g d.w./litre of Bioreactor IP volume and ~ 13 g d.w./litre of Bioreactor IA volume when culturing C. roseus plant cells according to the standard protocol used. These biomass capacities may change depending on the plant cell type being cultured.

A final point worthy of notice concerns the responsiveness of the SIPC biofilm to chemical stimulation. This was noted previously in Section 3.3.1.3. The responsiveness of a growing SIPC biomass appears to differ from that of a stationary phase biomass. This may be plant cell type and/or inoculum age dependent. Consequently,

optimizing the SM production of surface immobilized cultured plant cells will require evaluating this factor.

4.3 The Production of Secondary Metabolites from SIPC.

Surface immobilized C. roseus cells cultured in shake flasks and in Bioreactor IA were shown in Section 3.4 to biosynthesize indole alkaloids to concentrations at least comparable to that of plant cells of the same line (MCR17) cultured in shake flask suspensions under identical conditions. The comparison of these results to others is difficult since the kinetics of SM biosynthesis by SIPC was not determined and this production is significantly affected by the cell line and culturing conditions used (see Section 1.2.2 and 1.2.3).

In addition, some alkaloids were detected in the medium of SIPC culture IA6 contrary to suspension cultures. This secretion of SM by immobilized plant cells, and in particular Ajmalicine and Serpentine from C. roseus cells, has been reported for gel entrapped IPC (133,168). Comparable concentrations of Ajmalicine in the medium were detected (50 ug/L per litre of medium for SIPC as compared to 80 ug/L of culture (168)). As discussed (168), this product release was probably not caused by cell lysis when considering the general aspect of the SIPC culture (clear medium, viable and attached plant cells) and the stability of the pH. The secretion patterns of the SIPC culture (Figure 3.44) followed surprisingly well the biosyntnesis

trends of the same products measured in the biomass of the shake flask suspension cultures (Figure 3.43). The secretion pattern parallels most probably the metabolic trend, including catabolism (?).

The highest total quantity of Serpentine released in the medium (at day 19) was ~ 11% of the total quantity measured in the immobilized biomass at the end of the IAo culture (day 26). The quantity of Serpentine released in the medium at day 26 was ~ 0.4% of the intracellular concentration of the SIPC biomass. Some kind of metabolic and/or mass transfer equilibrium may have existed between these intracellular and extracellular concentrations. If this was the case, it would be interesting to evaluate the biosynthesis trends of SIPC since extrapolation of the above results suggests high concentrations of SM produced by a SIPC biomass (~ 10000 ug/g d.w. or 1%). This is 3 to 12.5 times higher than the maximum quantities biosynthesized by a suspended plant cell biomass cultured in shake flasks under identical conditions.

The significant, rapid and gentle stimulation (by a factor of 10 within 110 minutes) of the SIPC secretion mechanism shown in Figure 3.44 was most probably caused by osmotic and/or ionic shock (106,117,118). This approach should improve the economics of this process.

The unsuccessful schemes listed in Table 3.21 confirm the last point made at the end of the previous Section, that optimizing a production process performed within the better culture system developed in this work represents another major research project.

4.4 The Potential of the SIPC Culture Technique.

The immobilization of plant cells has been suggested as a better way of culturing plant cells. Its advantages were discussed at length in Section 1.2.4. However, all the immobilization techniques used to culture plant cells were

- difficult to perform under sterile conditions, especially upon scale up (entrapment).
- chemically interfering with the composition of the medium (calcium alginate entrapment).
- growth uncontrolled (gel entrapment).
- biomass capacity limited (see Table 4.2).
- mass transfer restricted (see Table 4.2) and/or
- difficult to scale up within a suitable and reliable system.

In addition, the precious secondary metabolites biosynthesized by the cultured plant cells are not secreted in the medium.

