UNIVERSITÉ DE MONTRÉAL

A STUDY OF INDOLE ALKALOID PRODUCTION BY SURFACE IMMOBILIZED CATHARANTHUS ROSEUS PLANT CELLS

par

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ÉCOLE POLYTECHNIQUE

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UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE

Ce memoire intitulé:

A STUDY OF INDOLE ALKALOID PRODUCTION BY SURFACE IMMOBILIZED CATHARANTHUS ROSEUS PLANT CELLS

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en vue de l'obtention du grade de: M.Sc.A.

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SOMMAIRE

Utilisant *Catharanthus roseus* comme modèle biologique, la production d'alcaloïde indoles ont été étudiées, en suspension de 200 mL et en cultures immobilisées sur textile technique dans des réacteurs de 2 L. Deux procédé de production ont été évalués pour leurs effets sur les productivités en suspension et en cultures immobilisées. Le premier procédé de production fait appel à une seule étape où les cellules de plantes croissent dans un milieu[20] de production d'alcaloïde (APM) durant 15 jours. Le deuxième procédé de production se fait en deux étapes. Les cellules sont cultivées au préalable, dans un mileu de croissance de base avant de poursuivre la phase de production dans l'APM durant 15 jours.

Aussi bien, pour les cultures en suspensions qu'immobilisées, le procédé à deux étapes favorise une production plus élevée d'alcaloïdes indoles par rapport au regime à une étape. Les rendements maximaux sont 6 fois plus élevés en deux stages pour les cultures en suspension et 4 fois plus élevés pour les cultures immobilisées par rapport aux estimations de la totalité des alcaloïdes indoles (TIA). La production de chaque espèce d'alcaloïde est également d'un ordre de grandeur plus élevée (10^3 vs 10^2 µg/g p.s.) en suspension et en culture immobilisée pour le procédé en 2 étapes.

Pour le procédé à une étape, les cellules en suspension et les cellules immobilisées ont des niveaux de production similaires (50 - 53 mg/L). En revanche, les cultures en suspension de 200 mL sont 4 fois plus productives (700 mg/L) que les cultures immobilisées (185 mg/L) dans le procédés en deux étapes.

Les alcaloïdes ont été détectés dans le milieu de culture. De 20% (1-étape) à 30% (2-étapes) des alcaloïdes produits dans les cultures en suspension sont libérés dans le milieu.

Les cellules immobilisées quant à elles, larguent de 50 à 60%. Les rapports sont calculées par rapport à l'estimation de la totalité des alcaloïdes indoliques (TIA) et ne comprennent pas les concentrations due à la lyse cellulaire.

Il a été établi que la lignée cellulaire *Catharanthus roseus* MCR17 produit le précurseur de la tryptamine et 10 alcaloïdes qui appartiennent à 6 familles d'alcaloïdes indoliques: strictosidine lactam, ajmalicine, serpentine, yohimbine, tabersonine, lochnerinine, epivindolinine, vindolinine, catharanthine et vallesiachotamine. Ni la vindoline, ni les alcaloïdes dimériques, vinblastine et vincristine n'ont été détectés lors de ces cultures.

La production des alcaloïdes n'est en général mesurable qu' après trois jours quand le milieu de culture est totalement épuisé en nitrates. L'analyse des résultats a montré que la conductivité du milieu, aussi bien dans les cultures en suspension que dans les culture immobilisées, est une fonction linéaire de la concentration en ions nitrate. Ces paramètres peuvent être exploités comme indicateurs de l'évolution métabolique de la biomasse pour la production de métabolites secondaires.

ABSTRACT

Indole alkaloid production was investigated in 200 mL suspension and 2-L surface immobilized plant cell cultures using *Catharanthus roseus* as the study model. Two production processes were evaluated for their effect on the productivity of the suspension and immobilized cultures. The first production process was a 1-stage regime which consisted of growing the plant cells in alkaloid production medium[20] (APM) for 15 days as a control experiment. The second process was a 2-stage regime where the cells were cultured in basal growth medium for 6 days (growth stage) prior to the 15-day production phase in APM.

The 2-stage regime was found to encourage higher indole alkaloid production, as compared to the 1-stage regime, for suspended and immobilized plant cells. Peak yields were 6-fold higher for the suspension cultures (40 mg/g dw) and 4-fold higher for the immobilized (18 mg/g dw) cultures with respect to total indole alkaloid (TIA) estimations. Individual alkaloid yields were also 1 order of magnitude higher (10^3 vs 10^2 μg/g dw) in suspension and immobilized cultures performed according to the 2-stage process.

For the 1-stage regime the suspended and immobilized cells produced similar quantities (50 - 53 mg/L). The 200 mL suspension cultures were about 4 times more productive (700 mg/L) than the 2-L immobilized cultures (185 mg/L) in the 2-stage process.

Alkaloids were detected in the medium of the cultures. Excluding the occurrence of partial cell lysis, about 20% (1-stage) to 30% (2-stage) of the alkaloids produced in the suspension cultures were released to the medium, according to TIA estimates. The 1 and 2-stage immobilized cultures released 50% to 60% of total alkaloids produced on a TIA basis.

Catharanthus roseus cell line MCR17 was found to produce the precursor tryptamine and

10 alkaloids belonging to 6 indole alkaloid families: strictosidine lactam, ajmalicine, serpentine, yohimbine, tabersonine, lochnerinine, epivindolinine, vindolinine, catharanthine and vallesiachotamine. No vindoline, nor the dimeric alkaloids, vinblastine and vincristine, were detected in any of theses cultures.

Pronounced alkaloid production usually occurred about 3 days after nitrate was depleted from the culture medium. The experiments showed that nitrate concentration was linearly correlated to the medium conductivity in suspension and immobilized cultures. These parameters could be used as indicators of the plant biomass' metabolic status and with regard to the induction of secondary metabolite production.

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NOMENCLATURE

AB5:	Gamborg's[50] basal growth medium supplemented with 1.0 mg/L (1 μM) indole acetic acid and 20 g/L sucrose.
APM:	Zenk's[20] Alkaloid Production Medium.
BRI:	Biotechnology Research Institute.
CAS:	Ceric Ammonium Sulfate.
dw:	Biomass dry weight.
FAB-MS:	Fast Atom Bombardment Mass Spectrometry.
f.w.:	Biomass fresh weight.
HPLC:	High Perfomance Liquid Chromatography.
IAA:	Indole Acetic Acid.
K:	Conductivity cell constant (cm^{-1}).
k_La:	Oxygen mass transfer coefficient (h^{-1}).
MCR17:	*Catharanthus roseus* cell line number.
μ:	Specific biomass growth rate (d^{-1}).
μsiemens:	unit of conductance (inverse of resistance).
NMT:	N-methyl transferase enzyme.
PBI:	Plant Biotechnology Institute.
PicA:	Amphoteric pairing ion reagent for liquid chromatography (Waters Scientific).
PID:	Proportional Integral Derivative.
PSI:	Pounds per square inch.

r:	Linear correlation coefficient.
RIA:	Radioactive Immunological Assay.
R_f:	Ratio of displacement between alkaloid spot and solvent front in thin layer chromatography.
RPM:	Revolutions per minute.
Re_i:	Impeller Reynold's number.
SF:	Shake flask.
SIPC:	Surface Immobilized Plant Cell[22].
SS:	Strictosidine synthase enzyme.
t:	time (days).
TDC:	Tryptophan decarboxylase enzyme.
TIA:	Total Indole Alkaloid estimate.
TLC:	Thin Layer Chromatography.
UV:	Ultra-violet.
V_m:	Volume of matrix.
V/V:	Volume-to-volume basis.
VVM:	Volume of gas injected per volume of culture per minute.
W_I:	Net weight of wet immobilized plant biomass (g f.w.).
W_T:	Weight of wet biomass-loaded structure (cage, matrix, plant cell biomass) (g).
W_{mc}:	Weight of matrix-covered immobilization cage (g).

W/W: weight-to-weight basis.

X: Plant biomass concentration (g d.w./L culture).

1B5: Gamborg's[50] basal growth medium containing 1 mg/L (4.5 μM) 2,4-dichlorophenoxy acetic acid.

2,4D: 2,4-dichlorphenoxy acetic acid.

CHAPTER I

1.0. INTRODUCTION

1.1. NATURAL PRODUCTS FROM PLANTS

Species of the plant kingdom are constantly involved in our daily lives whether they be for nutritive, medicinal or decorative purposes. Plant-derived products are used principally by the pharmaceutical, cosmetic and food industries[1,2]. In 1986, natural products comprised 25% of the pharmaceutical prescriptions in the United States which accounted for 3 to 8 billion dollars in sales[3,4,7]. The cosmetic industry seeks fragrances such as jasmine and the attar of roses, while the food industry uses natural products such as vanillin and mint for flavouring, or anthocyanins for coloring. Natural products are sought to replace the potentially carcinogenic chemical substitutes which inundate every aspect of our lives[1,2,5,6].

The active agents, which colour flower petals or grapes, stimulate the nervous systems of organisms[8], or taint the arrowtips of tribesmen, belong to a vast class of compounds known as secondary metabolites[9,10]. Primary metabolism involves the biochemical reactions that produce components essential for survival. Secondary metabolism procures compounds that serve no clear function to the organism, yet they derive from common precursors found in the primary metabolic pathways[9]. The plant's propensity to produce secondary metabolites probably evolved from environmental pressures during the course of natural selection[11,12]. Secondary metabolites emerged in plants to serve as defense molecules against microbial or fungal invasions, insect repellants/attractants[8,11,12].

Alkaloids are a class of secondary metabolites which are principally characterized by a heterocyclic organic ring containing a nitrogen atom[10,13]. They are predominantly synthesized in higher plants and can be found in some lower inverterbrate organisms and animals[10]. They manifest physiological reponses in the nervous systems of organisms which is why the pharmaceutical industry is interested in certain plant-derived products. More than 6000 alkaloids have been characterized from plants and a large number of them contain the monoterpenoid skeleton[10,13]. Indole alkaloids contain the basic skeleton (Figure 1.0) where the indole moiety originates from the amino acid tryptophan and the terpene moiety derives from mevalonic acid[14].

The Periwinkle flower of Madagascar (*Catharanthus roseus*) used to be ingested by native people for its anti-hypertensive and anti-hypoglycemic properties[15,16]. This plant contains about 100 different indole alkaloids of which a few are of great commercial value. The dimeric alkaloids, vincristine and vinblastine have anti-leukemic properties and were valued at 5 million dollars per kilogram[15-18]. More common biologically active indole alkaloids of *C.roseus* are the monomers ajmalicine and serpentine which are used for circulatory disorders[7,19].

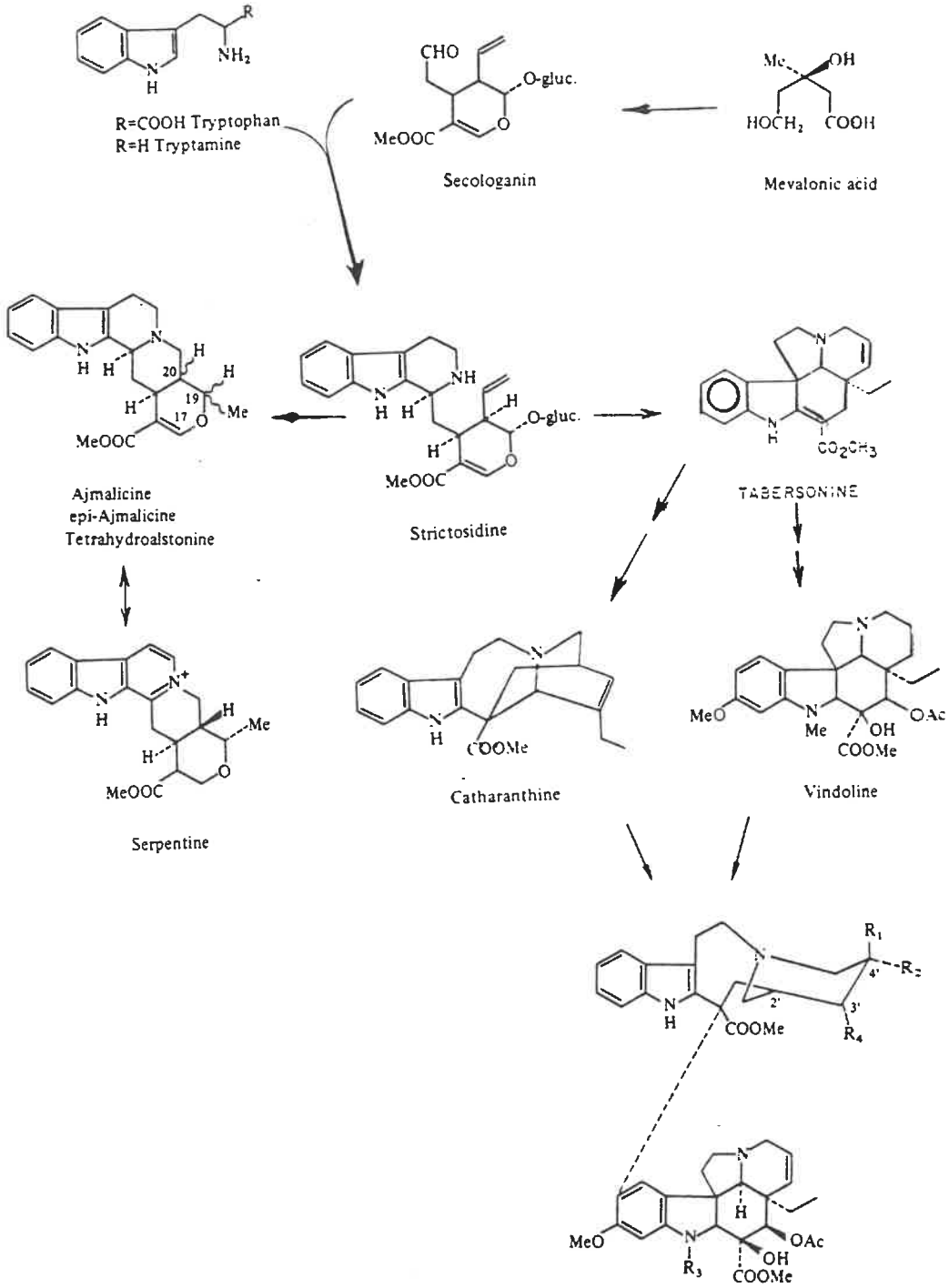


FIGURE 1.0 Schema of the indole alkaloid pathway leading from tryptamine and mevalonic acid.

1.2. PLANT TISSUE AND CELL CULTURE

In vitro culture techniques for plant tissues were developed since the 1950's for plant tissues in order to provide alternate but stable sources of plant-derived products[1,2,8]. Whole plants require long cultivation periods (months to years) and they are susceptible to capricious environmental conditions (blight, drought, etc.). In addition to the aforementioned factors, geographical and political restraints limit the accessibility of certain plant-derived products[1,2,8]. The advent of plant tissue culture techniques circumvented these problems in order to maintain environmentally-controlled and stable productive cell lines.

Nonetheless secondary metabolite synthesis by plant tissue cultures has been low (ca. 0.001 to 0.01% w/w) due to lack of knowledge of the complex biosynthetic pathway regulation and the sensitive nature of plant cell physiology (compared to microbial cultures)[1,2,5,6]. Yet for certain species, product yields could be significantly increased by 3 to 400-fold (catharanthine and shikonin, respectively)[20,21] in plant cell cultures as compared to intact plants[12].

Chemical, physical and bioengineering aspects of plant cell culture had to be re-evaluated[22] in order to accommodate the plant cells for large scale production[23,24] of secondary metabolites. Necessary improvements encompassed the development of production media, novel bioreactor configurations[6,24-26] as well as the advent of immobilization techniques[22-24,26,27]. Biological approaches were also adopted to select high-producing plant cell strains and to elucidate the enzymological controls of secondary metabolite synthesis in plant cell cultures[1,2,5,12,28,29].

Studies revealed that secondary metabolite production in plant cells was generally a growth-dissociated phenomenon[5,6,12,23,30,31]. Some media compositions of various types and concentrations of phytohormones, inorganic nitrogen and phosphate and carbohydrate sources, were found to promote less growth and induce production[20,32,33]. Culture techniques, in which the cells could sustain maximum intercellular contact and morphological stability, lent to improved secondary metabolite production.

Successful scale-up of plant cell cultures for secondary metabolite production was impeded by problems due to the rheological properties of these cultures: excessive foaming, mass transfer and viscosity problems[8,22,34]. Immobilized cells exhibited improved product yields and volumetric productivities, as well as prolonged viability compared to suspension cultures[22,24,35]. Techniques that had been developed to immobilize plant cells have been used since the 80's: gel entrapment, surface immobilization via cross-linking and adhesion to various matrices such as hollow fibres and poly-urethane foam particles[22,24]. However these techniques could have proven difficult to scale up[22,36].

In addition to the development of production media and novel culture techniques, culture process regimes had to be devised so that secondary metabolite production could be economically feasible upon large-scale production. The productivity of a culture process would be dependent on the 3 above-mentioned parameters. The re-use of biomass and secretion of products to the medium (ie. as in a fed-batch or continuous process), and on-line harvesting could also enhance the productivity, thus lower the cost of a culture process. Multiple product harvesting could also amortize the cost of production of low yield products; if not, then production of high market value plant-derived products would have

to be envisaged in order to justify the cost of large scale plant cell culture processes[2,8].

In 1987 a novel immobilization technique was patented[22]. Bioreactors of 2 and 6 L capacities were designed by taking into consideration the shear sensitivity of plant cells and their tissue-forming tendency[12,22]. The surface immobilization of plant cells highlights a spirally-wound inert geotextile onto which the cells adhere to, thus segregating the biomass and the culture medium. Medium exchanges are easily executed and the bioreactor configuration has improved hydrodynamics, easier scale-up potential and handling as well as prolonged asepsis potential as compared to the previously mentioned techniques[22-37].

The objective of this project was to evaluate the productivity of 2-L surface immobilized plant cells, using *Catharanthus roseus* as the study model, for indole alkaloid production. Two production processes were compared for the immobilized cultures. In addition, shake flask-grown suspension cultures were compared to the immobilized cultures using both culture processes.

This project was performed in close collaboration with Barbara Jardin whose master's project was to determine how to induce indole alkaloid secretion by immobilized cultures of *C.roseus*. Collaborative work was executed to determine the growth curve of immobilized cells, optimization of the 2-stage process and preliminary studies of indole alkaloid production patterns in both culture regimes.

1.3. PROJECT OBJECTIVES

The aim of this project was to evaluate the production of indole alkaloids in surface immobilized *Catharanthus roseus* plant cells. To do so, the following factors were

determined:

1. characterization of growth of *C. roseus* in suspension (0.2 L) and immobilized cultures (2L).

2. selection of an optimum moment during the growth cycle of the culture in order to induce secondary metabolite production in 2-L surface immobilized cultures.

3. Comparison of 1-stage (production only) and 2-stage (growth followed by production) production regimes in surface immobilized cultures.

4. Comparison of indole alkaloid production in a 2 L immobilized and in a 0.2 L suspension cultures using both production regimes.

1.4. LITERATURE REVIEW

1.4.1. IN VITRO PLANT CELL TECHNIQUES

With the advent of microbial and mammalian culture techniques it was conceivable that plant cells could be cultured similarly to provide for an alternate source of plant chemicals[1,2,5,6,8]. Plant cells can be cultured under controlled environments and any plant species can be maintained for many years. The agricultural industry uses plant cell culture techniques for crop improvements[1,2,8]. Whole plants can take up to 2 - 3 years to accumulate small quantities (shikonin: 2 - 3% dw) of products while cultured plant cells could potentially produce larger yields (23% dw for shikonin) in less than 1 month[1,2,8,3,38].

1.4.2. THE PLANT CELL

A plant cell (ca. 10^2 μm diameter) is almost a thousand times more voluminous than a bacterial cell (ca. 1-2 μm). It contains a plasma membrane enveloped within a cellulosic cell wall. Water comprises about 95% of the cell and by virtue of its large volume it behaves like a water balloon that is sensitive to shearing forces. In mature plant cells the vacuole occupies 90% of the cell space[6,22,25,34,35,39].

Plant cell metabolism is slower than for microbial cells. It takes about 20 minutes for microbes to divide while for plant cells it is in the order of 40 to 60 hours. Microbial culture periods last from 1 to 2 days while plant cell cultures last from 3 to 5 weeks[8,25,28,29,34,40]. The respiration rate has been reported to be in the range of 0.2 to 3.6 mmole O_2/g dw·h

where it is about 0.5 to 570 mmole O_2/g dw·h for bacterial cells[6]. While some bacteria release products to the medium most plant cell products of interest remain sequestered in the vacuole and are seldomly released[12,34,35,41-44].

1.4.3. CELL LINE GENERATION

The development of plant cell lines, from any part of the intact plant, involves de-differentiation into cellular masses that exhibit no morphological characteristics of the excised plant tissue. Basal media must supplement growth hormones, mineral nitrogen, phosphate and carbohydrate[45-49] in order to support the growth of these heterotrophic plant cells[5,6,17,21,34,50-52]. The cells can however be redifferentiated into any plant organ depending on the balance between the auxins and cytokinins (phytohormones) in the medium, since they all are contain the same genetic make-up. Thus the dedifferentiated plant cells are deemed totipotent. With respect to secondary metabolite synthesis, leaves are usually the selected organs to generate cell lines[14,53-56] for the majority of enzymatic activities localized therein (Dr. H. Khouri, pers. comm., BRI, Montreal). Young tissues such as the embryos from plant seeds are also used to generate cell lines[50].

It takes 4 to 6 weeks to de-differentiate plant tissue into compact friable cell masses (callus) on solid agar nutrient medium[50] which is described for a *C.roseus* strain in Zenk et al.[20] (Figure 1.1). From the agar medium the callus is transferred to liquid growth medium to form plant cell suspension cultures. Plant cells tend to grow in aggregates of 2 - 10 cells[25,34,53].

Suspension cultures can contain heterogenous cell populations with respect to growth and secondary metabolite production capacity[7,20,57-60]. With loss of morphological differentiation, product yields were inferior (ie. ajmalicine, serpentine) or non-existant (vindoline, vinblastine, vincristine), as compared to the intact plant[8,12,20,34,45,56,61-65]. However some plant cell cultures could produce more than the intact plants (shikonin, berberine, catharanthine).

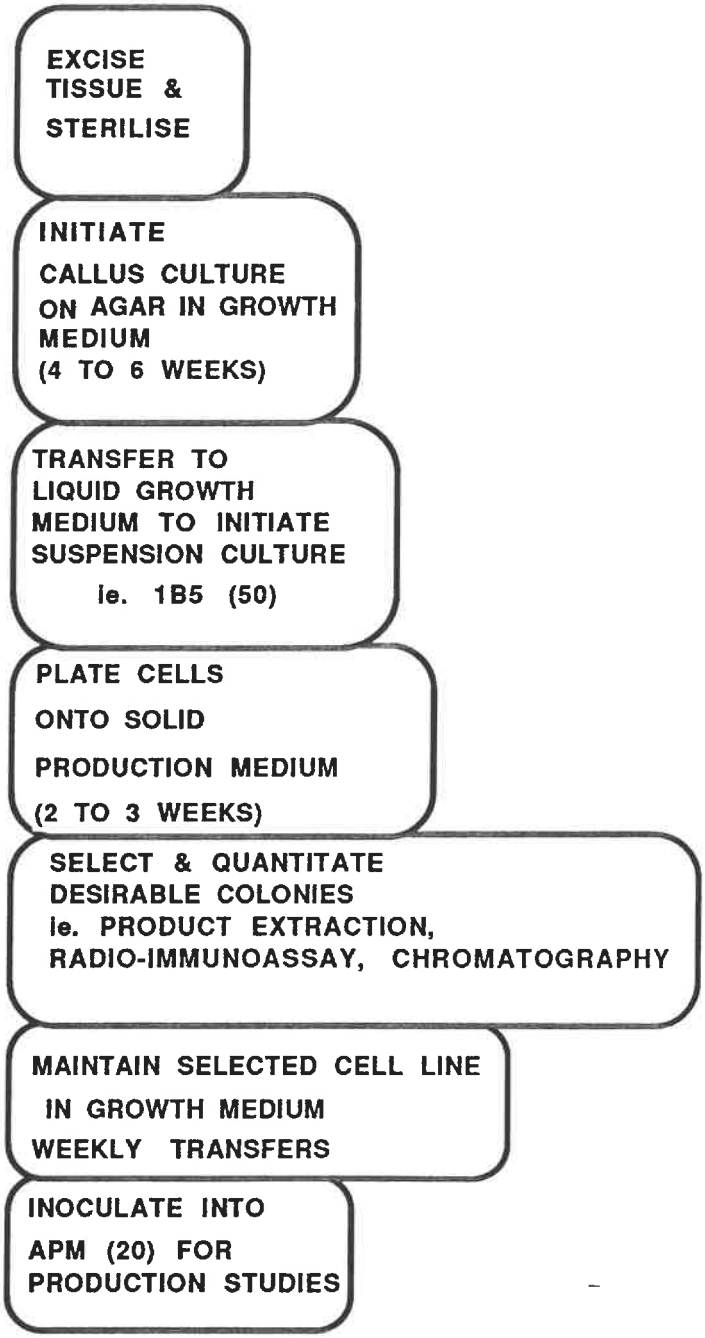


FIGURE 1.1 Scheme for plant cell line generation from excised plant tissue.