Surface immobilization has been developed in this research project as a new culture technique for plant cells. This technique, from its development in flask systems to its experimentation in laboratory size bioreactors, is an easy and efficient culture mode for plant cells. The inoculated biomass is readily and naturally attached to the immobilizing matrix. The

TABLE 4.2

BIOMASS CAPACITY OF PLANT CELL CULTURING SYSTEMS.

Culture System	Plant Cells	Biomass Capacity	Reference	Notes
Surface Immobilization				
- Shake flasks	C. roseus	20 mg d.w./cm^2 or 34.8 g d.w./L of wet biomass + matrix	This work	
- Bioreactor IA	C. roseus	11.2-13.3 g d.w./L of reactor volume or 25.8 g d.w./L of wet biomass + matrix	This work	Total 2 side thickness: 0.75 mm
- Bioreactor IP	C. roseus	8.6-10.4 g d.w./L of reactor volume	This work	
	Glycine max	9.4 g d.w./L of culture	This work	Cell Line SB1
	Nicotiana tabacum	11.2 g d.w./L of culture	This work	Cell Line Su582
Calcium Alginate Gel Entrapment	C. roseus	<15 g d.w./kg of beads	46,48,131, 153.187 and others	O_2 starved at beads OD ~ 5 mm
Hollow Fiber Entrapment	C. roseus	15 g d.w./L of dialysis tubing	135	O_2 starved (fiber OD: 6.5 mm)
Polyurethane Foam Particles Entrapment	C. frutescens	32.4 g d.w./L of empty foam particles	206	Particle of 1 cc
Suspension Culturing				
- Shake Flasks	C. roseus	32.5 g d.w./L of suspension	2	
- Airlift Bioreactor	C. roseus	14-20 g d.w./L of suspension	2,52,73	Foaming Problems
- Stirred Tank Reactor (Modified Impeller)	C. tricuspidata	30 g d.w./L of suspension	66	

biomass-support bond is strong enough to resist bioreactor culturing conditions. The formation of the foam and biomass froth characteristic of plant cell cultures is significantly reduced. The overall system allows complete separation of the biomass from the gas and liquid phases, making its operation more flexible and its mass transfer processes easier to control.

The good operating characteristics of the SIPC technique represent important process advantages over the traditional suspension culture of plant cells. This technique is also easier and more efficient than all other plant cell immobilization techniques used and discussed in Section 1.2.4. The attachment process cannot be gentler and easier. No physical and mass transfer barrier is introduced between the immobilized plant cells and the medium. This makes this technique particularly compatible to the important new technology of plant cell elicitation for the production of valuable compounds. The immobilization material is inert, sterilizable with the medium, formable, easily available and inexpensive. Growth of the immobilized biomass is allowed without matrix disruption. This growth can be controlled, at least for C. roseus plant cells, by using a suitable stationary phase inoculum and sufficient medium and immobilizing area without affecting the viability of the biomass. This

represents a significant advantage of this technique which will need to be optimized for production. The SIPC technique has been successfully scaled up in efficient and reliable laboratory size bioreactors.

The biomass capacity of the SIPC technique is higher (10-130%) than all other plant cell immobilization techniques used presently, as shown in Table 4.2, with much less oxygen transfer problems. It compares better than these techniques to plant cell biomass concentrations which can be attained by suspension cultures performed in bioreactors (~ 13 g d.w./L against 15-20 g d.w./L for the latter culture system).

It is important to observe that the overall biomass concentration of all plant cell immobilization techniques, calculated over the entire liquid volume of the culture system, is inferior or barely reaching suspension culture biomass concentrations. This is contrary to published discussion (132 and others). These concentrations represent less than 4% of the culture mass on a dry weight basis. This fact stems from the large size and high hydration level (≥ 95%) of cultured plant cells and the additional volumetric requirements of the immobilizing matrix relative to the gas and liquid phases.

The advantage of the immobilization technology applied to the culture of plant cells is mainly in the much improved efficiency of the resulting system. Foaming and mass transfer control is easier. Biomass retention allows easy medium changeover and continuous cooperation. More important, but still largely unknown, is the effect of the biomass homogeneity, morphological rest and microenvironment on the plant cell physiology, and more specifically, on its secondary metabolite production capability.