1.4.4. LARGE SCALE CULTURES

Shake flask-grown cultures (100 - 200 mL) have been used as basic models to study growth and production. It had been shown that plant cell growth and productivity was diminished or lost in larger scale cultures when compared to the shake flask systems[12,24,34,40,66]. Scale up of plant cell cultures (>200 - 1000 mL) require careful control of aeration and agitation conditions due to the nature of these shear-thinning fluids[22,25,40]. As plant cells increase in biomass the suspension becomes more viscous thus impeding efficient mixing and transfer of gases and nutrients to the cells. Plant cells are large particles (ca 10^5 μm^3) and mixing must be sufficient to maintain an evenly distributed suspension. Insufficient mixing would allow the particles to sediment rapidly and concomitantly impede efficient mass transfer of gases and nutrients to the cells[8,34,67].

In stirred tank cultures, high shearing rates exist and because plant cell suspensions are shear thinning fluids, cell destruction is prominent in the vicinity of the impellers. However toward the walls of the vessel less shear and mixing are experienced by the cells and stagnant zones occur[25]. Helical ribbon impeller[25] and airlift bioreactors[34,40] have been suggested as alternative culture vessels, for viscous plant cell suspensions, due to lower shear rates and better aeration[25,40].

The oxygen mass transfer coefficient (k_La) for plant cell cultures had been reported[68,153] to be in the order of 5 to 20 h^{-1}. The minimum oxygen demand for *C.roseus* plant cells is 10 to 1000 times less (2 mg O_2/L) than for microbial organisms. Increased aeration rates (0.4 - 1.5 VVM), thus increased k_La values (30 - 50 h^{-1}), failed to optimize the growth rates

of *C.roseus* plant cells (0.055 - 0.03 g/L·h) in a 10-L airlift reactor[69]. At a higher rate (0.86 VVM) *C.roseus* growth was repressed[66]. Many researchers believed that oxygen toxicity, at high aeration rates, led to diminished plant cell growth[66,69-72]. Later, it was found that at high aeration rates, essential volatile cell surface gases, primarily carbon dioxide, were stripped off[5,6,73]. These findings were corroborated when 2 - 5% CO_2 supplementation to the air supply enhanced *C.roseus* cell growth in a 7-L airlift[70,72].

IMMOBILIZATION OF PLANT CELL CULTURES

Immobilization techniques were conceived in the 1970's to circumvent the problems associated with batch suspension cultures[24,27,35,74-77]. The rationale for turning to immobilized cultures was based on factors related to the non-newtonian behavior of the viscous plant cell suspensions. Immobilized plant cells could be mechanically stabilized[23,77] from the turbulent shear stresses encountered in conventional stirred tank reactors. From a physiological standpoint, the cells could be subject to increased intercellular contact, thus accommodating their tissue-forming tendency. Segregation of the biomass and medium could enhance the productivity of the culture system with repetitive medium changeovers (fed-batch or continuous), thus permitting the re-use of the biomass and lowering the cost of inoculum preparation[8,23,24,26,27,36,75,76].

If the immobilized plant cells could be induced to release their secondary metabolites to the medium[41,42,78], production could conceivably be increased by in situ product adsorption using neutral polymeric adsorbents (ie. amberlite, activated charcoal, chitosan)[74,79,80]. On-line product removal would relieve feedback inhibition and possibly toxic effects of the

secondary metabolites on the cells[74,79,81-83].

Three types of immobilization methods were devised. Plant cells were first entrapped[26,27,36,76,84] in calcium alginate beads and in foam cubes (1 cm^3)[25,26]. Plant cells were chemically cross-linked to solid carriers as medium washed through them yielding nutrients and carrying toxic products away from them[22,60]. Adhesion was the gentlest technique where plant cells attached to membranes, tubes and cartridges[22,60,76].

These techniques however, were not problem-free as difficulties were observed in each. Cell leakage frequently occurred as expanding plant cells burst out of their gel beads. Oxygen mass transfer limitation occurred when the bead diameters exceeded 3 mm. The same problem was encountered by plant cells which were entrapped in foam cubes. Microenvironments formed and necrosis occurred for these compromised plant cell cultures[6,22,26,36]. Chemical cross-linking (with glutaraldehyde) of plant cells to solid supports was the least preferred technique due to the toxicity and harshness of the treatments[22,26,27,74-76].

Surface immobilization exploits the adhesion of plant cells onto an inert fibrous geotextile[22]. Bioreactors of 2 and 6-L capacity were designed. This immobilization technique segregates the plant cell biomass from the medium, giving morphological and physical stability to the cells. Unlike the entrapped cells and batch suspension cultures, the surface immobilization technique allows controllable mass transfer (of oxygen and nutrients), thus controllable plant cell growth. Repetitive medium changes can be effected while product release stimulation[41,42] and on-line product removal can be potentially exploited from this system[22,36].

1.4.5. INDOLE ALKALOIDS FROM *CATHARANTHUS ROSEUS*

It has proven impossible to date[7,14], to produce the highly valued dimeric alkaloids, vincristine and vinblastine, by plant cell cultures[8]. However there have been reports of vinblastine production in differentiated callus cultures[55,56,85]. One of the monomers that comprises half of vincristine and vinblastine, catharanthine, has often been isolated by in vitro culture techniques. The other monomeric half, vindoline, has not been found in undifferentiated plant cell cultures[5,56] for it required morphologically-related enzymes for its conversion[14,55]. Other indole alkaloids (Figure 1.0) such as ajmalicine and serpentine have been more frequently isolated in *C.roseus* cultures[7,14].

Chemical synthesis of the dimeric alkaloids had proven to be difficult in light of the complex stereochemistry of the molecules. Differences between any 2 alkaloids can be as subtle as the spatial arrangement of a chemical group which can only be discerned by product-specific enzymes[9,86]. Plant tissue and cell cultures were used to obtain the enzymes to elucidate the regulation of indole alkaloid synthesis in *C.roseus*[87-92].

Numerous enzymatic studies have partially revealed the biosynthetic pathway of indole alkaloids belonging to the genus *Catharanthus* and 8 families[18,28,29] of alkaloids were classified (Figure 1.2, eburnan family not shown). The amino acid tryptophan becomes decarboxylated to tryptamine via tryptophan decarboxylase (TDC) when culture conditions become growth-limiting. The enzyme appears to be inactive until phosphate concentrations fall below 10 mM[33,62,93-95]. Acetyl-CoA condenses to mevalonic acid which is further converted to secologanin[9]. Tryptamine and secologanin condense together by strictosidine synthase

(SS)[28,91,96] to become strictosidine (Figure 1.0) which is the principle alkaloid that precedes all the other indole alkaloids[87]. It is usually identified in cultures as strictosidine lactam due to the extraction procedure[28,97]. It usually constitutes a large portion (ca. 60%) of the alkaloids extracted from *C.roseus* plant cell cultures[22,28,pers. comm. Dr. R. Tyler, PBI].

The corynanthé alkaloids (ie. ajmalicine, serpentine) are synthesized after strictosidine through a reduced pyridine nucleotide-dependent reaction involving NADH or NADPH. If these cofactors are absent then ajmalicine, serpentine and yohimbine cannot be synthesized and an intermediate, cathenamine, accumulates instead[9,14,87,89,90,98,99]. In intact plants the corynanthé alkaloids are found in the roots[14]. These alkaloids accumulate to peak concentrations (as a form of feedback signalling) before subsequent alkaloid transformations occur[18,28,29]. After the corynanthé alkaloids peak, strictosidine is shunted, via geissoschizine, to produce preakuammicine and stemmadenine of the strychnan family. Following the appearance of stemmadenine, the aspidospermatan and ibogan alkaloids are synthesized[9,28] (Figure 1.2).

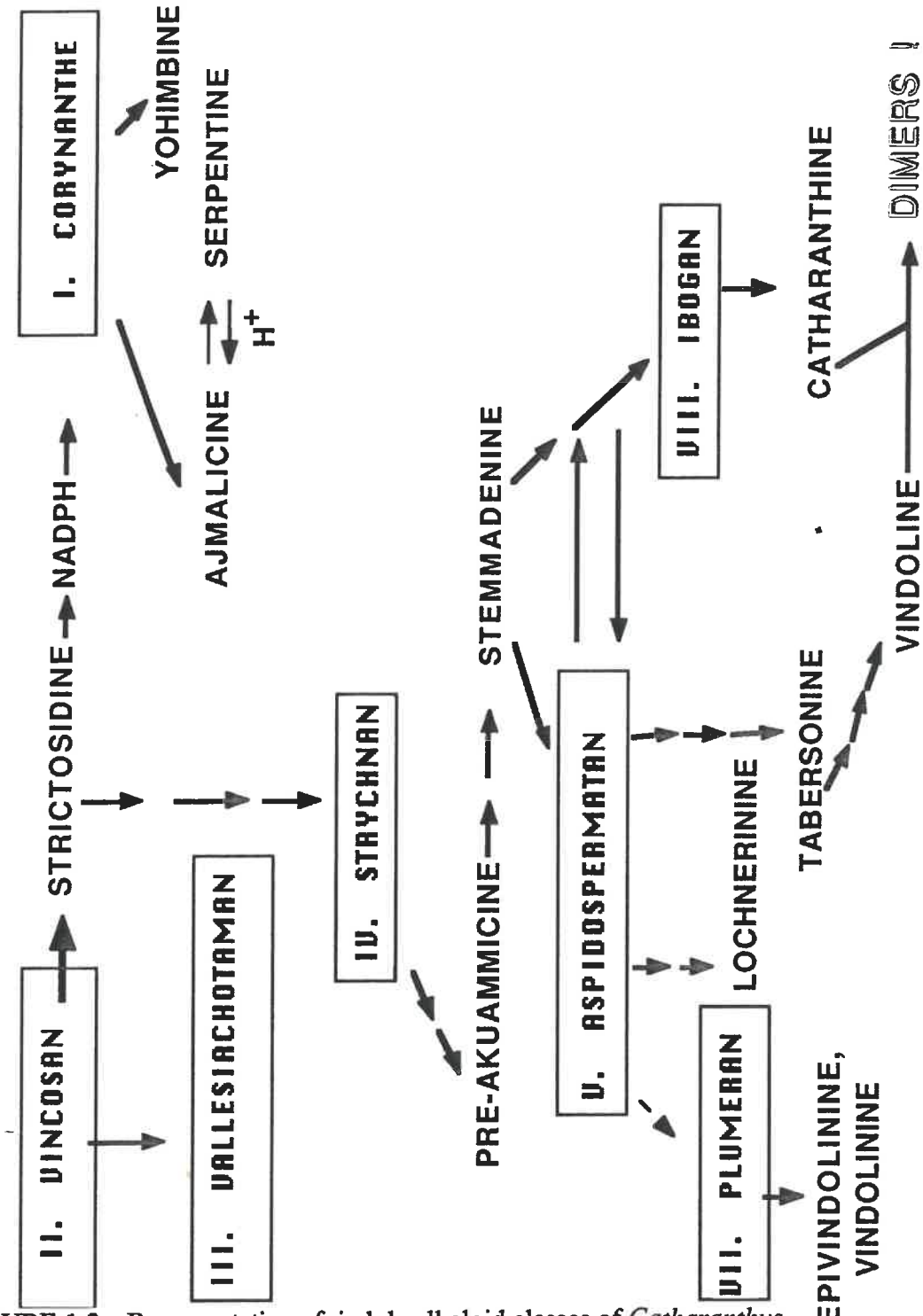


FIGURE 1.2 Representation of indole alkaloid classes of *Catharanthus roseus.*

Tabersonine (300 $/kg) is the commonly found alkaloid which is the closest related product to vindoline. To date, the conversion of tabersonine to vindoline has rarely been observed in plant cell cultures[14,55]. Etiolated (pale instead of green) seedlings of *C.roseus*, that were grown in the dark and then exposed to light, revealed the presence of a chloroplast-associated enzyme, N-methyl transferase (NMT). N-methyl transferase is the fourth enzyme in a 6-enzyme series that transforms tabersonine into vindoline. This enzyme is not present in *C.roseus* suspension cultures thus tabersonine often accumulates without conversion into vindoline. The 2 enzymes after NMT are absent from *C.roseus* suspension cultures also. All the other enzymes leading to tabersonine and preceding alkaloids were isolated from the cytosol of *C.roseus* cells grown in suspension cultures[14,63,64,92,100].

1.4.6. PRODUCTION TECHNIQUES

In vitro secondary metabolite production depends on the cultural environment and how it provokes the genetic make-up of the plant cells[7,29]. When cells are generated from certain plant tissues they may or may not[11,59] engender the capacity to produce secondary metabolites. Plant cells are notorious for their genetic instability and they are often heterogenous with respect to their metabolic and productive capacities, even if they originate from the same explant[7,58]. Productivity can be lost over repetitive sub-culturing[20,57].

CELL LINE SELECTION

Screenings of more than 2000 plant cell lines generated from *C.roseus* plants revealed that production varied between cell lines with respect to alkaloid family representation as well as yields. Maximal alkaloid yields found in *C.roseus* suspensions ranged from 0.1 to 1.5%dw (1 to 15 mg/g dw) while 32% of the cell lines generated produced no alkaloids at all[29]. Screening and selection techniques[101] for stable high yielding *C.roseus* cell lines resulted in yields that exceeded those of the plants by 4-fold[20] for ajmalicine and serpentine.

PRODUCTION MEDIA

In addition to selection of high-producing variants, other studies were concerted on increasing the plant cells' productivity via chemical control of the cultures[21,31,102]. Combined nutritional and hormonal factors were found to enhance secondary metabolite synthesis[20,34,38] by *C.roseus* suspension cultures[20,31,102-107].

Zenk et al[20] formulated the classical alkaloid production medium (APM), which differed from basal plant cell growth media, with respect to carbohydrate and mineral concentrations and phytohormone composition. Sucrose was found to be the preferred carbohydrate/energy source to promote growth[50,103,104] and secondary metabolite production by plant cells[20,38,45,47-49,62]. Elevated sucrose concentrations of 50 to 80 g/L[20,45] (as opposed to 20 to 30 g/L for growth) generally increased ajmalicine and serpentine yields by 9 and 4-fold respectively[7]. Increased sucrose concentrations in the production medium also led to higher biomass yields and increased alkaloid yields[7,20].

Phosphate is rapidly taken up and stored by plant cells in the first few days of growth

in order to build energy sources (ATP), membrane components and nucleic acids[7]. In the presence of inorganic phosphate, plant cells generally concentrate their energies toward growth activities. Sasse et al.[62] formulated phosphate-deficient media which induced 15-fold increases in ajmalicine yields.

Mineral nitrogen (nitrate and ammonium) depletion from the medium coincided with decreasing growth activities and entry into the stationary phase. Nitrate-ammonium ratio values less than 1 were correlated to the onset of alkaloid production as *C.roseus* cells entered the stationary growth phase[7,51].

Depending on the balance of growth auxins and cytokinins in the medium, the phytohormone composition was found to induce or repress certain metabolic acitvities, in addition to the degree of morphological differentiation of the plant cells[20,108,109], The synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4D) promotes culture growth for most plant species[50,110] but it is not catabolized by *C.roseus* cells. Zenk's production medium[20] replaces 2,4D with the natural growth auxin indole acetic acid (IAA) since the former strongly inhibits alkaloid production[32,73,111-115]. It was speculated that 2,4D exerted its inhibitory effect by blocking an enzymatic step in the mevalonate pathway leading to secologanin[21]. Higher ajmalicine and serpentine yields were observed in 2,4D-deficient media[116]. A concentration of 1 μM IAA and 5 μM of the cytokinin 6-benzylaminopurine (BAP) was established in Zenk's APM so cell growth and alkaloid production could be sustained in the same medium[20]. Abscisic acid (ABA) was found to increase ajmalicine and catharanthine accumulation when it was added to *C.roseus* suspension cultures[113]. Table 1.1 shows how ajmalicine and serpentine yields were affected by different media compositions

for different *C.roseus* cell lines.

TABLE 1.1 COMPARISON OF AJMALICINE AND SERPENTINE YIELDS IN DIFFERENT STRAINS OF *C.ROSEUS*.

CELL LINE	AJMALICINE (mg/g dw)	SERPENTINE (mg/g dw)	CULTURE CONDITIONS	REF.
953	-	-	batch-suspension	28
		-	shake flask	"
200GW	0.06	-	suspension	"
N/A	0.60	-	induction media[1]	62
C20	0.80	0.38	suspension	51
N/A	1.0	0.34	3.5-L batch[2]	116
N/A	0.46	0.22	3.5-L/1B52	"
C11C	-	2.0	250 mL SF (APM)[4]	111
	-	0.08	250 mL SF/1B5[3]	"
C87	-	1.7	250 mL SF/M3[5]	"
N/A	-	2.5	10-L airlift	8
N/A	0.01	1.17	SF/MS[6]	20
A-STRAIN	10.2	2.96	SF/APM	"
S-STRAIN	-	8.10	SF/APM	"

1. induction medium with no inorganic phosphate.
2. Gamborg`s[50] 1B5 medium containing 50 g/L sucrose.
3. Gamborg`s[50] 1B5 medium containing 20 g/L sucrose.
4. Zenk`s[20] alkaloid production medium.
5. modified Murashige and Skoog production medium.
6. Murashige and Skoog growth medium., SF = shake flask-grown suspension culture.

ENVIRONMENTAL CONDITIONS

The degree of secondary metabolite synthesis by plant cell cultures depends on how the environment affects the plant cell, even if a productive cell line and production medium have been selected. External factors such as pH, temperature and light influence secondary metabolite synthesis by plant cell cultures.

The culture pH is indicative of the culture conditions, whether the plant cells are assimilating nutrients or are lysing[7,34]. Alteration of the culture pH (via alkalinization or acidification) has been exploited in order to trick the plant cells into releasing their vacuolarly-bound products to the medium[41,42,117].

Temperatures ranging from 20 to 28°C were found to favor plant cell growth[7,61,147]. *C.roseus* cultures grow optimally at 28 °C while they die at 35 °C. When the temperature was decreased to 16 °C growth was strongly inhibited while indole alkaloid accumulation appeared to be 3-fold higher than at the temperatures favoring growth[7,46,112]. However, equivalent alkaloid accumulation could be attained at elevated temperatures when nutritional and osmotic factors were exploited without compromising biomass growth[112].

Plant cell cultures are considered heterotrophic for they cannot grow without an exogenous carbohydrate source[20,57-60]. The effects of light on secondary metabolism in plant cell cultures is varied[5,21,45]. The monomer vindoline and the dimers vinblastine and vincristine are usually located in *C.roseus* leaves but none of them have been found in suspension cultures. Unlike heterotrophic cells, photoautotrophic plant cells contain chloroplasts which allow them to use light to transform carbon dioxide into utilisable carbohydrates (photosynthesis) to grow on[52]. However a photoautotrophic *C.roseus* suspension culture at

the Plant Biotechnology Institute (PBI) did not produce vindoline nor the dimeric alkaloids[14, Dr. R.Tyler, pers.comm.] Light exposure was found to inhibit catharanthine production in a heterotrophic *C.roseus* cell line[118] yet for another it was a potent effector on serpentine production in medium-induced cultures. It was suggested that light exposure was beneficial for indole alkaloid and anthocyanin production in heterotrophic cultures for it blocked the inhibitory effects of phosphate and mineral nitrogen on alkaloid synthesis[7,60,94]. Ajmalicine, the biogenetic precursor of serpentine, is usually a root alkaloid. Table 1.2 shows how ajmalicine yields are affected by light in differentiated and undifferentiated *C.roseus* cultures[119].

TABLE 1.2 AJMALICINE PRODUCT YIELDS IN DIFFERENTIATED PLANT TISSUE CULTURES OF *C.ROSEUS* CULTIVAR LITTLE DELICATA[119]

CULTURE CONDITIONS	AJMALICINE YIELD (mg/g dw)
LEAF	0.03
STEM	0.01
ROOT	0.34
SHOOT/DARK/SC#1	0.46
SHOOT/DARK/SC#2	0.62
SHOOT/LIGHT	0.10
ROOT/DARK	0.30
SUSPENSION/LIGHT	0.22
SUSPENSION/DARK	1.15

1.4.7. ALTERNATIVE PRODUCTION METHODS

Alternative induction schemes to improve secondary metabolite synthesis and productivity by plant cell cultures were evaluated. Two plant bacteria, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, have been employed to infect plant cells in a process called transformation. After a successful infection with one of these bacteria, the plant cells would have received a foreign plasmid that conferred an uncontrollable growth characteristic to the cells (ie. cancer-like growth). They become prototrophic which means that they produce their own hormones and no longer need external supplementation[7,88]. Infection by the latter bacterium induces them to produce adventitious outgrowths known as hairy roots. It was believed that the plant cells, with the added morphology (hairy roots), would be more productive[85,119]. Hairy root *C.roseus* cultures were reported to produce about 1 μg/g dw of vinblastine and related dimers[85]. Intact *C.roseus* plants produce about 5 μg/g dw of vinblastine[7].

There are however, a few draw-backs in working with transformed plant cell cultures. It is difficult to obtain successful infection and transformation of callus cultures and they do not necessarily produce higher product yields as compared to intact plant organs (Table 1.2). Large-scale cultures of transformed cultures would be difficult to realize due to mixing and mass transfer problems related to filamentous cultures[12,56].

Elicitation is a species-specific, invader-host interaction where upon microbial or fungal infection, the plant cell defends itself by producing molecules called phytoalexins, in order to kill the intrusive species[120]. The elicitation process is relatively new (since 1980) and it

is not well-understood.

An elicitor, which is often comprised of a fungal homogenate (ie. *Pythium aphanidermatum*), is added to the plant cell suspension culture to induce secondary metabolite synthesis. Product spectra and yields are affected by many factors: the elicitor concentration, exposure duration, age of the plant cell culture and the type of fungal species used[120-122]. *C.roseus* cells produced phenolics, which browned the culture, and higher ajmalicine, tabersonine and catharanthine yields (amongst other alkaloids), 18 hours after exposure to a homogenate of *P.aphanidermatum*[120,122]. Forty-two percent of the ajmalicine produced was recovered from the medium after elicitation[7]. Elicitation does not necessarily kill the cells[120-123] and it is possible to re-elicit the culture[120].

It has been suspected that the components of the fungal cell wall are ß-glucans which exert their effect through a receptor-mediated response to induce the secondary metabolite enzymes (ie. TDC and SS)[124]. Elicitors such as *Pythium aphanidermatum*[120-122], nigeran of *Aspergillus niger*[126], and oligosaccharins[125], are categorized as biotic elicitors[126].

Abiotic elicitors include a variety of basic salts such as potassium salts, vanadate salts[126], and metallic ions[123]. It has been suggested that these elicitors induce secondary metabolite synthesis by changing the nature of the plasma membrane with respect to permeability and electrochemical gradient[123,126,127].