The scale up of the SIPC technique involved a compact vertical spiral wound configuration of the immobilizing structure installed in laboratory size vessels of suitable hydrodynamics patterns. Bioreactor IP is a direct offspring of the magnetically stirred flask system. The main objective in designing this system was to provide for a small (2 L), easily handled, laboratory size system more representative ($A/V \sim 1.0\ cm^{-1}$) of the surface immobilization technique than the original flask system. It allowed also decoupling the effect of mixing from aeration which was not possible in Bioreactor IA. The limited scale up potential of Bioreactor IP was recognized early, especially considering its low H/D, which may represent mechanical restrictions. In addition, the necessary transition from magnetic to strictly mechanical mixing

means may reduce its (sterile) reliability. Bioreactor IA has better scale up potentials.

The laboratory size systems developed were efficient and reliable. They were optimized for uniform and maximum biomass content. Their experimentation illustrated more clearly than all other immobilization techniques the significant operational advantages of using this technology to culture plant cells for the production of valuable fine chemicals

The slowness of plant cell metabolism makes the growth phase of this process performed in the SIPC systems not mass transfer limited at mild operating conditions determined as compared to shake flask suspension cultures. A similar non-dependency on mass transfer is anticipated for its production phase. This phase may rather be limited by the secretion of the products by the plant cells, by the limited solubility of these compounds in the biofilm and in the medium and/or by their uptake and catabolism by the biomass (Figure 3.44).

The scale up of the SIPC technique to industrial size bioreactors can be considered, and no major problems are anticipated. The immobilizing matrix can be formed in an efficient vertical spiral wound configuration with a layers

spacing of ~ 1.2 cm and a H/D ~ 1.6, which may require further optimization. This will improve the biomass content by at least 10%, rendering this system more competitive to suspension culturing. The design of this structure represents the only serious mechanical problem of the bioreactor. Possible solutions to this problem include a cartridge type arrangement, the use of some light, strong, inert and sterilizable (reinforced plastic) structure similar to the one used for the laboratory SIPC bioreactors or some roll-in configuration of the matrix material with a widely open mesh structure to ensure sufficient spacing between the immobilizing layers.

This immobilizing structure will need to be installed in a low H/D vessel operated as the laboratory SIPC bioreactors. This vessel can be a mechanically stirred reactor, similar to Bioreactor IP, equipped with a large (D_i/D_T ~ 0.6) marine impeller operated at low speed for efficient vertical pumping of the culture broth and with a compatible air sparger. A more (sterile) reliable and better hydrodynamically configured alternative would be a modified airlift vessel similar to Bioreactor IA. This reactor will require a profiled bottom to minimize dead spaces. The required mild culture conditions of such a process, except for the strict sterility requirement, should not cause serious operational difficulties. They

would be determined as for the laboratory bioreactors. It is noteworthy to mention that the economic size of such a bioreactor may be limited by (sterile) reliability considerations and/or by market size constraints (1), which are price dependent and consequently flexible, especially when considering a continuous flow operation.

Predicting, controlling and optimizing the behaviour of a SIPC biofilm for the production of fine chemicals from this type of biomass represent as great a challenge as its scale up, especially in the context of the (genetic) complexity and versatility of plant cells cultured in vitro. The particular growth pattern observed for SIPC using a complete medium, especially the occurrence of the early stable biomass stationary phase without complete carbohydrate exhaustion from the medium, is unique to this culture mode. It represents an interesting approach to growth control of the immobilized plant cells. The dependency of this phenomenon on the plant cell type and line being cultivated, on the inoculum quality and on the particular nutrients uptake behaviour(s) of plant cells stresses the complexity of culturing this particular biological system.