CHAPTER II

2.0. MATERIALS AND METHODS

2.1. MAINTENANCE OF THE PLANT CELL LINE

From the leaf of a Periwinkle plant (*Catharanthus roseus*) cell line MCR17 was generated in 1984[22]. Since its initiation, the cell line was maintained as a suspension culture in Gamborg's[50] basal growth medium 1B5 (Appendix I) containing 4.5 μM of the growth auxin 2,4 dichlorophenoxyacetic acid (2,4D) and 20 g/L of sucrose. The cells were transferred on weekly intervals at 10% (V/V) ratio into 200 mL of 1B5 in 500 mL DeLong flasks. They were agitated on rotary shakers operating at 150 RPM under constant illumination at 28 °C. These were the same operating conditions (agitation, temperature) used for 1 and 2-stage shake flask experiments. All media (growth and production) were adjusted to pH 5.5 with 0.1 N KOH and then were autoclaved for 60 minutes, at 121 °C and 15 psi throughout the duration of the project.

2.2. ALKALOID PRODUCTION REGIMES

2.2.1. THE 1-STAGE REGIME

The 1-stage regime consisted of inoculating MCR17 cells at a 20% (V/V) ratio, from 200 mL 1B5-grown suspensions, into 200 mL of Alkaloid Production Medium[20] (APM, Appendix II) in 500 mL DeLong flasks. The inoculum consisted of late exponential phase cells aged at 5 or 6 days at 12 g dw/L. This 1-stage process lasted for 15 days.

2.2.2. THE 2-STAGE REGIME

The 2-stage regime consisted of the 15-day production stage in APM which was preceded by an additional 6-day growth stage in a modified basal growth medium, AB5, instead of 1B5. The former growth medium contained 1 μM of the natural growth auxin indole-3-acetic acid (IAA) instead of 2,4D; the latter auxin was known to inhibit alkaloid production[113]. The IAA concentration was arbitrarily chosen to be the same as that used in the APM composition. The duration of the growth stage (6 days) had been determined in preliminary studies using immobilized cultures.

To 200 mL of AB5 medium in 500 mL DeLong flasks, a 10% (V/V) inoculation of 1B5-maintained cells (also at 12 g dw/L) was done to start the 6-day growth stage. On the 6th day about 90% of the AB5 medium was aseptically decanted from the cells and was replaced by APM for the 15-day production stage. Experiments for each production regime were done in 4 replicate shake flasks where the average values varied by ±10-30%. Table 2.0 shows the principle differences between the media 1B5, AB5 and APM, with respect to hormone, carbohydrate and mineral concentrations.

TABLE 2.0

PRINCIPAL SUBSTRATES & HORMONES	1B5[1]	APM[2]	AB5
HORMONE (μM)	4.5 2,4D	1.0 IAA	1.0 IAA
SUCROSE (g/L)	20	50	20
NITRATE (mM)	25	9.4	25
AMMONIUM (mM)	1.0	9.0	1.0
INORG. PHOSPHATES (μM)	1.1	0.50	1.1

1: O.L. GAMBORG, 1967

2: M.H. ZENK, 1977

2,4D: 2,4 DICHLOROPHENOXY ACETIC ACID

IAA: INDOLE ACETIC ACID

2.3. SURFACE IMMOBILIZATION OF PLANT CELLS

2.3.1. THE SIPC BIOREACTOR

The Surface Immobilization of Plant Cells (SIPC) in a 2-L bioreactor was performed according to Archambault et al[22]. The principle features of the reactor are presented in Figure 2.1 while Figure 2.2 depicts a top view of the bioreactor configuration. An inert geotextile (#7605 of Texel Inc.) of about 0.16-0.17 cm width was cut into strips of 90 x10 cm to yield an average immobilization area 1845 ±135 cm^2. The strip was wound around a stainless steel cage (12.5 x12.5 x10 cm) into a spiral configuration. The matrix-covered cage was placed into a 2-L glass jar with a conical profile bottom. Associated accessories consisted of a glass cover, holding clamp, a viton rubber ring seal, a sintered glass air sparger connected to a glass wool air filter, a magnetic stirring bar (7.7 x13 cm), and a glass sampling tube. The reactor was filled with 1.2 L of medium and was sterilized at 121 °C, 15 psi for 1 hour.

The sterile bioreactor was inoculated at 10 to 20% (V/V) of its working volume and additional sterile medium was added to fill the reactor to an initial volume of about 1.9 L. A sterile glass Friedrich condensor was added to the bioreactor to minimize evapouration. To the exterior wall of the bioreactor a surface resistance temperature probe[128] was attached (not shown in figure) which was connected to a proportional integral derivative (PID) regulator (Model TC300 by Viconics Inc.) controlled by the heating module of the magnetic stirring plate (Thermolyne, Sybron Inc.).

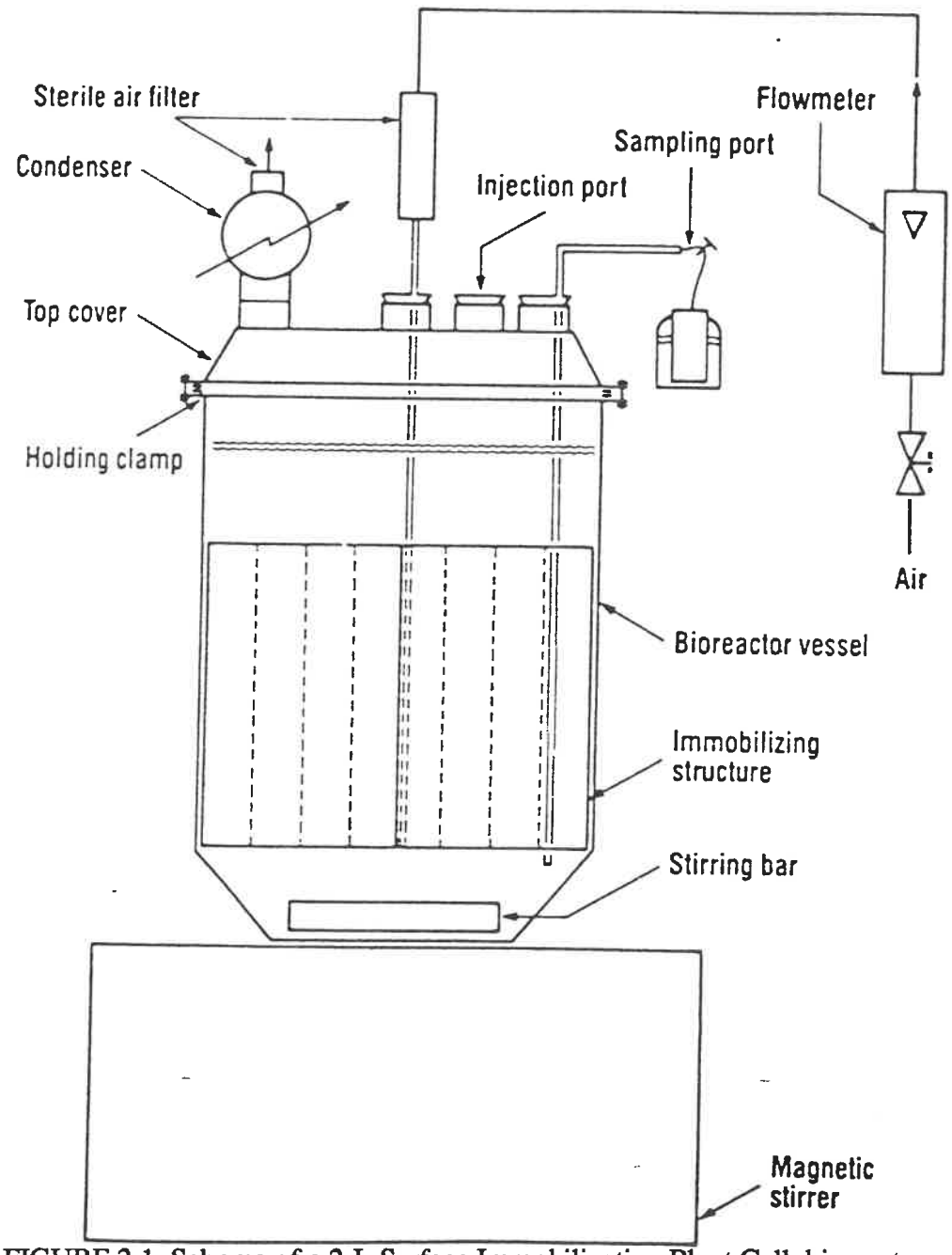


FIGURE 2.1 Schema of a 2-L Surface Immobilization Plant Cell bioreactor.

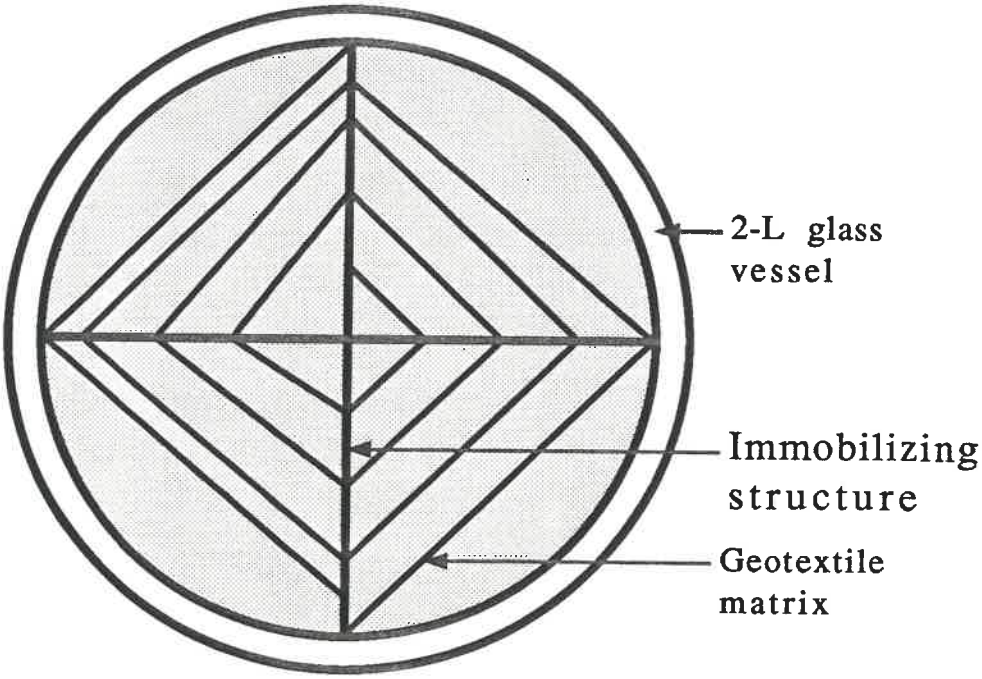


FIGURE 2.2 Top view of the 2-L surface immobilization plant cell bioreactor.

VESSEL

Inside diameter:	13 cm
Internal height:	20 cm
Max. liquid volume:	1.9 L (incl. matrix)

IMMOBILIZING STRUCTURE

Height:	10 cm
Geotextile thickness:	0.15 cm
# of spiral turns:	4

Agitation was sustained at 300 RPM which corresponded to a Reynold's impeller number of 29460 and an impeller tip speed of 120 cm/s. Air was supplied at about 0.05 to 0.07 VVM (k_La 5-7 h^{-1})[37] through the glass sparger and the immobilized cultures were constantly illuminated.

The k_La, being the oxygen mass transfer capacity of the culture system to the cells, was not measured in this project since it had been previously determined by Archambault et al[37]. The oxygen uptake rate, defined by OUR = QO2*X, could not be determined at the time the experiments were done. Ther was no oxygen probe included nor a dissolved oxygen controller designed for this particular culture system at the time. Moreover, the immobilized plant biomass concentration, X could only be measured at the end of the culture, thus rendering continuous biomass and OUR measurements difficult to determine for this immobilized culture technique.

2.3.2. ALKALOID PRODUCTION IN SIPC CULTURES

The 2 alkaloid production regimes were tested in 2-L SIPC cultures as was done for the 0.2 L shake flask-grown suspsension cultures. For the 1-stage regime the 2-L SIPC bioreactor, containing APM medium, was inoculated at approximately 20% of its initial volume (V_i) with 2-200 mL suspension cultures that were raised in 1B5; the inoculum cells were similarly 5-6 days old at about 12 g dw/L for all experiments. Five bioreactors were parallely inoculated and were dismantled every 3 days (for up to 15 days of production) for biomass determination, nutrients consumption and alkaloid content. Medium samples were

withdrawn from all the bioreactors (ca. 25 mL) every 2 days after inoculation to obtain intermediary points on the evolution of the pH, extracellular nutrients and conductivity changes, for the SIPC experiments.

As for the 2-stage shake flask experiments, the 2-L SIPC bioreactors containing growth media were inoculated at 10% of their initial volume (V_i) and the cells grew for 6 days to comprise the growth stage; sampling and harvesting were done similarly as previously described for the 1-stage SIPC cultures. On the 6th day about 90% of the growth media was aspirated out of the bioreactor and aseptically refilled with APM to initiate the production phase of the 2-stage regime. Seven bioreactors were parallely inoculated so that points could be obtained for the 6-day growth stage and 15-day production stage where a reactor was dismantled every 3 days.

2.4. BIOMASS DETERMINATION

The suspension cultures were sampled every 2 or 3 days (ca 40 mL). Suspended cells were separated from the medium by vacuum filtration (5 micron Whatman filter) and they were rinsed with distilled water. For biomass determination, 10 mL of the sample was treated by drying the separated cells in an oven (60°C, 24 h) to a constant weight. The rest of the separated cell sample was sacrificed for alkaloid content determination.

The residual medium sample was assayed for pH (Fisher Model 805MP) and conductivity using a Yellow Springs Instrument (YSI) glass dip cell connected to a YSI model 35 conductance meter. The nitrate ion content was quantified with an Orion nitrate electrode

(model 930700) connected to the same pH meter. Samples were diluted with 2 or 3 equal parts of distilled water to make a final volume of 4 mL and then they were treated with 80 μL of nitrate ionic strength adjustor (Orion 930711) prior to measurement. Ammonium ions were quantitated using an Orion ammonium ion electrode (model 951205) coupled to the same pH meter. Samples (2 mL) were treated with 40 μL of ammonium pH adjusting solution (Orion 951007) prior to measurement. Nitrate and ammonium ion concentrations as low as as 0.01 mM could be detected. The inorganic phosphate content was quantitated according to the ascorbic acid-phosphate analysis method described in the Standard Methods for the Examination of Water and Waste Water[129]. Samples (200 μL) were diluted with 4.8 mL of distilled water and treated with the ascorbic acid reagent. Absorbance readings were taken at a wavelength of 880 nm using a Varian UV-Visible DMS100 spectrophotometer.

Carbohydrates (sucrose, glucose, fructose) were measured by injecting 25 - 50 μL of pre-filtered (0.45 micron Millex) media samples into a Waters HPLC system equipped with: model U6K injector, model 590 pump, model 410 refractometer, and a Biorad Aminex carbohydrate column (HPX-87C). The column temperature was 80 °C and the eluant was pre-filtered (0.45 micron Millex) double distilled water. The flow rate was 1.0 mL/min. with a back pressure of 900 to 1300 psi. Readings were enabled by a Spectra Physics SP 4270 integrator.

2.4.1. BIOMASS DETERMINATION OF SIPC CULTURES

Media samples that were withdrawn from the SIPC bioreactors during the course of the cultures were assayed as previously described in section 2.4. The dry biomass concentration of SIPC cultures were determined according to Archambault[22]. At the end of each culture run the immobilization cage, loaded with plant cell biomass, was drained of excess medium and it was weighed (W_T) prior to extraction for alkaloids. The biomass was dried on the immobilization structure and matrix in an oven at 60 °C, 24 h to a constant weight. The net amount of immobilized biomass (W_I) raised in the bioreactor was calculated by subtracting the weight of the matrix-covered cage (W_{mc}) from the weight of the wet biomass-loaded structure (W_T). The weight of the liquid retained by the matrix was subtracted from W_T also according to Equation 1.

$$W_I = W_T - W_{mc} - 0.92V_m \qquad (1)$$

V_m = volume of matrix

0.92 = void volume of matrix[22]

The corresponding quantity of dried biomass was determined by dividing W_I by the wet-to-dry ratio of 32.9 that had been determined by Archambault[22].

2.5. INDOLE ALKALOID ANALYSIS

2.5.1. INDOLE ALKALOID EXTRACTION

Indole alkaloids were extracted from the biomass and medium according to a liquid-liquid partitioning method of Kutney et al.[28]. The immobilized biomass was immersed into 1.7 L of hot methanol (MeOH) at 53°C, 45-60 min. to permeablize the cells. The resultant methanolic extract was evaporated (Buchi rotoevaporator) and the residue was twice extracted with equal volumes (50 - 75 mL) with 1N hydrochloric acid (HCl,aqueous phase) and ethyl acetate (organic phase). The organic phase was acidified to pH2 and was twice extracted with equal volumes of 1N HCl. These steps permit separation of cell constituents from the alkaloids. The aqueous phases were pooled together and alkalinized to pH10 with 10 N sodium hydroxide (NaOH). The indole alkaloids were recuperated by extracting the aqueous phase with ethyl acetate (EtAc) twice. The resultant EtAc phase was evaporated and the resultant residue was dissolved in a solution of EtAc and MeOH (1:1) yielding about 2 - 5 mL of concentrated alkaloid extract.

Alkaloids from the medium were extracted by alkalinizing the medium with 10 N NaOH and extraction (2x) with EtAc. The resultant organic phase was evaporated and the resultant residue was recuperated in 2 - 5 mL of EtAc and MeOH.

2.5.2. TOTAL INDOLE ALKALOIDS

Three methods were used to quantify the indole alkaloids produced. By using a method described in Lee et al.[30], measurements at 280 nm roughly revealed the amount of UV-absorbing species (the indole moiety) present in the biomass and media extracts. Proteins have been measured at 280 nm but after the extraction procedure, no cellular proteins are present in the alkaloid extracts. In Table 2.1 the concentrations of the 14 available alkaloids that comprised the standard mixture to obtain the total indole alkaloid (TIA) contents of the biomass and media, while Table 2.2 shows the absorbances of various dilutions of the mixture at 280 nm in the Varian spectrophotometer. The absorbance was linearly correlated to varying concentrations of the mixture ($r = 0.99$) and the TIA content (mg/kg solution) of the biomass and media extracts could be correlated by Equation 2.

$$TIA = 0.64*AU + 0.0288 \qquad (2)$$

The alkaloid standards were generously provided by Mr. Brock Chatson at PBI in Saskatoon, Canada, except for vincristine and vinblastine (Sigma, St.Louis, Missouri).

TABLE 2.1 COMPOSITION OF THE STANDARD INDOLE ALKALOID SOLUTION FOR TIA MEASUREMENT.

INDOLE ALKALOID	CONCENTRATION (mg/kg solution)*
TRYPTAMINE	51.62
STRICTOSIDINE LACTAM	54.69
AJMALICINE	52.25
SERPENTINE	3.35
YOHIMBINE	59.38
TABERSONINE	72.19
LOCHNERININE	19.25
19-EPIVINDOLININE	34.43
VINDOLININE	37.32
VINDOLINE	35.06
CATHARANTHINE	63.63
VINBLASTINE	34.31
VINCRISTINE	33.06
VINCADIFFORMINE	53.25

*Solution was a mixture of the alkaloid standards dissolved in 1:1 methanol and ethyl acetate.

TABLE 2.2 INDOLE ALKALOID SOLUTIONS AND UV ABSROBANCES MEASURED AT 280 nm USED FOR TIA DETERMINATION.

TIA SOLUTION CONCENTRATION (mg/kg solution)	ABSORBANCE (relative units)
1.20	1.790
0.60	0.920
0.37	0.558
0.30	0.448
0.15	0.128

2.5.3. THIN LAYER CHROMATOGRAPHY

Qualitative identification of the alkaloid spectra was done according to the method of Farnsworth et al[130]. Extract samples (25 - 250 μL) were applied to J.T. Baker silica gel plates (#7001-00) that were pre-dried in a 60 °C oven for about 10 minutes. The origin for sample application was marked 4 cm from the bottom of the plate and the elution path was about 12 cm. Sample spot application was kept to about 5 mm in diameter and dried with oxygen-free nitrogen prior to development. The plates were put into an elution tank, filled to about 2 cm with 10% (V/V) methanol in ethyl acetate which was previously dried with anhydrous sodium sulfate. After 20 to 30 minutes the solvent front was marked in pencil and the plates were dried in a 60 °C oven for about 2 minutes. With the aid of a UV lamp at 254 nm the alkaloid components were marked to calculate their R_f values according to Equation 3.

$$R_f = \frac{\text{distance of component (cm)}}{\text{distance of solvent front (cm)}} \quad (3)$$

A developer solution of 1% (W/W) of ceric ammonium sulfate (CAS) in syrupy o-phosphoric acid was sprayed onto the plates to identify the alkaloids according to their chromogenic reactions[130].

2.5.4. HIGH PRESSURE LIQUID CHROMATOGRAPHY OF ALKALOIDS

The biomass and media extracts were injected (10-100 μL) into a Waters HPLC system (model 590 pump, model 680 automated gradient controller, model 490E UV detector, model U6K injector) equipped with a Brownlee Labs spheri-5 RP-8 column to quantify and compare the alkaloids with the available standards. The column temperature was 48 °C and the flow rate was 2 mL/min. with a back pressure of 3000 to 5000 psi. The detection wavelength was 254 nm and a linear recorder was coupled to the UV detector to detect absorbances at 280 and 330 nm in order to facilitate tabersonine and lochnerinine identification. Readings were enabled by a Spectra Physics integrator SP4270.

Preliminary studies were done under isocratic flow conditions using an eluant composition of 60% methanol with 2.5 mM of the pairing ionic reagent PIC-A by Waters. For the last half of the project alkaloid identification was facilitated by adopting a linear gradient HPLC method (starting at 55% methanol) according to Verzele et al[131]. All eluants were pre-filtered at 0.45 micron with Millipore filtres (#HV047).

CHAPTER III

3.0. RESULTS

3.1. INITIAL GROWTH STUDIES OF *Catharanthus roseus* CELL CULTURES

The growth and nutrient consumption patterns of *C.roseus* suspension cultures (200 mL) in 2 different growth media (1B5 and AB5), and in production medium APM were studied in 500 mL shake flasks (Figures 3.1 and 3.2). Growth characteristics summarized in Table 3.1 show that the cells grew slightly faster in AB5 than in 1B5 (0.46 vs 0.36 d^{-1}). The high sucrose concentration in APM (50 g/L) may have exerted a higher osmotic pressure on the cells and consequently slowed their growth rates relative to those in B5 media with 20 g/L sucrose. Furthermore, the lower availability of nitrate in APM (18mM) probably contributed to growth rates that were lower than found in the B5 media (25 mM). However, the increased sucrose in APM contributed to higher biomass concentrations and yields than in B5 media (Table 3.1). In APM the cells grew slower(0.28 d^{-1}) than in B5 but they attained the highest biomass concentration at 22 g dw/L.

Figure 3.1 shows that more biomass is produced in APM (22 g dw/L) in the presence of higher carbohydrate concentration (~5% w/v), as opposed to the B5 media (11 - 13 g dw/L in ~ 2% w/v). It would seem that APM would serve suitably as a growth medium but it is poorer than the B5 media in nitrate, thus it manifests a slower specific growth rate than the 1B5 medium. The reason APM was designated to be a production medium, rather than a growth/maintenance medium, was due to its carbon-to-nitrogen content (2.24 in B5, 7.74 in APM) which was not favorable to maintain growth, as compared

to B5, during repetitive sub-culturing. The cells would become lower in nitrogen and higher in carbohydrate constituents, thus leading to higher starch content which could increase cell agglomeration in the presence of increased polysaccharide secretion.

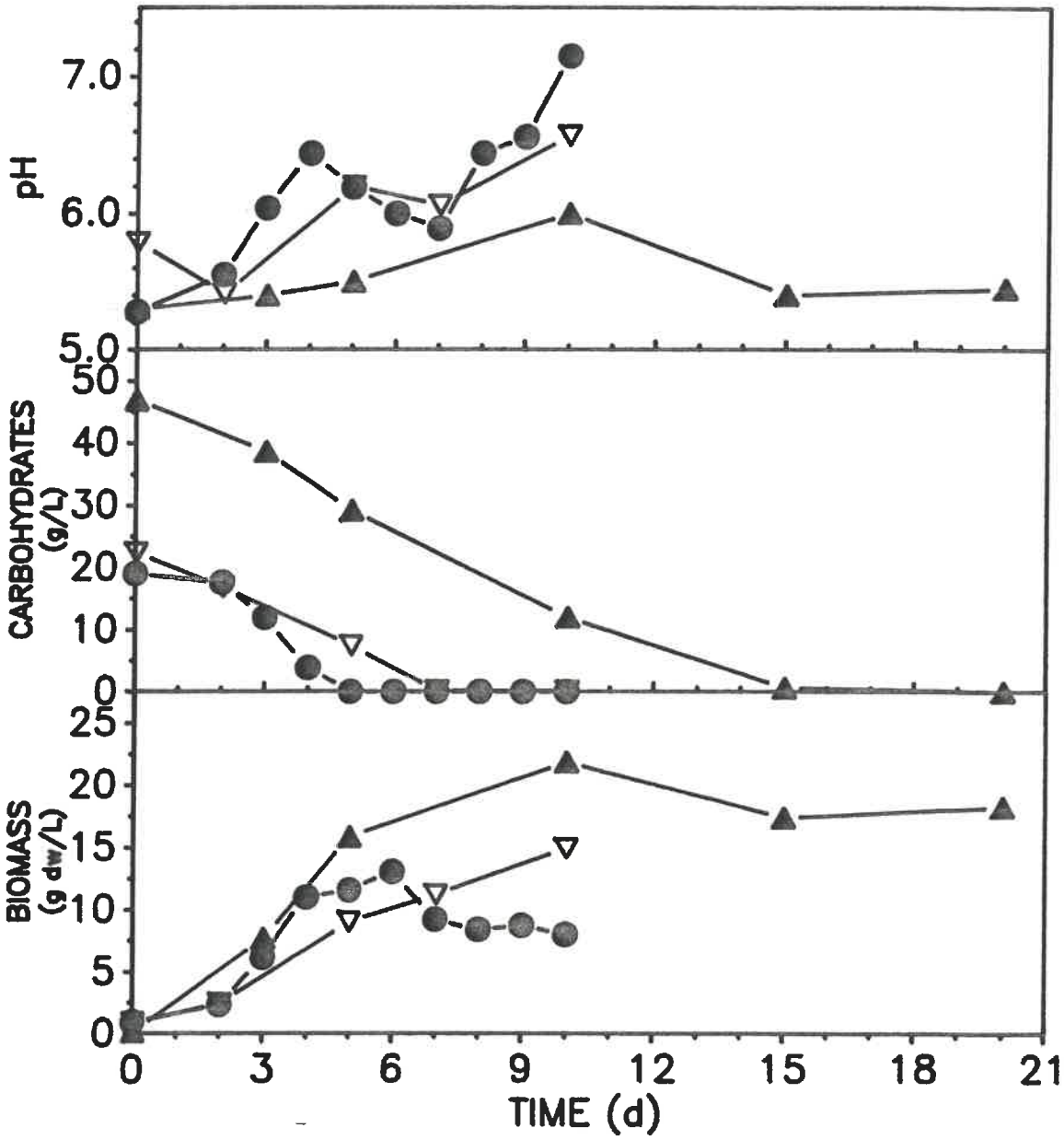


FIGURE 3.1 GROWTH CURVES OF CELL LINE MCR17 IN 3 DIFFERENT MEDIA FOR 200 mL SHAKE FLASK–GROWN SUSPENSION CULTURES. (▽) 1B5; (●) AB5 (100 mL); (▲) APM.

TABLE 3.1 GROWTH CHARACTERISTICS OF *CATHARANTHUS ROSEUS* CELL LINE MCR17 IN SUSPENSION AND IMMOBILIZED CULTURES.

CULTURE SYSTEM	200 mL SUSPENSION	200 mL SUSPENSION	100 mL SUSPENSION	2 L SIPC
MEDIUM	1B5	APM	AB5	1B5
SPECIFIC GROWTH RATE (d^{-1})	0.36	0.28	0.46	0.48
MAXIMUM BIOMASS (g dw/L)	11 +/- 2	22 +/- 2	13 +/- 1	6.8 +/- 0.5
AVG. BIOMASS YIELD (g dw/g sugar)	0.46	0.64	0.61	0.45
AVG. CARBOHYDRATE CONSUMPTION RATE (g /L d)	3.2	3.5	3.8	0.89
AVG. GROWTH RATE (g dw/L d)	1.5	2.2	2.3	1.7

The AB5 medium appeared to favor faster growth (0.46 d^{-1}) than the 1B5 medium (0.36 d^{-1}) while the maximum biomass concentrations were almost similar (13 g dw/L and 11 g dw/L). However, the biomass yield in AB5 was 0.60 g dw/g carbohydrate where the initial carbohydrate concentration was 19.06 g/L; therefore the maximum biomass concentration should have been only 11.4 g dw/L. The disparity between the actual and apparent maximal biomass concentration is probably due to experimental error since more biomass cannot be created from a limited carbon supply. If the 1B5-grown cells had been sampled more frequently (every 1 or 2 days) the 1B5 growth curve would have shown a similar increase, as compared to the APM and AB5-grown cells, from day 2 to 4 (Fig. 3.1).

A detailed plot of *C.roseus* suspension growth is depicted in Figure 3.2. For the first 2 days the cells are apparently in a lag phase while about 50% of the sucrose was being hydrolyzed into utilizable forms, glucose and fructose. By the third day all of the sucrose is hydrolyzed and the plant cells have entered exponential phase growth. Inorganic ions such as ammonium and phosphate are rapidly assimilated during the first 2 days while the nitrate consumption pattern appears to parallel the carbohydrate consumption. Both of these 2 latter nutrients are exhausted from the medium by the fifth day as the cells enter stationary phase growth. While no major nutrients exist in the medium the biomass dry weight decreases from day 6 to day 10. By the 10th day the re-appearance of ammonia (0.3 to 0.33 mM) and an increased pH value of 7.2 indicates a decline in cell viability.

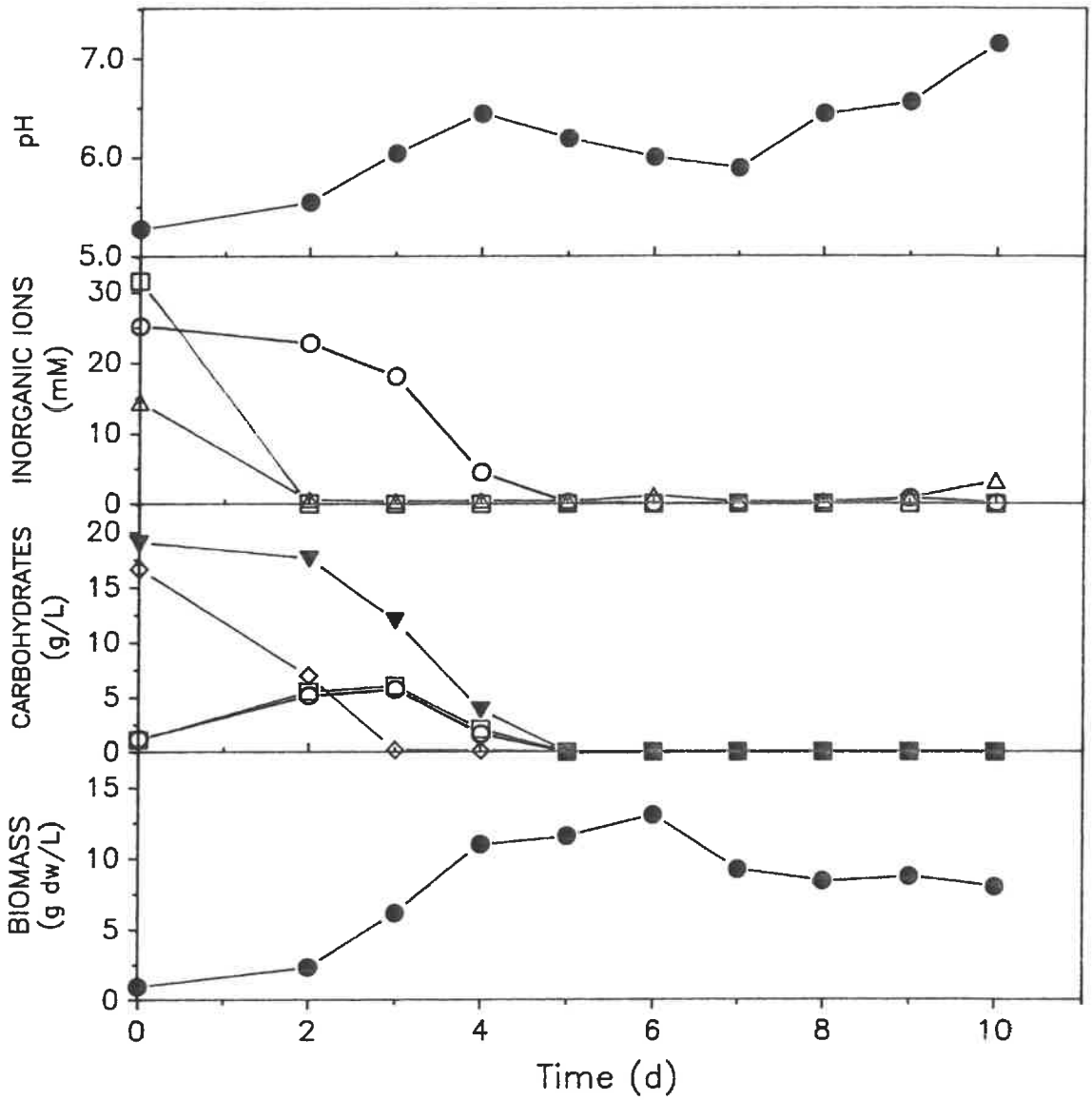


FIGURE 3.2 GROWTH AND SUBSTRATE CONSUMPTION CURVES OF 100 mL MCR17 SUSPENSION CULTURES IN AB5 GROWTH MEDIUM. (○) NITRATE; (△) AMMONIUM; (□) INORGANIC PHOSPHATE (x 10^{-2} mM); (◇) SUCROSE; (□) FRUCTOSE; (○) GLUCOSE; (▼) TOTAL CARBOHYDRATES.

During the stationary phase (day 6 - 10) the cells are probably respiring their stored nutrients, principally carbohydrates in the form of starch, in order to maintain themselves as they decreased in mass from 13 to 7 g dw/L.

The pH of the cultured *C.roseus* suspensions in all 3 media tended to increase as the cells grew and consumed nutrients (Figs. 3.1 & 3.2). A detailed study of growth and nutrient consumption in AB5 medium shows that the cells completely assimilated ammonium and inorganic phosphate just before the cells had entered exponential growth (day 2). Nitrate and carbohydrate assimilation parallelled the rapid biomass growth between day 2 and 4. As they were depleted by day 5 the cells entered stationary phase. After day 6 the biomass concentration decreased which was caused by cell lysis and was marked by a sharp pH increase (5.9 - 7.2) and the re-appearance of ammonium in the medium (0.03 - 0.33 mM, Fig. 3.2).

3.2. GROWTH OF IMMOBILIZED *C.roseus* CULTURES

A growth curve of cell line MCR17 in 2 L immobilization bioreactors in 1B5 medium is depicted in Figure 3.3. Using a 10% (V/V), 5-day old inoculum, many 2 L bioreactors were simultaneously inoculated and one reactor was harvested every 2 days for up to 16 days. The inoculum cells were observed to adhere to the immobilizing matrix in less than a day (4 - 6 h). The only way to determine the growth curve of this immobilized system was to harvest the plant cell biomass every 2 days.

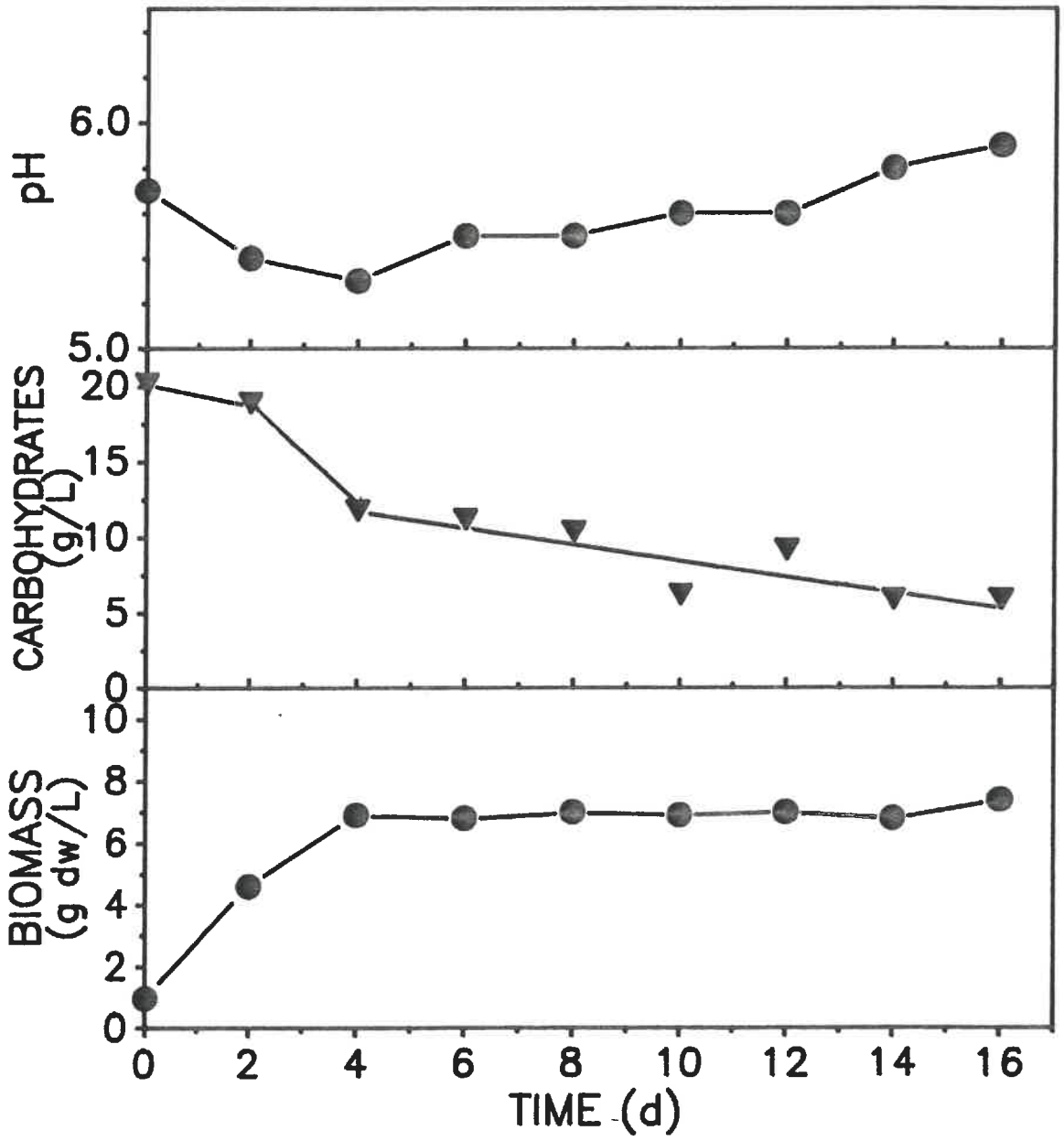


FIGURE 3.3 GROWTH CURVE OF A 2–L SURFACE IMMOBILIZED MCR17 CULTURE IN 1B5 GROWTH MEDIUM.

Each point on the growth curve represents a bioreactor. Unlike the suspension cultures immobilized cell growth was linear and exhibited no lag phase, which was previously observed by Archambault[22]. Perhaps the physical stabilization/protection of the cells by the matrix encouraged them to adapt to their environment more easily which was not evident in the suspension cultures. Nonetheless growth of the immobilized cells was limited by the spacing between the matrix layers, and due to their high hydrostatic volume the maximal biomass concentration attained was 6.8 ±0.5 g dw/L, or 6.1 ±1 mg/cm^2 (Fig. 3.3). It could be seen that with a smaller specific growth rate, the immobilized cells were not similarly utilizing their carbohydrates as in the suspension cultures which had higher specific growth rates.

While the carbohydrates were depleted by the end of the rapid growth phase in the suspension cultures, the immobilized cells consumed only 70% of the carbohydrates supplied to them. As the suspension cells continued to grow after day 4, the immobilized cells did not, since they utilized the carbohydrates for maintenance energy (ca. 0.07 g sugar/ g dw·d) between day 4 and 16 (Fig. 3.3). The carbohydrates were stored by the cells in the form of starch. Since the cells were no longer growing it is possible that some of the carbohydrates could have been directed toward alkaloid synthesis but measurements were not envisaged at this point of the project.

Table 3.1 shows that the immobilized cells in 1B5 had an average growth rate (1.7 g dw/L·d) that was about 25% slower than the suspension cultures in AB5 but 13% faster than in 1B5. Although the biomass yields were similar for suspension and immobilized cultures (0.46 g dw/g sugar) in 1B5 the former had a faster carbohydrate consumption rate

(3.2 g/L·d) than the latter (0.89 g/L·d, Table 3.1). For the first 4 days the pH decreased (5.7 - 5.3) as the cells increased in biomass and consumed carbohydrates at the greatest rate (4.1 g/L·d). After day 4 the average consumption rate was about 0.5 g/L·d as the pH steadily increased to 5.9 (Fig. 3.3).

3.3. DEVELOPMENT OF A 2-STAGE CULTURE REGIME

It was believed that a 2-stage process that permitted the build-up of sufficient biomass, followed by an induction stage would promote higher product yields by the cells than in a 1-stage system where the cells are grown and induced in the same production medium. To develop a 2-stage process, preliminary studies were done to determine the best time to induce alkaloid production in immobilized *C.roseus* cultures. Two parameters were evaluated in order to optimize the 2-stage process: the effect of the growth stage duration and the effect of the phytohormone on indole alkaloid production.

3.3.1. EFFECT OF GROWTH STAGE

After having determined the growth characteristics of the immobilized plant cells, it was desired to know what state, during the immobilized plant cell cycle, would be favorable to induce alkaloid production. Would the plant cells during the rapid growth stage (day 3) be more active to produce indole alkaloids, would it be at the transition point (day 5 and 6), from exponential to stationary growth, or would it be during the stationary

phase (ie. day 8) that the cells would be the most productive? Thus four periods (days 3,5,6,8) selected to allow the plant cells to grow in 1B5 medium followed by an exchange for alkaloid production medium (APM) to constitute the 15-day production phase. A 1-stage production process, where *C.roseus* cells were grown strictly in APM for 15 days, served as the control experiment to the 2-stage cultures. The 15-day production stage duration was arbitrarily chosen.

A comparison of alkaloid content between the 2-stage process (1B5 and APM) and the control (APM only) showed that the effect of any growth period using 1B5, prior to APM exposure, was not conducive to alkaloid production. The results in Table 3.2 show that the 1-stage production process in APM for 15 days contained the most alkaloids (14450 µg/reactor or 855 µg/g dw). Most alkaloids detected were intracellular where strictosidine lactam (11830 µg/reactor) was the prominent product (82% of identified alkaloids). Ajmalicine (2040 µg/reactor) was the second prominent alkaloid followed by serpentine (281 µg/reactor) and then tabersonine (193 µg/reactor). While no strictosidine lactam was detected extracellularly, 0.3% (ajmalicine) to 10% (serpentine) of identified alkaloids produced were released to the medium (Table 3.2).

TABLE 3.2 EFFECT OF CULTURE AGE ON ALKALOID PRODUCTION

GROWTH (d) 1B5	PRODUCTION (d) APM	STR. LAC. µg/reactor	AJM. µg/reactor	SERP. µg/reactor	TAB. µg/reactor	PDT. YIELD $\mu g.g_{dw}^{-1}$
0	15	11830	2111	312	197	855
3	15	-	-	6	-	0.4
5	15	-	9	2	-	1.0
6	15	38	18	8	-	3.5
8	15	-	-	-	-	0

STR.LAC.: STRICTOSIDINE LACTAM
AJM.: AJMALICINE
SERP.: SERPENTINE
TAB.: TABERSONINE

Alkaloid production was severely compromised when the 15-day production stage was preceded by the growth stage in which 1B5 was the growth medium used. The 3 and 5-day growth stage only produced traces (0.18 - 0.82 μg/g dw) of ajmalicine and/or serpentine while the 8-day growth stage procured no detectable amounts. The best product yield was 3.5 μg/g dw, in the 6-day growth (1B5)/15-day (APM) production regime; 2.08 μg/g dw of strictosidine lactam, 0.98 μg/g dw of ajmalicine, and 0.44 μg/g dw of serpentine were detected.

3.3.2. EFFECT OF PHYTOHORMONE

The phytohormone content in plant cell media has been shown to influence the plant cell's propensity to maintain growth activities or to commence secondary metabolite synthesis, which was previously mentioned in the literature review. It was not realized at the time of the experiment, (in section 3.3.1) that the presence of the auxin 2,4D was impeding the alkaloid production during the 2-stage process. The 2,4D auxin was replaced by the natural growth hormone, indole-acetic acid (IAA) at a concentration (1 μM) that was similarly found in the APM formulation. This latter hormone, which has a different chemical structure from 2,4D, was not found to inhibit alkaloid production. Product yields dramatically improved from 43 to 300-fold for 5 and 6-day growth stages, respectively, when the 2,4D in 1B5 was exchanged for IAA, thus becoming AB5. Intracellular yields of strictosidine lactam (436 μg/g dw), ajmalicine (38 μg/g dw), serpentine (162 μg/g dw) and tabersonine (398 μg/g dw) were the highest for the 6-day growth stage in AB5.

Extracellular quantities (65 - 90 μg/reactor) of the last 3 alkaloids were detected whereas they were absent when 1B5 (with 2,4D) was used (Table 3.3).

The experiment from section 3.3.1 was essentially repeated to ascertain the best time to induce alkaloid production. Induction in APM, after 6 days of growth in AB5, produced a similar alkaloid yield of 1049 μg/g dw as compared to the 15-day control experiment in APM only (855 μg/g dw). The APM-grown cells (1-stage process) produced at least 2 times more strictosidine and 4 times more ajmalicine than the most productive 2 stage process. However, more complex alkaloids such as serpentine and tabersonine were produced by the 6-day growth/15-day production process than in the 1-stage process (Tables 3.2, & 3.3). Therefore the 2-stage production regime was established as the 6-day growth period in AB5, followed by the 15-day production stage in APM. After having determined the optimal time to induce alkaloid production in the immobilized cultures, subsequent studies were concerted on evaluating the evolution of the alkaloid patterns in the 1 and 2-stage processes, using immobilized and suspension cultures.

TABLE 3.3 **EFFECT OF PHYTOHORMONE ON ALKALOID PRODUCTION IN 2 L SIPC CULTURES**

HORMONE IN B5	GROWTH STAGE (d)	PROD. STAGE (d)	STR. LAC. µg/reactor	AJM. µg/reactor	SERP. µg/reactor	TAB. µg/reactor	PROD. YIELD $\mu g.g_{dw}^{-1}$
IAA	5	15	133	38	484	-	43
2,4D	5	15	-	9	2	-	1
IAA	6	15	6365	644	2449	5865	1049
2,4D	6	15	38	18	8	-	3.5
IAA	8	15	-	14	5	-	1.6
2,4D*	8	15	-	-	-	-	0
control	0	15	11830	2111	312	197	855

IAA: 1.0 µM
2,4D: 4.5 µM
2,4D*: 1.4 µM 2,4D + 0.7 µM IAA

3.4. ALKALOID PRODUCTION STUDIES IN SUSPENSION CULTURES

Alkaloid production patterns were evaluated in 200 mL shake flask-grown *C.roseus* suspension cultures. These experiments served to compare against the 2-L immobilized cultures with respect to growth, nutrition and alkaloid production patterns in 1 and 2-stage production regimes. For each production regime 4 replicate flasks were used and samples were withdrawn every 3 days for alkaloid extraction.