The results presented in this work suggest a particular physiology of a SIPC biofilm which was shown to

differ from that of a suspension cultured plant cell biomass. This behaviour is imposed on the viable plant cells by this unidirectional growth space restricted culture technique and/or represents their adaptive response to this mode of cultivation. In fact, the surface immobilization of plant cells can be seen, more than any other culture mode, as closing the loop in the in vitro Tissue Culture of plant cells. This started with the generation of a dedifferentiated biomass callus of cells from a plant tissue. Plant cells from this solid cultivated callus were subsequently cultured in the form of fine biomass aggregates suspended in an agitated liquid medium. The SIPC technique restores the callus like growth of plant cells in a uniform and homogeneous biofilm fully submerged and bathed within a well controlled liquid environment. This is a more natural growth form (in tissue) for plant cells which offers the possibility for reinitiating intercellular communication and associated physiological behaviour(s). This process could be pursued under proper physical, chemical and/or hormonal controlled stimulation to any desired level of biochemical and even morphological differentiation. True artifical Plant Tissue Culture in a bioreactor can be achieved for fine chemicals production and for physiological studies.

5.0 CONCLUSION.

5.1 Contributions.

1- The major contribution of this research project consisted in the development of the surface immobilization technique as a new and superior culture mode for plant cells as compared to most existing cultivating techniques.

2- This technique was scaled up successfully to laboratory size bioreactors specifically designed for the culture of surface immobilized plant cells. These systems can be further scaled up.

3- The operation of these systems was optimized for biomass formation. This phase of the process was found not to be mass transfer limited above the mild operating conditions determined. It compared well to the growth of shake flask cultured plant cell suspensions.

4- The produced SIPC biomass was partly characterized. It complex and unique physiology, which is still largely unknown since plant cells were never cultured in this particular biofilm form, was found to depend on the plant cell type and line cultivated as well as on the quality of the plant cell suspension inoculum used.

5- The physiology of SIPC was found to differ from that of a suspension cultured biomass. It offered the possibility of controlling easily the growth of the immobilized biomass.

6- SIPC were shown to biosynthesize secondary metabolites to levels at least comparable to that of a shake flask cultured suspended plant cell biomass.

7- Compounds produced by SIPC were shown to be partly secreted in the medium, contrary to a suspension cultured biomass.

8- This secretion mechanism was shown to be rapidly, easily and gently stimulated.

5.2 Recommendations.

Further development work is required before the industrial application of the new SIPC technique can be fully evaluated. It is recommended that this be undertaken as soon as possible to maintain the Canadian lead in this field. The major areas of study include:

1- The application of this culture technique to other economically important plant cell types.

2- The optimization of the physiology of each type of SIPC biofilm for the chemicals biosynthesis according to suitable induction schemes (medium induction, elicitation, etc.).

3- The induced secretion of these compounds in the medium.

4- The development of suitable laboratory size processes and monitoring and control strategies using the SIPC technique.

5- The scale up of such processes to a pilot plant level for operational and economic feasibility studies of the production of SM from SIPC.

This undertaking can already be justified by preliminary economic evaluations similar to the crude estimate presented in Table 5.1. This preliminary study involves the production of the typical plant compounds Ajmalicine and Serpentine. These biochemicals derived from the plant C. roseus are used as pharmaceuticals for the treatment of circulatory (A) and hypertension (S) problems. The world market for each of these products is 3500 kg/y (1983) (1,84). Their updated (see below) selling prices ($Cdn 1987) are respectively ~ $2900/kg (1) and ~ $58,600/kg (84).

The base case was taken from Fowler (84) since this study represents the most reasonable economic analysis of

TABLE 5.1

PRELIMINARY ECONOMICS OF THE PRODUCTION OF THE INDOLE ALKALOIDS SERPENTINE AND AJMALICINE FROM CULTURED C. ROSEUS PLANT CELLS.