3.4.1. GROWTH OF 1-STAGE SUSPENSION CULTURES

Growth and nutrient consumption profiles are depicted in Figure 3.4 for 1-stage suspension cultures. The 1-stage experiments were inoculated at 20% (V/V) by 1B5-grown *C.roseus* cells into APM. The initial biomass concentration was 1.94 g dw/L. Major inorganic nutrients such as nitrate, ammonium, and phosphate were assimilated 6 days after inoculation. Ammonium (10 mM) and inorganic phosphates (85.5 μM) were depleted in 3 days while the medium conductivity decreased from 2300 to 750 μsiemens, and the pH increased from 4.3 to 5.9. By day 6 the cells began to enter stationary phase and nitrate (11 mM) had been depleted. The pH remained constant around 5.9 while the conductivity levelled off to about 300 μmho between day 6 and 15.

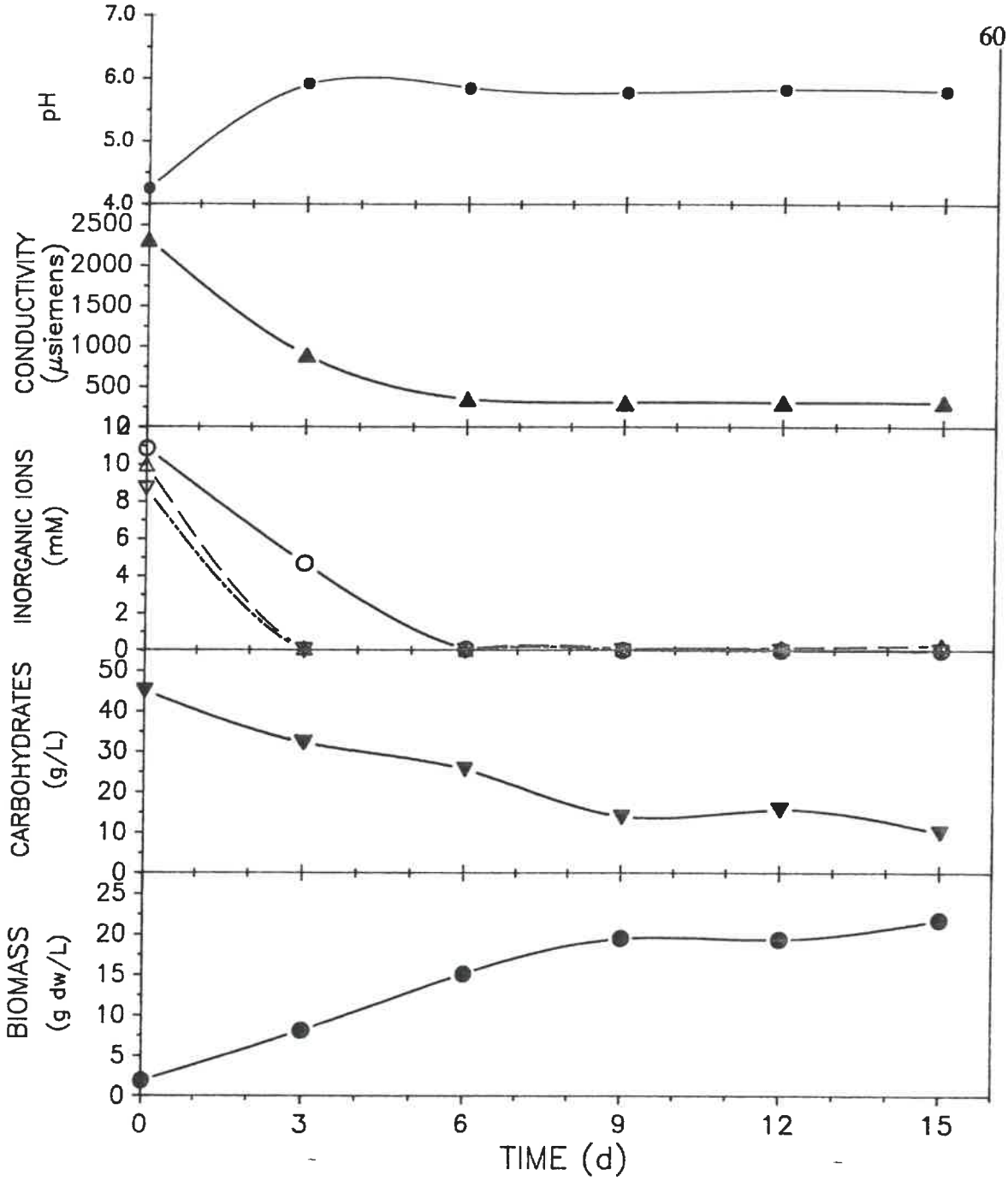


FIGURE 3.4 GROWTH AND SUBSTRATE CONSUMPTION CURVES OF 1–STAGE, 200 mL SUSPENSION CULTURES OF MCR17. (○) NITRATE; (△) AMMONIUM; (▽) INORGANIC PHOSPHATE (x 10^{-2} mM).

It could be seen from the experiments in suspension and immobilized cultures that the conductivity often remained around 300 μmho subsequent to major ion depletion, particularly nitrate. No other means were available to measure the ionic species such as calcium, chlorine, magnesium, and other ionic micronutrients. This basal conductivity value (300 μsiemens) could have been attributed to the presence of these aforementioned ions which were included as macro and micronutrients in the culture media (Appendices I and II). Carbohydrates were consumed at an average rate of 1.84 g/L·d (Table 3.4) 3 days after sucrose was completely hydrolyzed (not shown) into glucose and fructose. The cells grew most rapidly, with no apparent lag phase, for the first 6 days, at ca. 6.5 g dw/L d; sucrose was concomitantly hydrolyzed to glucose and fructose by the cells. For the first 9 days the carbohydrates were consumed at an average rate of 3.33 g/L d giving a yield of 0.57 g dw/g carbohydrate. After nitrate depletion on day 6, the growth rate decreased as internally stored forms of nitrate were consumed. This allowed the cells to grow to almost 22 g dw/L between day 6 and 15 as the carbohydrate concentration decreased to 10 g/L. Table 3.4 summarizes the growth parameters of the 1-stage cultures. Between day 9 and 15 the growth rate declined and cell lysis may have begun to take place by day 15 as ammonium ions were detected (0.3 mM) in the medium (Fig. 3.4).

TABLE 3.4 GROWTH CHARACTERISTICS OF *CATHARANTHUS ROSEUS* CELL LINE MCR17 IN 1 AND 2- STAGE SUSPENSION CULTURES.

CULTURE PROCESS	1-STAGE	2-STAGE	
MEDIUM	APM	AB5	APM
SPECIFIC GROWTH RATE (d^{-1})	0.25	0.43	0.18
MAXIMUM BIOMASS (g dw/L)	22.0	13.0	21.0
AVG. GROWTH RATE (g dw/L·d)	1.32	2.00	1.15
AVG. CARBOHYDRATE CONSUMPTION RATE (g/L·d)	1.84	4.17	4.44
AVG. BIOMASS YIELD (g dw/g sugar)	0.59	0.47	0.35
AVG. NITRATE CONSUMPTION RATE (mM/d)	1.80	4.15	5.15
AVG. AMMONIUM CONSUMPTION RATE (mM/d)	2.71	0.90	1.94

3.4.2. ALKALOID PRODUCT SPECTRA OF 1-STAGE CULTURES

Strictosidine lactam, ajmalicine, serpentine epivindolinine, and the precursor tryptamine were detected in the cultures. Total product and extracellular alkaloid spectra are illustrated in Figures 3.5 and 3.6, respectively. Tryptamine decreased (150 - 65 μg/g dw) between day 3 and 15 while strictosidine lactam increased overall (ca. 25 - 80 μg/g dw). Tryptamine was detected extracellularly (40 - 90 μg/L) between day 3 and 9. Epivindolinine, usually a late-synthesized alkaloid, was detected early on day 3 and 6 (15-35 μg/g dw) where 90 to 95% of total produced was detected in the medium (150 - 250 μg/L) but none was found later.

Ajmalicine and its oxidized form, serpentine, first appeared on day 9 (3 and 5 μg/g dw) as the cells entered stationary phase and when all inorganic nutrients had been depleted (Fig. 3.4). With partial cell lysis occurring on day 15, most of the ajmalicine was detected extracellularly (10 μg/L) while only 1% of the total serpentine produced on that day (5 μg/L) was found in the medium.

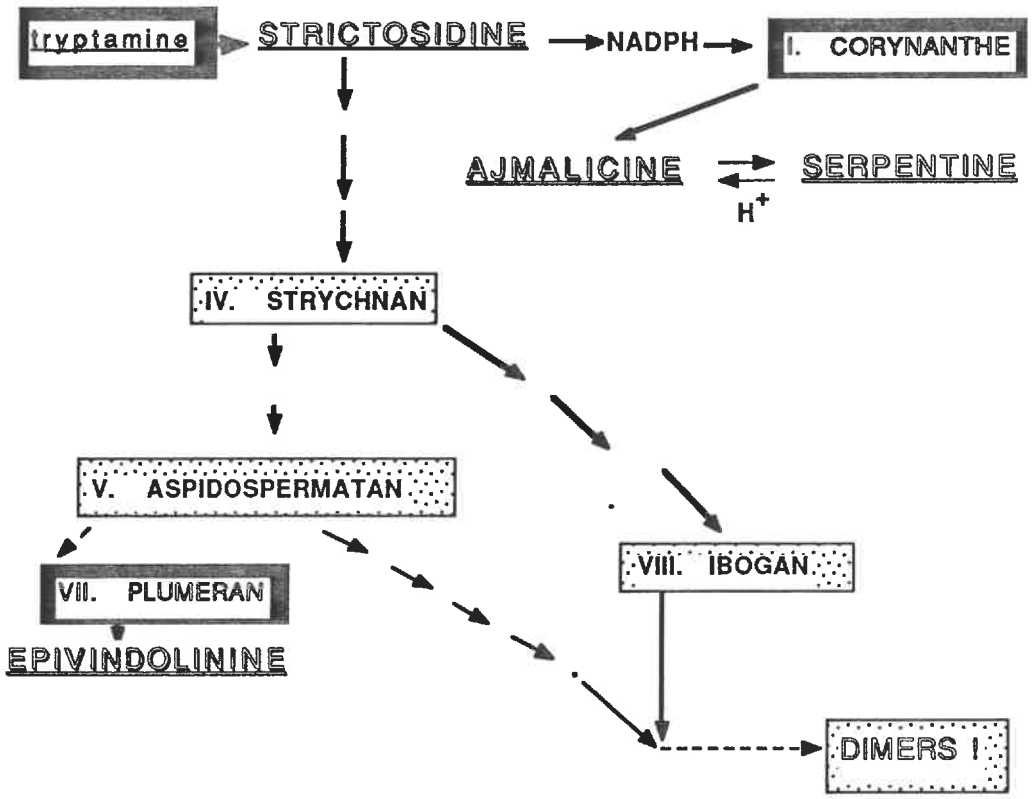
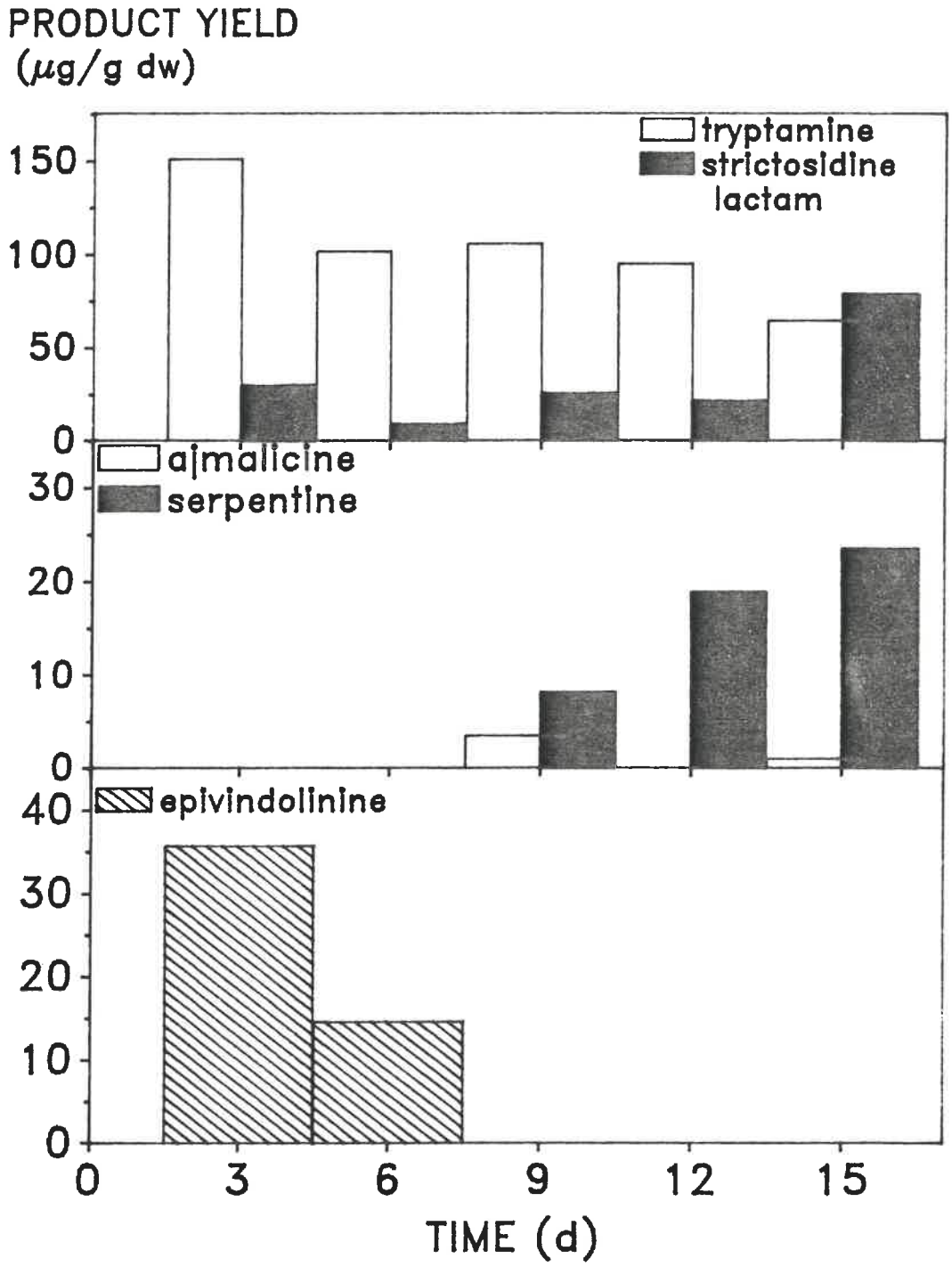


FIGURE 3.5 Alkaloid products detected (underlined) in 1-stage C.roseus suspension cultures.


PRODUCT YIELD
(μg/g dw)
tryptamine
strictosidine lactam
ajmalicine
serpentine
epivindolinine
150
100
50
0
30
20
10
0
40
30
20
10
0
0
3
6
9
12
15
TIME (d)

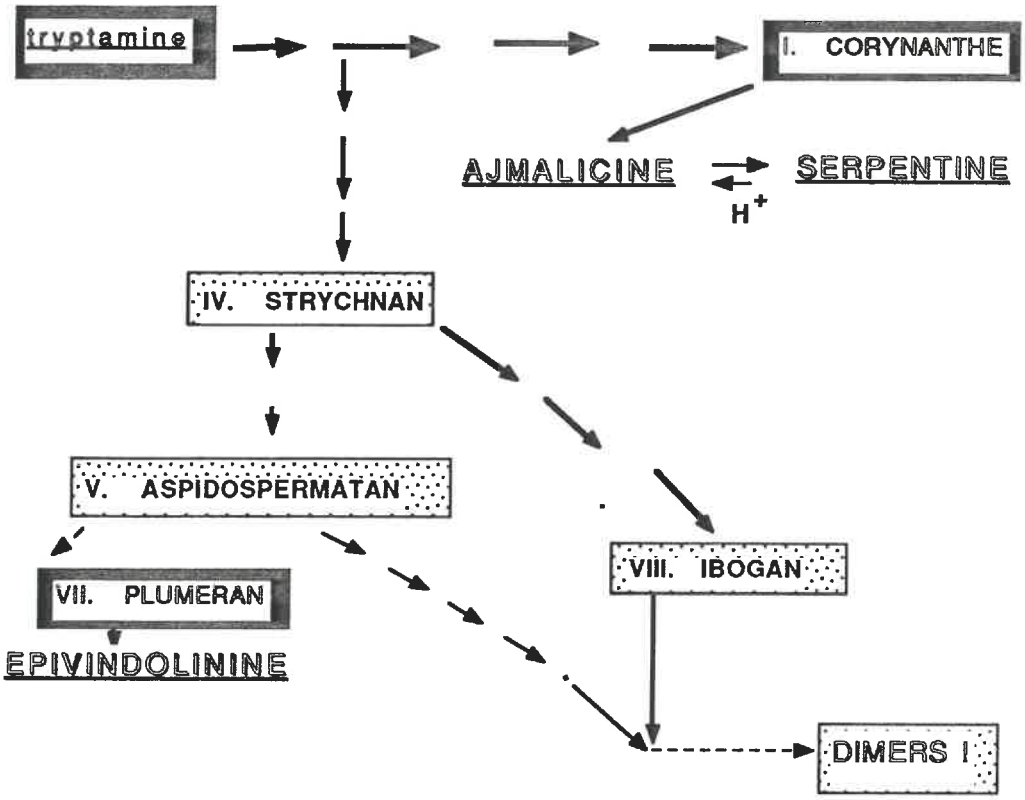
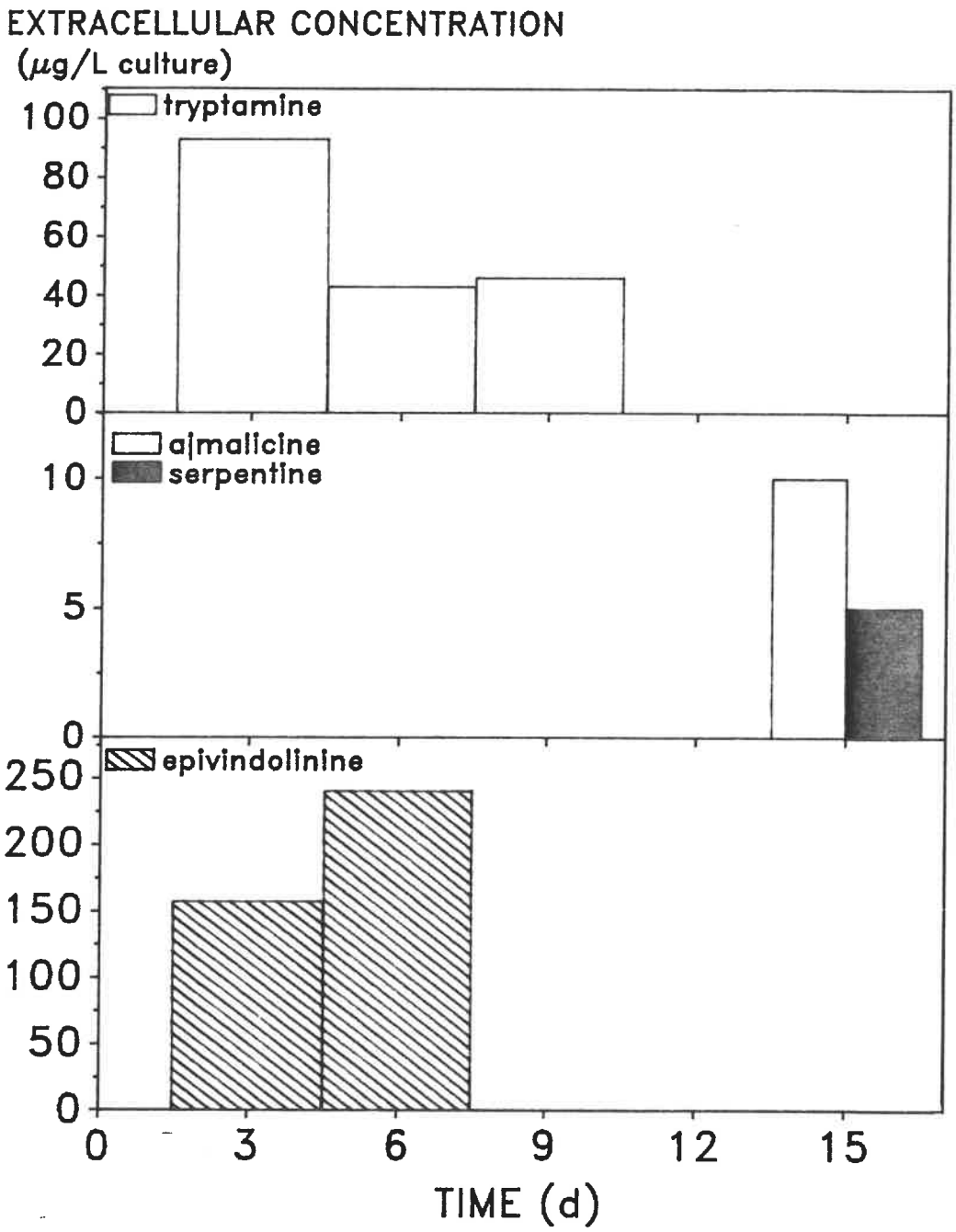


FIGURE 3.6 Extracellular alkaloid concentrations (underlined) detected in 1-stage C.roseus suspension cultures.


EXTRACELLULAR CONCENTRATION
(μg/L culture)
tryptamine
ajmalicine
serpentine
epivindolinine
100
80
60
40
20
0
10
5
0
250
200
150
100
50
0
0
3
6
9
12
15
TIME (d)

3.4.3. PRODUCTIVITY OF THE 1-STAGE PROCESS

A global estimate of alkaloid production was used by measuring the total indole alkaloids (TIA) at 280 nm previously described in Materials and Methods. Figure 3.7 depicts total alkaloid concentration in the cultures, total alkaloid content produced by the cells as well as extracellular quantities. The individual alkaloids identified in Figs. 3.5 and 3.6 represented about 2 to 8% of the TIA measurements shown in Fig. 3.7.

Table 3.5 shows a comparison of volumetric and specific productivities of the 1 and 2-stage suspension cultures. The volumetric productivity values were calculated to measure how productive the culture system as a whole would be with a given culture process. This parameter would eventually determine the cost-effectiveness of the culture process and system overall when larger-scale cultures are being considered. The specific productivity was calculated to evaluate the degree of active alkaloid production by the plant cells, under a given culture process and system (either shake flask or immobilized).

For the first 3 days the total alkaloid concentration increased to 55 mg/L culture which represented a peak total content of 6 mg/g dw. Between day 6 and 15 total alkaloid concentration varied by ±15 mg/L while the total product content remained at about 3 ±1 mg/g dw. Extracellular alkaloids released ranged from 10% (day 3) to 30% (day 15) of total alkaloids produced (Fig. 3.7).

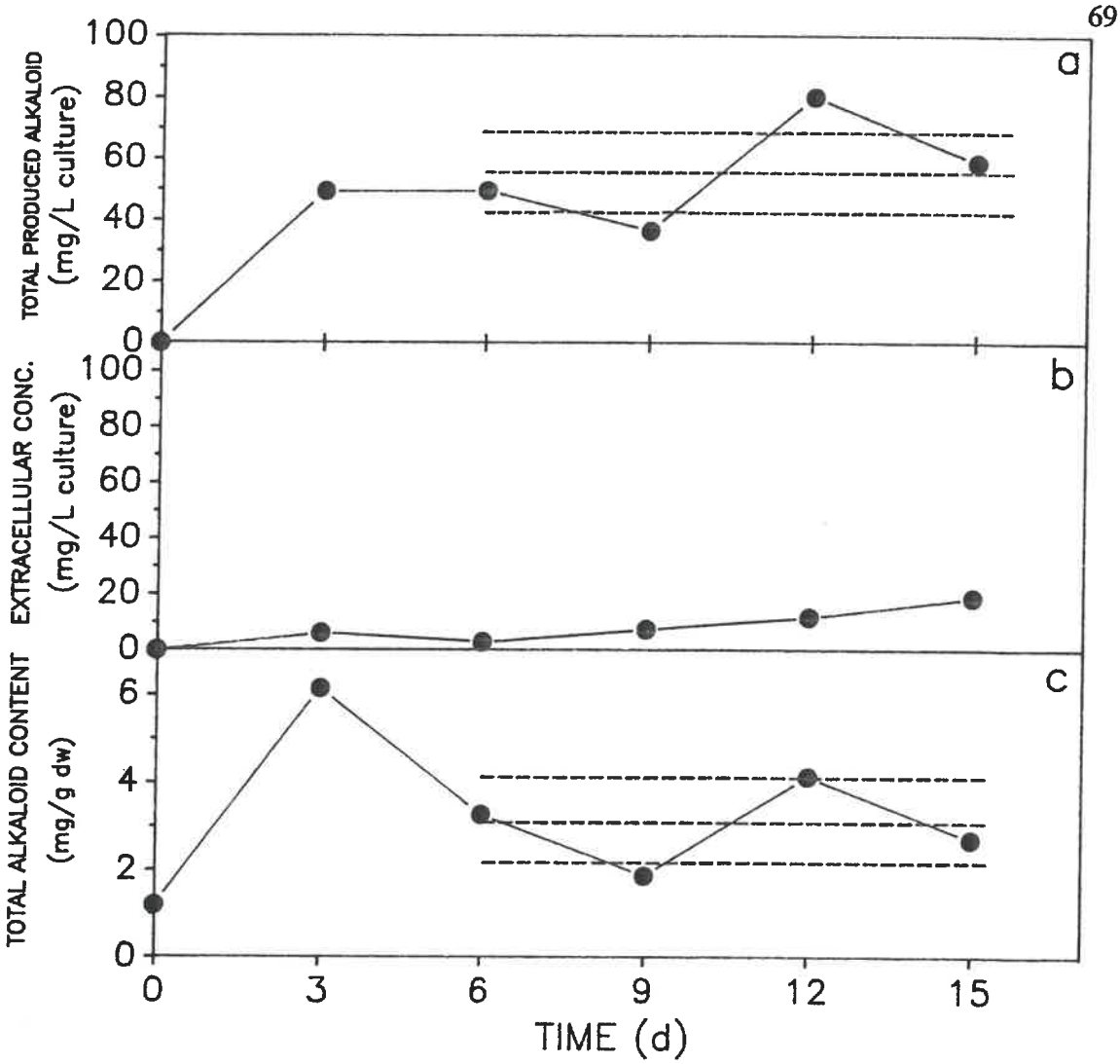


FIGURE 3.7 TOTAL INDOLE ALKALOID (TIA) QUANTIFICATION OF 200 mL MCR17 SUSPENSION CULTURES IN THE 1–STAGE PROCESS. a. TOTAL PRODUCED ALKALOIDS REPRESENT INTRA–AND EXTRACELLULAR QUANTITIES PRODUCED BY THE CELLS. b. EXTRACELLULAR CONC. IS EXPRESSED ON A PER LITRE CULTURE BASIS. c. TOTAL ALKALOID CONTENT IS THE PRODUCT YIELD OF THE INTRA–AND EXTRACELLULAR QUANTITIES PER GRAM DRY WEIGHT.

The highest concentration was about 20 mg/L culture on day 15, but some extracellular products may have been contributed by the onset of cell lysis which was indicated by ammonium re-appearance (Fig. 3.4).

The highest volumetric and specific productivities were 16.4 mg/L·d and 2.1 mg/g dw·d, respectively, on day 3. It is not certain why the values were the highest as early as 3 days into the culture (Table 3.5). As the *C.roseus* cells grew between day 3 and 6, the volumetric and specific productivities declined. No net alkaloid synthesis occurred between day 9 and 15 as the volumetric and specific productivities averaged about 4.9 ±1.6 mg/L·d and 0.25 ±0.1 mg/g dw·d, respectively. Usually it would have been expected that secondary metabolite synthesis occur only during the stationary phase, but it was not observable from the total indole alkaloid measurements. Some cell lines have been reported to produce alkaloids during the growth stage[20].

TABLE 3.5 COMPARISON OF VOLUMETRIC AND SPECIFIC PRODUCTIVITIES IN 200 mL SUSPENSION CULTURES OF *C. ROSEUS.*

PARAMETER	TIME (d)	1-STAGE PROCESS	2-STAGE PROCESS
VOLUMETRIC PRODUCTIVITY (mg/L · d)	3	16.4	18
	6	8.3	29
	9	4.1	22
	12	6.7	57
	15	3.9	49
	18	-	25
	21	-	19
SPECIFIC PRODUCTIVITY (mg/g dw · d)	3	2.1	2.5
	6	0.55	2.4
	9	0.21	1.2
	12	0.35	2.7
	15	0.18	2.6
	18	-	1.2
	21	-	0.99

3.4.4. GROWTH OF 2-STAGE SUSPENSION CULTURES

The 2-stage culture process using growth and production media was adapted for shake flask-grown suspension cultures according to the conditions that were established in preliminary studies discussed in section 3.3. The culture profile depicted in Figure 3.8 shows that the cells exhausted all inorganic nutrients and carbohydrates in both AB5 and APM media where the final biomass concentration remained similar (21 g dw/L) to that of the 1-stage production regime (22 g dw/L) shown in Figure 3.4

The 2-stage process provided 150% to 570% more inorganic nutrients and 150% more carbohydrates, with biomass yields lower than those of the 1-stage cultures. Table 3.4 shows that the nitrate and carbohydrate uptake were 50 to 230% faster, respectively, during the growth stage as compared to the 1-stage production process in APM only. Only the average biomass yields with respect to sugars (47% and 35%) and the average ammonium consumption rates (0.9 and 1.94 mM/d) were lower in the 2-stage process than the 1-stage process (72% and 2.71 mM/d, respectively). The second stage in APM had faster nitrate and carbohydrate uptake rates by 286% and 241%, respectively, as compared to the 1-stage regime in APM only (1.80 mM and 1.84 g/L·d, respectively).

The medium conductivity decreased with the consumption of inorganic ions, principally nitrate. No ammonium release was observed at the end of the culture which was previously observed in the 1-stage suspension cultures.

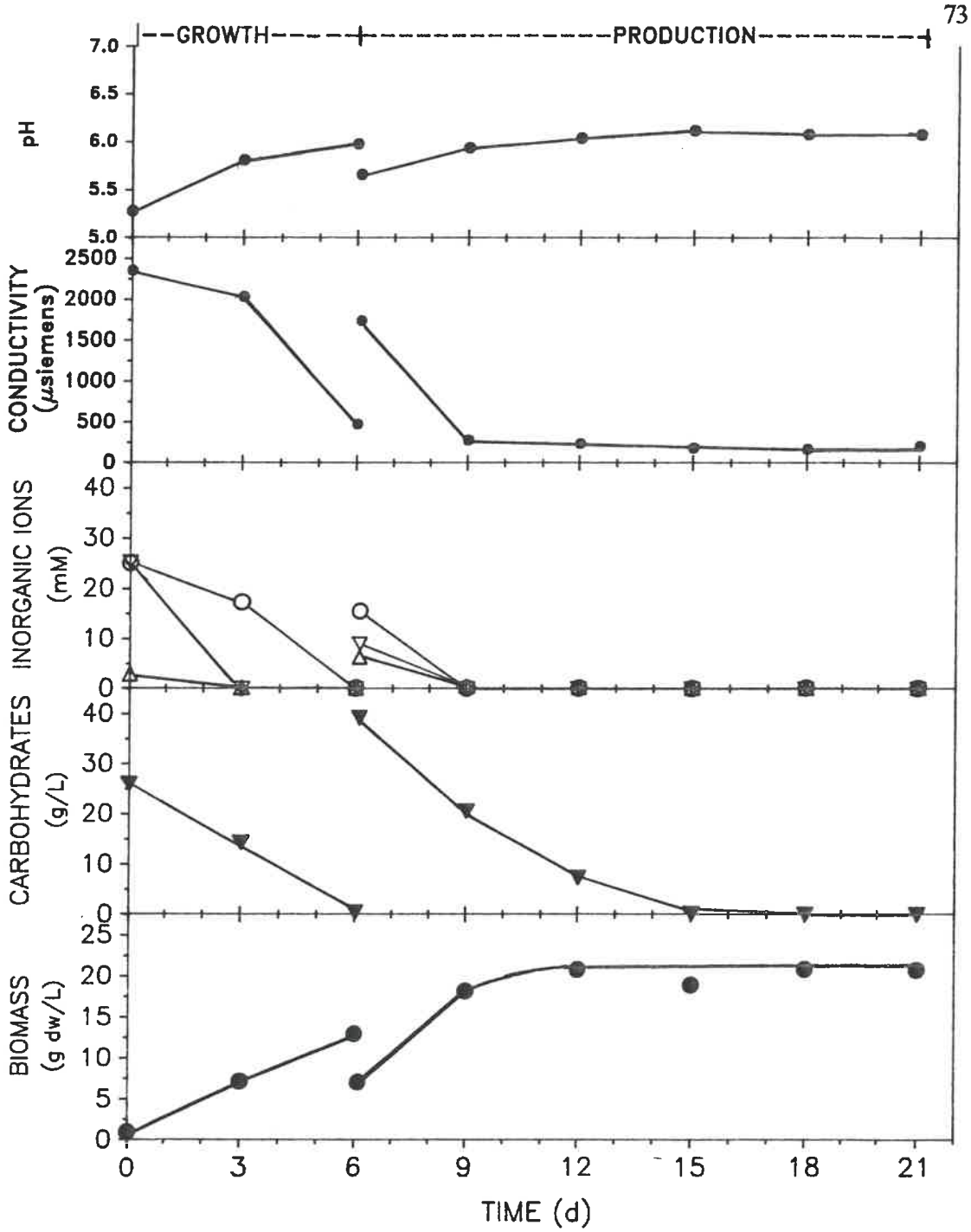


FIGURE 3.8 GROWTH AND SUBSTRATE CONSUMPTION CURVES OF 2–STAGE, 200 mL MCR17 SUSPENSION CULTURES. (○) NITRATE; (△) AMMONIUM; (▽) INORGANIC PHOSPHATE (x 10^{-2} mM).

The culture pH levelled off in both stages to 6.0 while the biomass concentration remained at about 21 g dw/L between day 12 and 21.

3.4.5. PRODUCT SPECTRA OF 2-STAGE CULTURES

Product yields (Fig. 3.9) and extracellular alkaloid spectra (Fig. 3.10) were broader in 2-stage *C.roseus* suspension cultures than in 1-stage cultures (Figs. 3.5 & 3.6). In addition to the precursor tryptamine, 4 other alkaloids were produced in the 2-stage cultures that were not detected in the 1-stage cultures: yohimbine, tabersonine, lochnerinine and catharanthine. The product yields were 30 to 40-fold higher than in the 1-stage process while the extracellular alkaloid concentrations were about 40-fold higher also.

During the growth stage (Fig. 3.9) no significant amount of alkaloids were produced except for trace amounts of strictosidine lactam, ajmalicine and serpentine. The latter 2 alkaloids were detected extracellularly on day 3 (50 μg/L ajmalicine) and day 6 (40 μg/L ajmalicine and 60 μg/L serpentine, Fig. 3.10). Three days into the production stage, coincident with inorganic ion depletion, strictosidine lactam (340 μg/g dw) and lochnerinine (50 μg/g dw) were at their peak yields. From day 9 to 21 the alkaloid yields increased in quantity and diversity as strictosidine lactam yields tended to decrease (680 μg/g dw serpentine, day 18; 150 μg/g dw tabersonine, day 21; 550 μg/g dw epivindolinine, day 21; 35 μg/g dw catharanthine, day 21).

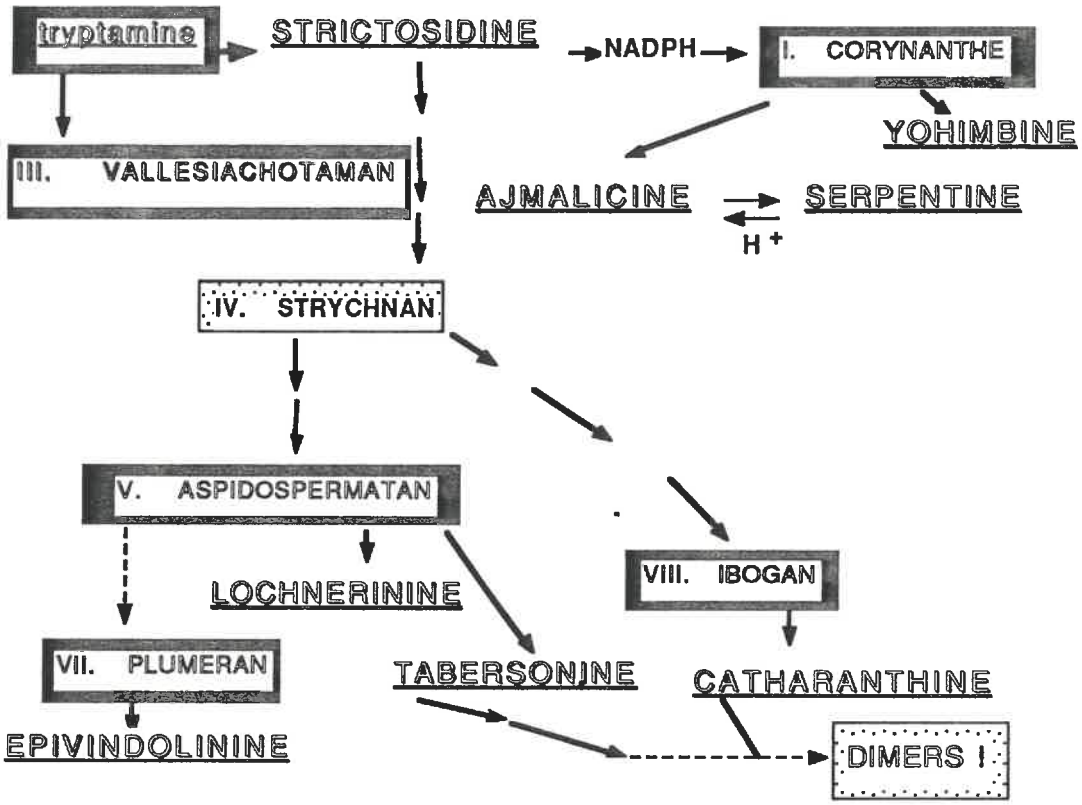
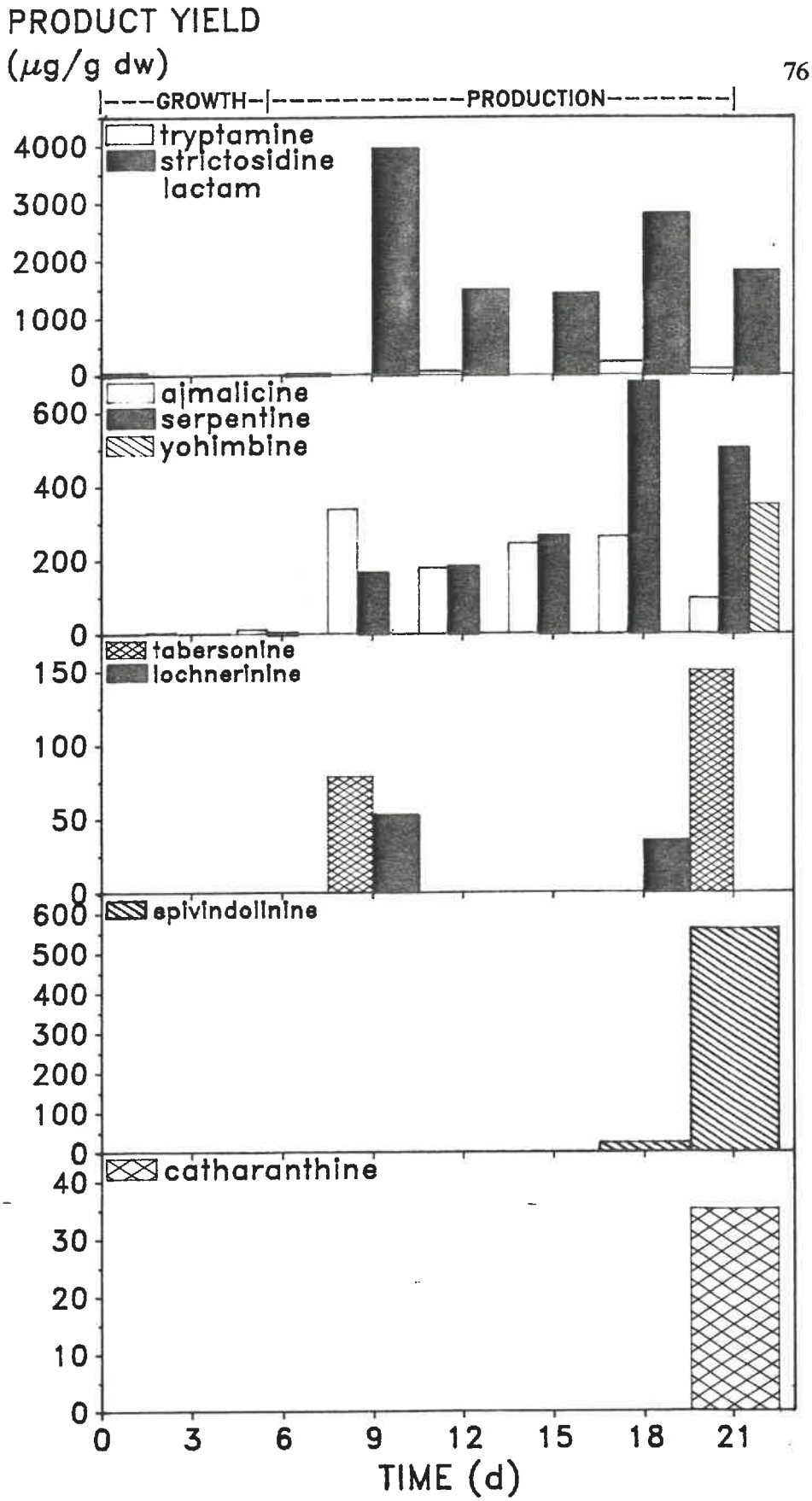


FIGURE 3.9 Alkaloid spectra detected (underlined) in 2-stage C.roseus suspension cultures.


PRODUCT YIELD
(μg/g dw)
GROWTH
PRODUCTION
tryptamine
strictosidine lactam
ajmalicine
serpentine
yohimbine
tabersonine
lochnerinine
epivindoline
catharanthine
4000
3000
2000
1000
0
600
400
200
0
150
100
50
0
600
500
400
300
200
100
0
40
30
20
10
0
0
3
6
9
12
15
18
21
TIME (d)

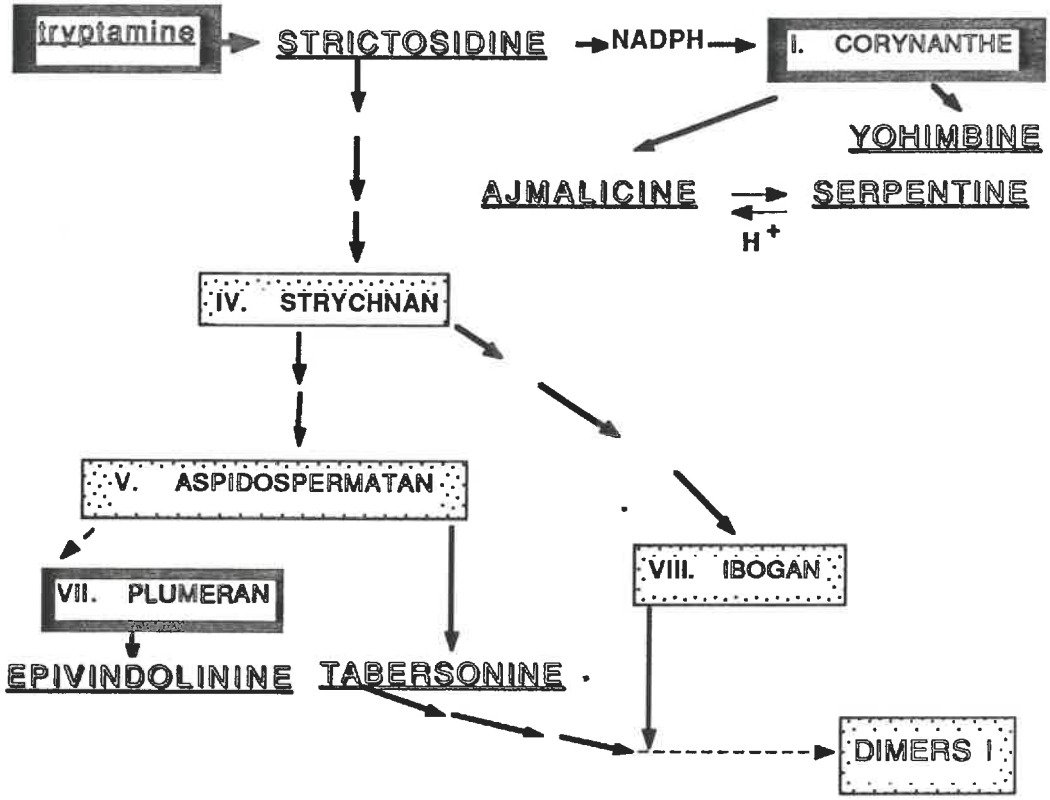
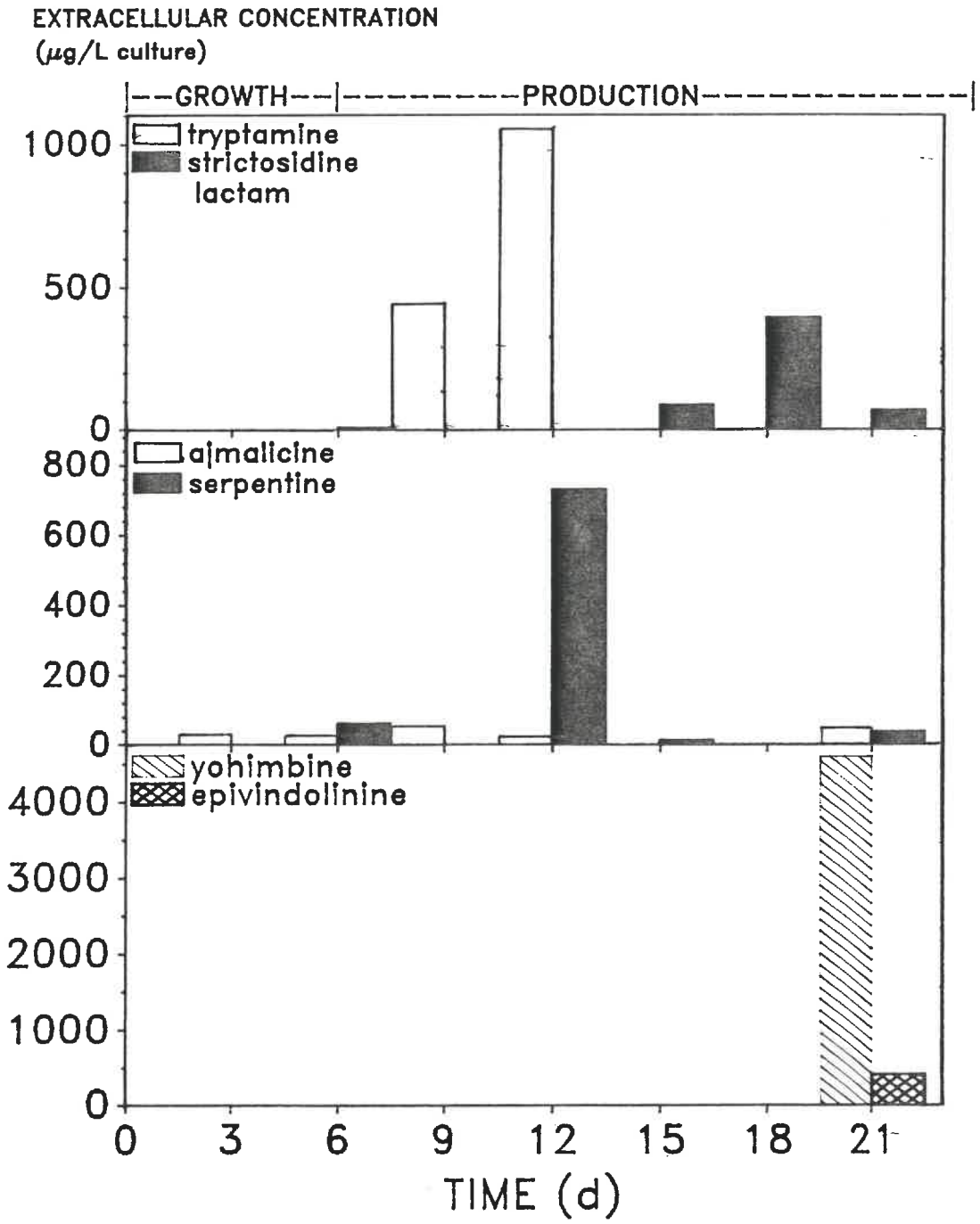


FIGURE 3.10 Extracellular alkaloids (underlined) detected in the medium of 2-stage C.roseus suspension cultures.