	Traditional Batch Suspension + Product Induction Technology (84).	Semicontinuous SIPC and Elicitor Technology.	
		Assumptions	
Production Cycle[6] Culture time	10 days	Preparation	8 days
		Growth	6 days
		Semicontinuous Prod.	24 weeks
Biomass Concentration	25g d.w./L	$\frac{9.0 \text{ mg d.w.}}{\text{cm}^2}$, $\frac{A}{V}$ ~1.4 cm^{-1}	12.6 g d.w./L
SM Produced	1% of d.w. per product(2) - in biomass - Total for 10d	1% of d.w. per product(2) - 75% in medium - per 2 day elicitation cycle.	
No of Prod. Cycle/year	30 (Ho:15% downtime)	(Ho:4% downtime)	2
Bioreactor Size (L)	50 m^3		50 m^3
Capital Cost (CC)	$23.4M[1] (Cdn 1987)	70% of base case	$16.4M
Operation Cost (OC)	$8.8M/y	50% of base case	$4.4 M/y
Raw Materials (RM)	$7.3M/y	50% of base case	$3.7 M/y
Breakeven Producer Price (BEPP)[2]	$16,400/kg	BEPP	$3820/kg

[1]M: 10^6.

[2]BEPP: $CF=0=(BEPP*V_p-OC-RM-CC/8)(1-t_a) + CC/8$.

Vp: Quantity of Chemicals produced and sold.
CC/8: Depreciation (8 year life of plant).
t_a: Taxes (20%).

this production of the few available in the literature. This case involves the traditional suspension culture of plant cells and production induction by an APM-type medium. It was modified to include the production of Ajmalicine at an assumed rate similar to that of Serpentine. The cost figures were updated by assuming a $2 Cdn/L STG exchange rate and an average 10% inflation rate from 1983 to 1987.

The immobilization case assumed using the SIPC and elicitation (114) technologies. A six month semi-continuous operation was also assumed with the biomass loading, biosynthesis and secretion rates as shown. The semi-continuous and product secretion schemes justified the assumed reasonable lowering of costs presented. According to this evaluation of a wholly Canadian owned technology mostly available today, the more advanced production process has a 4 to 1 cost advantage over the traditional process for a world market share of ~ 23% for each product. This breakeven producer price is within the (updated $Cdn 1987) selling price range of a certain number of interesting plant products ($2900 → $7600 → $9.5 x 10^4/kg) (1). This means that any further technological advances, production scale up and/or diversification or a more accurate economic evaluation can make this biotechnological process profitable in a very near future.

It is also recommended that this gentle, easy, efficient and advantageous surface immobilization technique be evaluated for other cultures. The surface immobilization of microbial and fungal biomasses should not represent particular problems. In fact, the surface immobilization of the fungus Rhizopus arrhizus was performed successfully in the magnetically stirred flask system using a milk whey salts supplemented medium. A 3% inoculum was fully immobilized within 4 hours. A three day growth period gave a loading of 16.4 mg d.w./cm^2 and full sporulation. A more intriguing and interesting application would be the culture of animal cells in this system.

Finally it is recommended that the SIPC technique be presented to, and evaluated by, phytoscientist as a means of recreating in vitro under closely controlled conditions, plant tissues for physiological studies.

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Appendix 1 - Measurement of the Rheological Properties of a Plant Cell Suspension.

The rheological properties of the C. roseus suspension culture mentioned in Sections 1.2.1.2 and 2.7 were determined from the results presented in Table A1.1. They were correlated according to Equation (2.1) as follows (74).

$$\theta = \alpha_{\theta} K W^{n} \qquad (2.1)$$

$$\ln \theta = n \ln W + \ln \alpha_{\theta} K \qquad (A1.1)$$

Correlation of the results of Table A1.1 for each spindle gives:

Spindle 1:

$$\ln \theta = 0.77 \ln W + 3.04$$

No of points: 4 $r = 0.99$ (A1.2)

Spindle 2:

$$\ln \theta = 0.70 \ln W + 1.45 \qquad (A1.3)$$

No of points: 7 $r = 0.99$

Spindle 3:

$$\ln \theta = 0.70 \ln W + 0.49 \qquad (A1.4)$$

No of points: 7 $r = 0.99$

The last term of Equations A1.2 to A1.4 (B_{i1}) represents the $\ln \alpha_{\theta i} K$ term of Equation A1.1. This can be written as

TABLE A1.1

MEASUREMENT OF A C. ROSEUS CELL SUSPENSION VISCOSITY.