EXTRACELLULAR CONCENTRATION
(μg/L culture)
GROWTH
PRODUCTION
tryptamine
strictosidine lactam
ajmalicine
serpentine
yohimbine
epivindolinine
1000
500
0
800
600
400
200
0
4000
3000
2000
1000
0
0
3
6
9
12
15
18
21
TIME (d)

About 350 µg/g dw yohimbine, an hydroxylated form of ajmalicine, was detected on day 21.

Most of the tryptamine produced was in the medium on day 9, at 0.44 mg/L, and on day 12 at 1.05 mg/L. Strictosidine lactam was detected in the medium between day 15 and 21 (70 to 400 µg/L) which represented a little less than 1% of its total produced (Fig. 3.10).

Extracellular ajmalicine and serpentine were found in approximately equivalent quantities during the growth and production stages (40 - 50 µg/L) except for day 12 where 19% of the total serpentine produced was detected (680 µg/L). Yohimbine (4.6 mg/L) and epivindolinine (0.4 mg/L) were detected on day 21 only and they represented 43% and 2% of their total produced, respectively (Fig. 3.10).

3.4.6. PRODUCTIVITY OF THE 2-STAGE CULTURES

Measurements of the total indole alkaloid contents produced and released by the cells showed that the cells were 10-fold more productive (Fig. 3.11) than the 1-stage cultures (Fig. 3.7). The 2-stage cultures released about 2 times more alkaloids to the medium than the other culture regime.

The alkaloids identified in Fig. 3.10 represented as little as 0.2% on day 15, and at most 13% on day 21, of the extracellular TIA estimations. Alkaloid synthesis occurred during the growth stage until nutrient depletion.

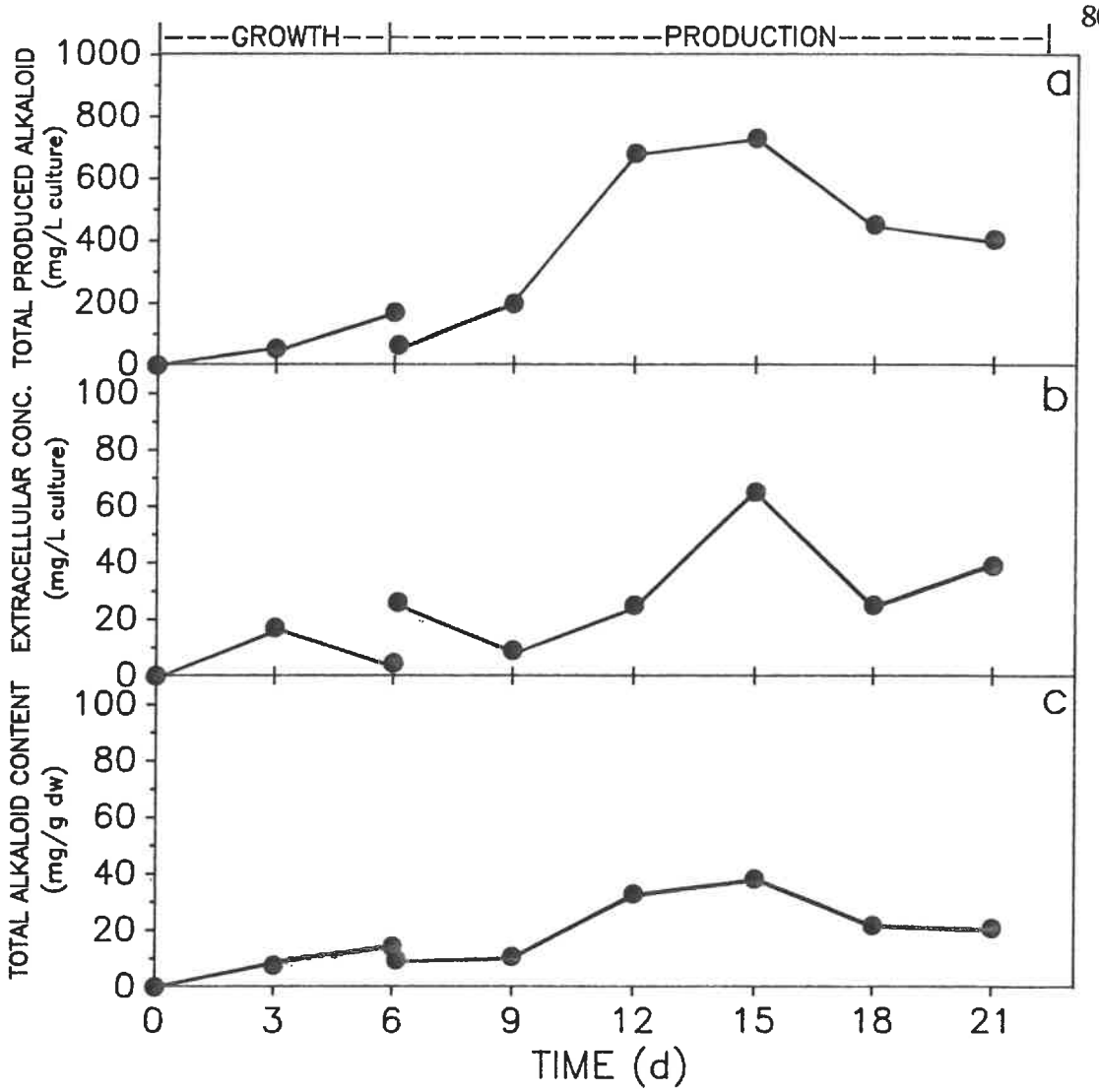


FIGURE 3.11 TOTAL INDOLE ALKALOID (TIA) QUANTIFICATION OF 200 mL SUSPENSION MCR17 CULTURES IN THE 2–STAGE PROCESS. a. TOTAL PRODUCED ALKALOIDS REPRESENT INTRA–AND EXTRACELLULAR QUANTITIES PRODUCED BY THE CELLS. b. EXTRACELLULAR CONC. IS EXPRESSED ON A PER LITRE CULTURE BASIS. c. TOTAL ALKALOID CONTENT IS THE PRODUCT YIELD OF THE INTRA–AND EXTRACELLULAR QUANTITIES PER GRAM DRY WEIGHT.

When APM was introduced on day 6 production remained low until day 9, at 100 - 200 mg/L culture, as newly replenished nutrients were consumed (Fig. 3.11a). From day 9 to 15 total alkaloid concentration increased 35-fold (20 to 700 mg/L) while the total alkaloid content increased about 3.5-fold (10 to 35 mg/g dw). By day 15, only traces of carbohydrates were detectable and the medium remained nutrient poor until day 21 (Fig. 3.8). After day 15 total alkaloid concentration and content declined by about 50% until day 21, as well as for the extracellular alkaloids (Fig. 3.11). Total products released represented about 10% of the total alkaloids produced (Fig. 3.12). Table 3.5 shows that the *C.roseus* cells were most active 3 days after inorganic nutrients depletion (day 9) when the volumetric and specific productivities were 57 mg/L·d and 2.7 mg/g dw·d, respectively. A comparison of volumetric and specific productivities shows that the 2-stage cultures were almost 10 times more productive than the 1-stage cultures, for the same given culture period (57 vs 6.7 mg/L·d and 2.7 vs 0.35 mg/g dw, day 12). No substantial alkaloid synthesis occurred after day 15 due to product degradation and/or conversion into other complex alkaloids. Figure 3.12 summarizes the 10-fold difference in production capacity between 1 and 2-stage cultures in the cells and medium.

3.5. ALKALOID PRODUCTION STUDIES IN IMMOBILIZED CULTURES

Once the preliminary studies had established the operating conditions for the 2-stage production regime (section 3.3) indole alkaloid production studies were performed in 2-L immobilized *C.roseus* cultures. Using the 6-day growth, 15-day production scheme, bioreactors that were simultaneously inoculated were dismantled every 3 days for up to 21 days (up to 15 days for the 1-stage process) to harvest the indole alkaloids from the biomass and the medium (Materials and Methods, section 2.0). Each dismantled reactor represented a point on the growth curve. The nutritional constituents were analyzed to yield complete culture profiles for 1 and 2-stage production processes.

The 1-stage, 15-day production regime which served as the control to the 2-stage production regime, was modified. Whereas a 10% (V/V) inoculum (200 mL) was used in the preliminary studies (section 3.3), a 20% (V/V) inoculum was used to inoculate the bioreactors in the 1-stage experiments to see if improved growth and/or production would result as compared to the 2-stage cultures using the 10% inocula.

3.5.1. GROWTH STUDIES OF THE 1-STAGE IMMOBILIZED CULTURES

The growth and nutrient consumption curves of 2-L surface immobilized *C.roseus* cells are presented in Fig. 3.13. Nitrate was depleted at an average rate of 3.73 mM/d by day 6 as was similarly shown in Fig. 3.4 for 1-stage suspension cultures.

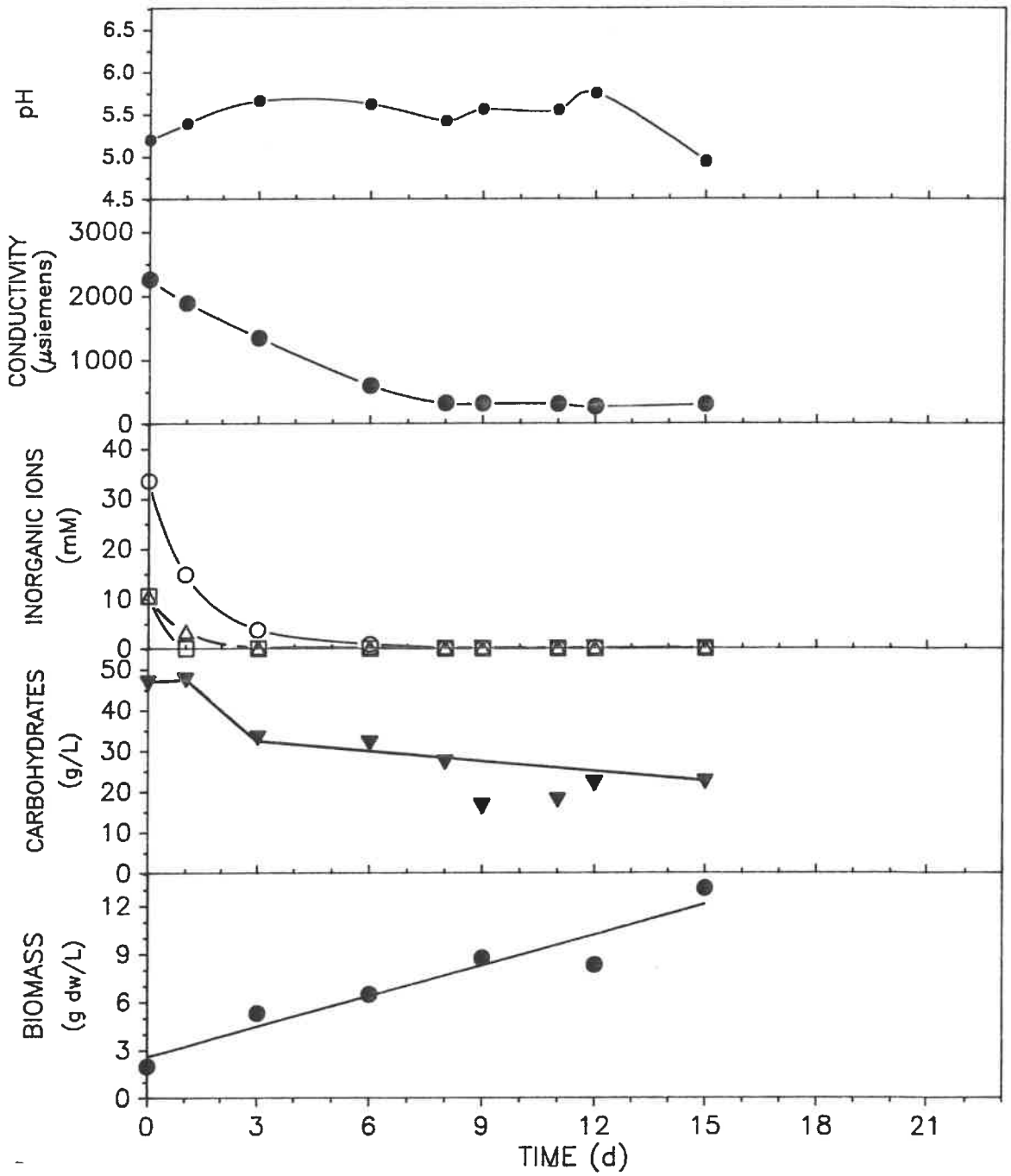


FIGURE 3.13 GROWTH AND SUBSTRATE CONSUMPTION CURVES OF 1–STAGE IMMOBILIZED MCR17 CULTURES. (○) NITRATE; (△) AMMONIUM, (□) INORGANIC PHOSPHATE (x 10^{-2} mM).

Inorganic phosphates (0.106 mM) were completely assimilated 1 day after inoculation while ammonium was consumed (3.45 mM/d) by day 3. The medium conductivity decreased linearly from 2300 to 600 μsiemens by day 6. It levelled off to 300 μsiemens until day 15 while no detectable inorganic nutrients were left in the medium except for the carbohydrates. The carbohydrates were taken up most rapidly (ca 4.67 g/L·d) for the first 3 days as sucrose was completely hydrolyzed to glucose and fructose (not shown in Fig. 3.13). After day 3 the cells consumed the sugars at about 1.68 g/L·d until 48% of the carbohydrates remained in the medium on day 15. The culture pH increased for 3 days (5.2 - 5.6) and fluctuated slightly around 5.6 for 9 days then it dropped to 4.85 on day 15. The pH (5.6) of the immobilized cultures was generally lower than that of the 1-stage suspension cultures (pH 6.0) between day 3 and 12.

The immobilized cultures attained a maximum biomass concentration of about 13 g dw/L, whereas the suspension cultures had attained 22 g dw/L, within 15 days. The immobilized cells grew linearly at a rate of 0.63 g dw/L·d (Table 3.6) which was 58% faster than for immobilized cells in 1B5 medium at 0.40 g dw/L·d (Table 3.1), but 2.1 times slower than 1-stage suspension cultures (Table 3.4). The average carbohydrate consumption rate, which was 1.64 g/L·d, and the average biomass yield (41%) were 12% and 76% lower than the 1-stage suspension culture values. However the average nitrate (373 mM/d) and the ammonium consumption rates (3.45 mM/d) were 200% and 127% faster in the immobilized cultures, respectively, than in the suspension cultures.

TABLE 3.6 GROWTH CHARACTERISTICS OF 1 AND 2-STAGE IMMOBILIZED CULTURES OF *CATHARANTHUS ROSEUS.*

GROWTH PARAMETER	1-STAGE	2-STAGE	
	APM	AB5	APM
MAXIMUM BIOMASS CONCENTRATION (g dw/L)	13.0	6.10	10.5
SPECIFIC GROWTH RATE (d^{-1})	0.15	0.22	0.04
AVG. GROWTH RATE (g dw/L · d)	0.63	0.85	0.24
AVG. CARBOHYDRATE CONSUMPTION RATE RATE (g/L · d)	1.64	2.70	1.67
AVG. BIOMASS YIELD (g dw/g sugar)	0.41	0.31	0.26
AVG. NITRATE CONSUMPTION RATE (mM/d)	3.73	2.68	1.01
AVG. AMMONIUM CONSUMPTION RATE (mM/d)	3.45	0.92	0.33

3.5.2. INDOLE ALKALOID SPECTRA OF 1-STAGE IMMOBILIZED CULTURES

The 1-stage immobilized cultures produced 6 indole alkaloids and the precursor tryptamine (Fig. 3.14) whereas the 1-stage suspension cultures yielded only 4 products and the precursor (Fig. 3.5). Maximum alkaloid yields were higher in immobilized cultures for strictosidine lactam (3.5 μg/g dw), ajmalicine (80 μg/g dw) and serpentine (20 μg/g dw). The maximum tryptamine yield was 25 μg/g dw on day 3 which was 6 times lower than in suspension cultures.

No significant amount of alkaloids were detected for the first 6 days except for tryptamine (25 μg/g dw) on day 3 and serpentine (10 μg/g dw) on day 6. The suspension cultures had already produced large amounts of tryptamine (100 - 150 μg/g dw), strictosidine lactam (25 μg/g dw) and epivindolinine (15 - 35 μg/g dw) for the same given period. When the immobilized cultures were exhausted of inorganic nutrients by day 6, simple and increasingly complex alkaloids appeared between day 9 and 15. Tabersonine (15-40 μg/g dw) and vindolinine (1.1 -1.7 mg/g dw) appeared on day 12 and 15 while they were absent in the 1-stage suspension cultures. Only a trace amount of epivindolinine (20 μg/g dw) was detected on day 15, which was 43% lower than in suspension cultures (Fig. 3.5).

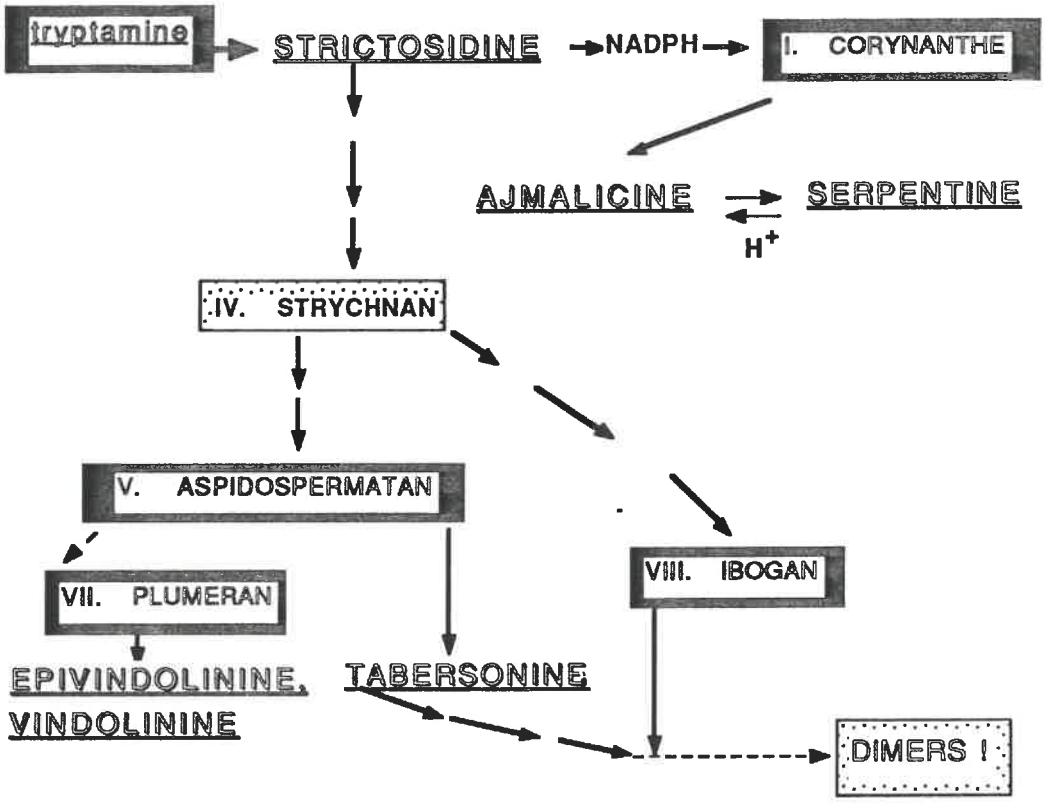
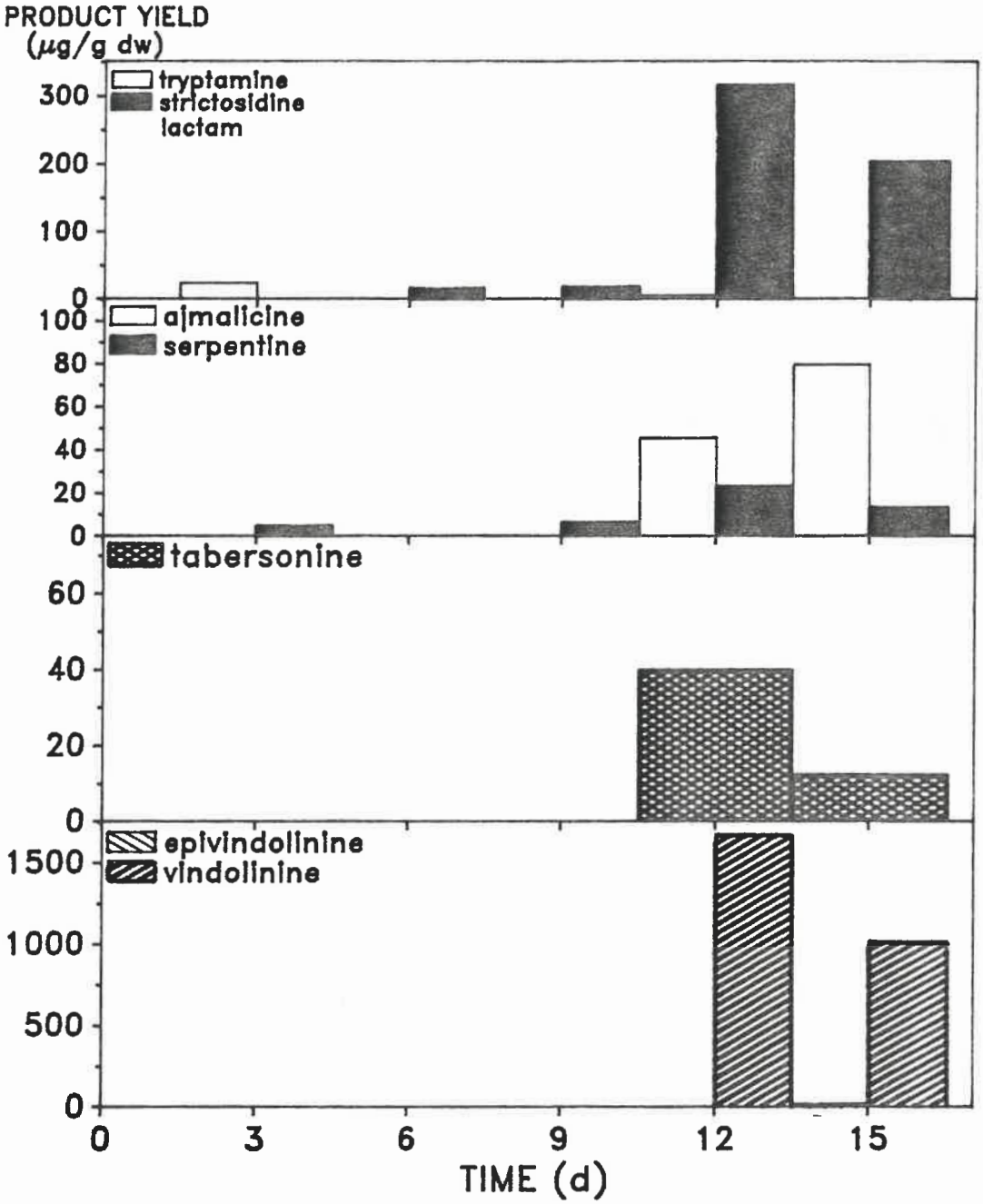


FIGURE 3.14 Alkaloid spectra detected in 1-stage surface immobilized C.roseus cultures.