Spindle #	S	Viscometer speed (RPM)	Average viscometer reading [-]	Apparent viscosity (cps)
1	1.0	0.5	12.0	2400
		1.0	21.7	2170
		2.5	43.0	1720
		5.0	71.0	1420
2	4.0	0.5	2.6	2080
		1.0	4.3	1720
		2.5	8.3	1328
		5.0	13.1	1048
		10.0	21.5	860
		20.0	36.0	720
		50.0	64.8	518
3	10.0	1.0	1.6	1600
		2.5	3.1	1240
		5.0	5.1	1020
		10.0	8.0	800
		20.0	13.7	685
		50.0	26.0	520
		100.0	38.1	381

$$B_{1,1} = \ln \alpha_{s,1} K \qquad \text{(A1.5)}$$

$$B_{1,1} = \ln \alpha_{s,1} + \ln K \qquad \text{(A1.6)}$$

A correlation between $\alpha_{s,1}$ and $B_{1,1}$ gives a consistency index K at no flow.

$$B_{1,1} = -1.11 \ln \alpha_{s,1} + 3.02 \qquad \text{(A1.7)}$$

No of points: 3 $\quad r = 0.99$

Consequently the value of the Power Law Model parameters for this plant cell suspension culture are:

Flow behaviour index $n = 0.72$

Flow consistency index $K = 20.6$ g/cm sec^{1-n}.

$$\tau = 20.6 (du/dr_1)^{0.72} \qquad \text{(A1.8)}$$

Appendix 2 - Plant Cell Culture Media Composition.

A2.1 - Composition of the Growth Medium 185 (43.214)

Nutrient	Concentration (mg/L)
KNO_3	2500
$CaCl_2 \cdot 2H_2O$	150
$MgSO_4 \cdot 7H_2O$	150
$(NH_4)_2SO_4$	134
$NaH_2PO_4 \cdot H_2O$	150
Inositol	100
Nicotinic Acid	1.0
Pyridoxine . HCL	1.0
Thiamine . HCL	10.0
Sucrose	20000
KI	0.75
H_3BO_3	3.0
$MnSO_4 \cdot H_2O$	10.0
$ZnSO_4 \cdot 7H_2O$	2.0
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
EDTA ferric salt	43.0

Nutrient	Concentration (mg/L)
2.4 Dichlorophenoxyacetic acid (2.4 D)	1.0

Notes

1- All chemicals were of reagent grade.

2- The growth medium NB5 was of the same composition as 1B5 except for the two following changes:

a) The auxin hormone 2.4D was replaced by the α-naphthalene acetic acid at the same concentration.

b) 8 g of agar was added per litre of medium.

A2.2 - Composition of the Alkaloid Production Medium (APM) (43.97).

Nutrients	Concentration (mg/L)
KNO_3	950
NH_4NO_3	720
$CaCL_2 . 2H_2O$	220
$MgSO_4 . 7H_2O$	185
KH_2PO_4	68
EDTA ferric salt	55.9
Glycine	2.0
Inositol	100.0
Nicotinic Acid	5.0
Pyridoxine . HCL	0.5
Thiamine . HCL	0.5
Sucrose	50000
KI	0.375
H_3BO_3	2.4
$MnSO_4 . H_2O$	7.0
$ZnSO_4 . 7H_2O$	4.0
$(NH_4)_6Mo_7O_{24} . 7H_2O$	0.0925
$CuSO_4$	0.001

Nutrient	Concentration (mg/L)
Folic Acid	0.5
Biotin	0.05
Indole-3-Acetic Acid	0.175
6-Benzylamino-purine	1.125

All chemicals were reagent grade.

Appendix 3 - Measurement of the Biomass Content of a Bioreactor Loaded Immobilizing Structure.

Prior to its formation in the spiral configuration, the cleaned (in boiling distilled water for 5 minutes, then in methyl alcohol) and dried immobilizing material was weighed and its dimensions were measured. The material was formed in the spiral structure, which was weighed (W_s).

At the end of the culture, the biomass loaded structure was treated as mentioned in Section 2.6. The net wet immobilized biomass weight (W_w) was determined according to the following equation.

$$W_w = W_{Tw} - W_s - M_w * V_m \qquad (A3.1)$$

where W_{Tw}: total wet weight of the biomass loaded structure (g).

W_s: total immobilizing structure weight prior to culture (g).

V_m: immobilizing material original unloaded volume (cm^3).

M_w: immobilizing material liquid absorptivity (g of absorbed water per unit volume of material).

The M_w of the immobilizing materials used was determined by sterilizing measured strips in distilled water. This M_w

was found to be 0.92 g/cm³ (average of 5 results. s = 0.07 g/cm³) for the AXY Materials used.

The net immobilized biomass dry weight (DW) was determined according to the following equation.

$$DW = W_{TD} - W_B - (M_w * V_m * CHO) \qquad (A3.2)$$

where W_{TD}: total dry weight of the biomass loaded structure (g).

CHO: medium final residual carbohydrate concentration (g/L).

No correction other than for the immobilizing matrix assumed equilibrium absorption of the medium (last term on the right hand side of Equation A3.2) was used to take into account the effect of unconsumed carbohydrates on the biomass dry weight measurement.

Appendix 4 - Materials Tested for the Surface Immobilization of Plant Cells.

Materials Tested for SIPC.

Material	Supplier
1-Copper magnet wire with a polyester imide coating	Westinghouse Inc.
2-Aluminium surface with an organic anti-corrosion coating	Hexel Inc.
3-Polyimide surface	Hexel Inc.
4-Ultipor GF - Membrane	Pall Inc.
5-Ultipor GF + Membrane	Pall Inc.
6-Polyvinylidene fluoride surface	Porex Technology Inc.
7-High density polyethylene porous surface (250 um)	Porex Technology Inc.
8-Polypropylene surface	Porex Technology Inc.
9-Ultrahigh molecular weight polyethylene porous sheet (20 um)	Porex Technology Inc.
10-Polystyrene surface	Monsanto Inc.
11-High density polyethylene surface	Dow Chemicals Inc.
12-High density polyethylene surface coated with an antistatic agent	Dow Chemicals Inc.
13-Crushed ceramic	Norton Inc.

14-Crushed ceramic	Centre de recher-che industrielle du Quebec
15-Zeosil SiO_2 powder (20-300 um)	Rhone Poulenc S.A.
16-Hydroculture oil shale. clay pellets and vermiculite	Hydroponic Inc.
17-Cellulose fiber melamine resin bound material	AMF Cuno Inc.
18-Cellulose fiber phenolic resin bound material	AMF Cuno Inc.
19-Acrylic fiber phenolic resin bound material (Q8 Material).	AMF Cuno Inc.
20-Acrylic/ravon fiber phenolic resin bound material (W8 Material)	AMF Cuno Inc.
21-Cellulose/glass fiber phenolic resin bound material	AMF Cuno Inc.
22-Raw glass fiber # 108A	Manville Inc.
23-Celite catalyst carrier # CS30R. #R610. #R626 and #R630	Manville Inc.
24-Asbestos cloth	Manville Inc.
25-Geotextiles 7607.7609 and 7612 (Unwoven polyster based).	Texel Inc.
26-Geotextile T500 and T1000	ICI Inc.
27-Geotextile Mirofi	Celanese

28-Purasin HR activated absorbing carbon beads (0.07 mm) — Union Carbide

29-Fibrous materials DM65. DM65I and M1S175 —

30-Incolov 800 —

31-Monel —