PRODUCT YIELD
(μg/g dw)
tryptamine
strictosidine lactam
ajmalicine
serpentine
tabersonine
epivindolinine
vindolinine
300
200
100
0
100
80
60
40
20
0
60
40
20
0
1500
1000
500
0
0
3
6
9
12
15
TIME (d)

The immobilized cultures released 5 products to the medium (Fig. 3.15) while the suspension cultures had released 3 (ajmalicine, serpentine, epivindolinine). Both systems released tryptamine where the latter had 2 times more (90 μg/L) than in immobilized cultures. With the exception of tryptamine, the extracellular alkaloid concentrations were the highest on day 15 after the immobilized cells had generally produced maximal yields on day 12. Released alkaloids represented 17%, 1.4%, 17%, 15% and 3% of total strictosidine lactam, ajmalicine, serpentine, tabersonine, and vindolinine produced, respectively, in the immobilized cultures. The beginning of cell lysis in the suspension cultures may have contributed to the low level of extracellular ajmalicine and serpentine (15 and 10 μg/L, resp.) in the medium. No ammonium was detected at the end of the culture for the immobilized cells which had been observed in the former cultures.

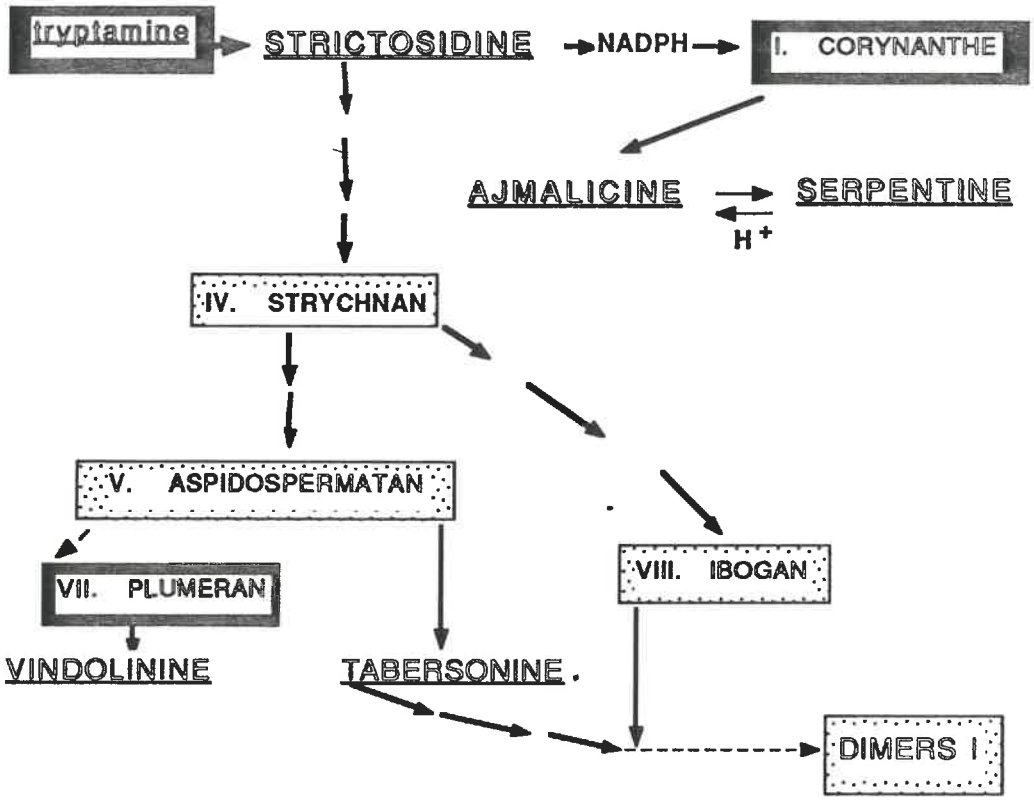
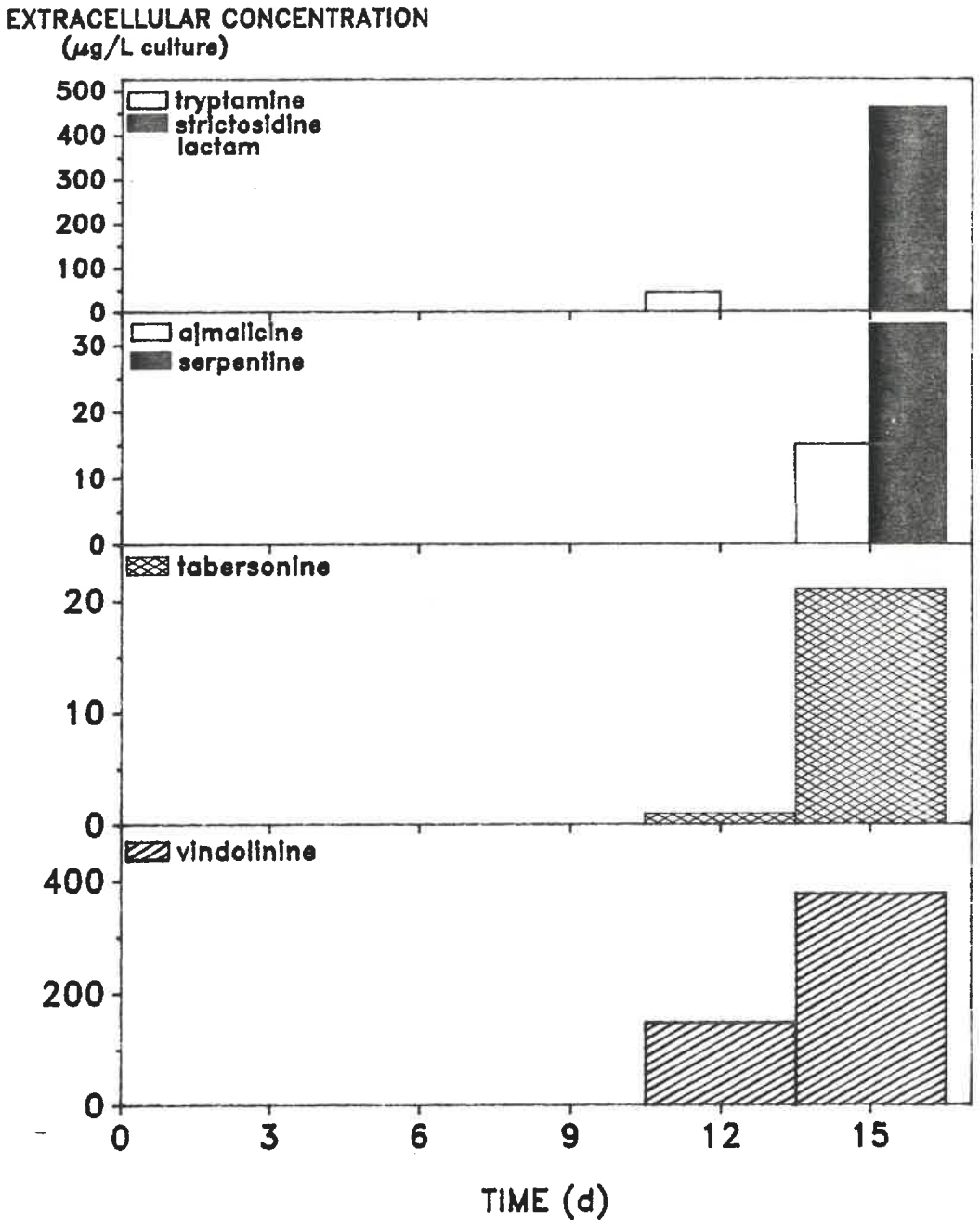


FIGURE 3.15 Alkaloid spectra detected (underlined) in 1-stage surface immobilized C.roseus cultures.


EXTRACELLULAR CONCENTRATION
(μg/L culture)
500
400
300
200
100
0
tryptamine
strictosidine lactam
30
20
10
0
ajmalicine
serpentine
20
10
0
tabersonine
400
200
0
vindolinine
0
3
6
9
12
15
TIME (d)

3.5.3. PRODUCTIVITY OF 1-STAGE IMMOBILIZED CULTURES

The TIA estimates of the 1-stage immobilized cultures showed that total alkaloid production increased at a rate of 3.3 mg/L·d, (correlation coefficient r = 0.99) as depicted in Fig. 3.16a. The summation of identified alkaloids (ca. 2167 μg/g dw) in section 3.5.2 represented, at most, up to 41% of the TIA value (ca. 5000 μg/g dw) on day 12.

The TIA content was 25% greater on day 15 in the immobilized cultures, as compared to the 1-stage suspension cultures, as it increased linearly (r= 0.80) from 2 to 5 mg/g dw (Fig. 3.16c). TIA values increased with the immobilized biomass concentrations and the volumetric productivity varied at 3.6 ±0.1 mg/L·d while specific productivities were 0.37 ±0.06 mg/g dw·d between day 9 and 15 (Table 3.7). The volumetric productivity was 36% greater and the specific productivity was 48% less than the values of the 1-stage suspension cultures.

The extracellular TIA values represented 17%, 67%, 30%, 19% and 10% of the total TIA produced from day 3 to 15, inclusively (Fig. 3.16b). The identified alkaloids detected in the medium (ca. 921 μg/L) represented, at most, 0.02% of the TIA value (ca. 5 mg/L) on day 15. The extracellular TIA values were of the same order of magnitude in 1-stage suspension and immobilized cultures at around 20 mg/L.

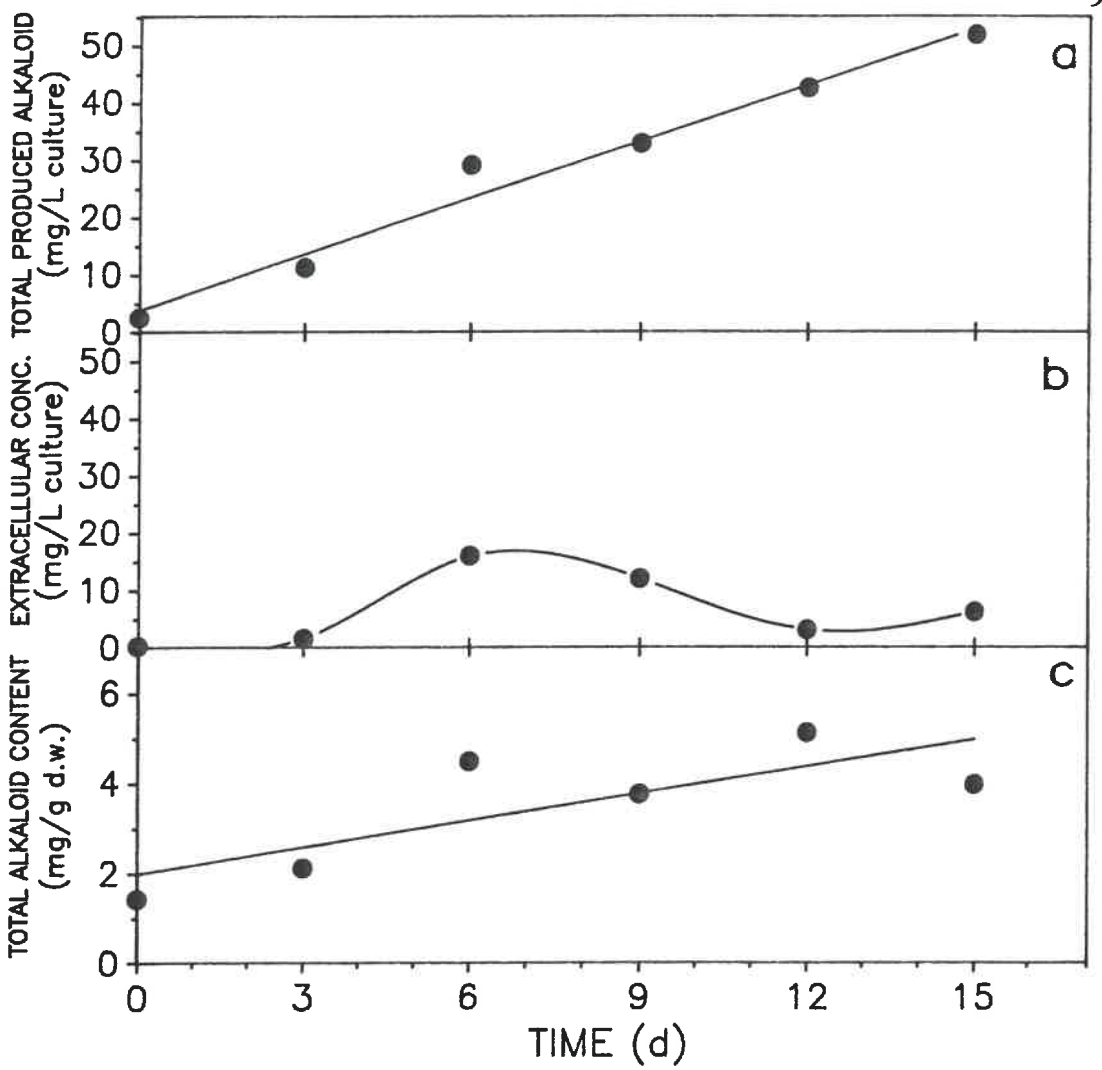


FIGURE 3.16 TOTAL INDOLE ALKALOID (TIA) QUANTIFICATION OF 2-L IMMOBILIZED MCR17 CULTURES IN THE 1-STAGE PROCESS. a. TOTAL PRODUCED ALKALOIDS REPRESENT INTRA-AND EXTRACELLULAR QUANTITIES PRODUCED BY THE CELLS. b. EXTRACELLULAR CONC. IS EXPRESSED ON A PER LITRE CULTURE BASIS. c. TOTAL ALKALOID CONTENT IS THE PRODUCT YIELD OF THE INTRA-AND EXTRACELLULAR QUANTITIES PER GRAM DRY WEIGHT.

TABLE 3.7 COMPARISON OF VOLUMETRIC AND SPECIFIC PRODUCTIVITIES IN 2 L IMMOBILIZED CULTURES OF *C. ROSEUS.*

PARAMETER	TIME (d)	1-STAGE PROCESS	2-STAGE PROCESS
VOLUMETRIC PRODUCTIVITY (mg/L • d)	3	3.8	20.0
	6	4.9	5.2
	9	3.7	4.0
	12	3.6	3.9
	15	3.5	3.4
	18	-	10.0
	21	-	2.2
SPECIFIC PRODUCTIVITY (mg/g dw • d)	3	0.7	4.4
	6	0.7	0.8
	9	0.4	0.5
	12	0.4	0.6
	15	0.3	0.4
	18	-	1.0
	21	-	0.2

3.5.4. GROWTH OF 2-STAGE IMMOBILIZED CULTURES

During the 6-day growth stage the immobilized cells increased to 6.10 g dw/L at an average growth rate of 0.85 g dw/L·d. Figure 3.17 shows the growth curve and nutrient consumption profiles while Table 3.6 shows the growth characteristics of the 2-stage immobilized cultures. One day after inoculation the conductivity increased by about 700 µsiemens (2400 - 3100 µsiemens), which coincided with a pH drop of 0.1 unit. This was not observed in the suspension culture experiments nor in the 1-stage immobilized cultures. The pH and conductivity changes observed on the first day could be explained by the release of acidic vacuolar contents and other ionic constituents from the plant cell inocula when they were transferred to fresh nutrient-rich AB5 medium; this event has been previously documented by Van der Heijden et al.[7]. Nonetheless the inorganic phosphate (253 µM) and ammonium (2.76 mM) uptake was complete by day 1 and they did not concur with the increased conductivity (Fig. 3.17).

Between days 1 and 6, the conductivity declined to about 1500 µsiemens while 80% of the nitrate (2.68 mM/d) and 65% of the carbohydrates (2.7 g/L·d) were consumed by the cells. The average biomass yield (0.31 g dw/g sugar) was 24% less, during the growth stage, than the yield (0.41 g dw/g sugar) of the 1-stage immobilized cultures.

Complete sucrose hydrolysis was observed 24 hours after APM had replaced the spent AB5 medium on day 6 and the carbohydrate consumption rate was the greatest (12 g/L·d) in this period.

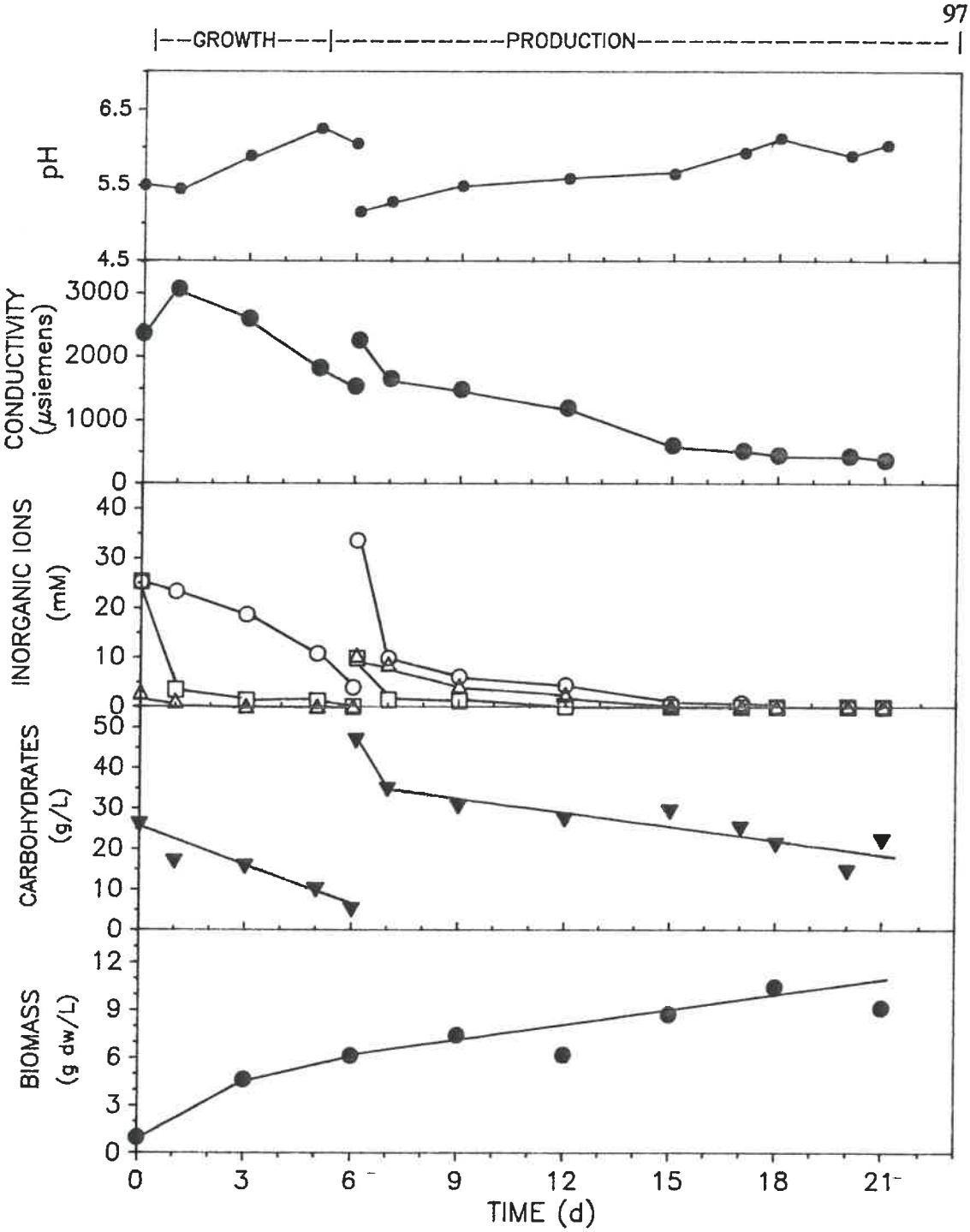


FIGURE 3.17 GROWTH AND SUBSTRATE CONSUMPTION CURVES OF 2-STAGE IMMOBILIZED MCR17 CULTURES. (○) NITRATE; (△) AMMONIUM; (□) INORGANIC PHOSPHATE (x 10^{-2} mM).

From day 7 to 21 the carbohydrate consumption rate declined to an average of 0.93 g/L·d. The cells had also assimilated 70% of the nitrate (23.5 mM), 90% of the inorganic phosphate (74 μM) 24 hours after APM transfer. Nitrate was depleted on day 15 at a rate of 0.67 mM/d while ammonium was consumed at 0.33 mM/d. The rest of the inorganic phosphate (8.2 μM) had been consumed by day 12 (6 days after APM transfer, Fig. 3.17). The medium conductivity decreased as the nitrate was assimilated by the cells. After the inorganic nutrients were exhausted from the medium on day 15, the conductivity levelled off to 300 μsiemens until day 21.

The immobilized biomass increased by 72% (6.1 to 10.5 g dw/L) at an average growth rate of 0.24 g dw/L·d. The specific growth rate of the cells during the 15-day production stage (0.04 d^{-1}) was 4 times slower than during the 6-day growth stage (0.22 d^{-1},Table 3.6). Although the 2-stage cultures were better supplied with carbohydrate (235%), nitrate (175%), ammonium (133%) and inorganic phosphates (316%), the biomass yields and consumption rates were generally less than the 1-stage cultures. The average biomass yields, nitrate and ammonium consumption rates (averaged of growth and production stages) were 30%, 50% and 82% less than those of the 1-stage immobilized cultures, respectively. The growth and consumption rates were 200% to 300% less in the immobilized cultures than in the suspension cultures.

3.5.5. INDOLE ALKALOID SPECTRA OF 2-STAGE IMMOBILIZED CULTURES

The alkaloid spectra was broader and higher in yield by 3 to 15-fold in the 2-stage immobilized cultures than in the 1-stage cultures. Whereas 6 alkaloids were found in the latter cultures, 10 indole alkaloids were produced by the 2-stage cultures (Fig. 3.18). Vallesiachotamine, which was detected in 2-stage suspension and immobilized cultures, could only be qualitatively identified (by its chromagenic reaction in TLC) since no standard was available to quantitate it by HPLC. The precursor tryptamine and simple alkaloids such as strictosidine lactam, ajmalicine, serpentine and yohimbine, were produced in increasing quantities during the growth stage, as nutrients were depleted from the medium. Most of the yohimbine (ca. 70 μg/g dw) produced on day 6 was found in the medium (410 μg/L, Fig. 3.19). On day 15, when nitrate was consumed, more complex alkaloids such as tabersonine, lochnerinine, epivindolinine, vindolinine and catharanthine, were produced during the production stage. Maximum alkaloid yields occurred on day 18 for 7 out of 10 alkaloids that were found in the cultures (Fig. 3.18). The catharanthine yield (5 μg/gdw) was maximal on day 15 while the lochnerinine yield (30 μg/g dw) was the greatest on day 21. Table 3.8 shows the days when maximal alkaloid yields and extracellular concentrations (Table 3.9) occurred for suspension and immobilized cultures in both production processes.

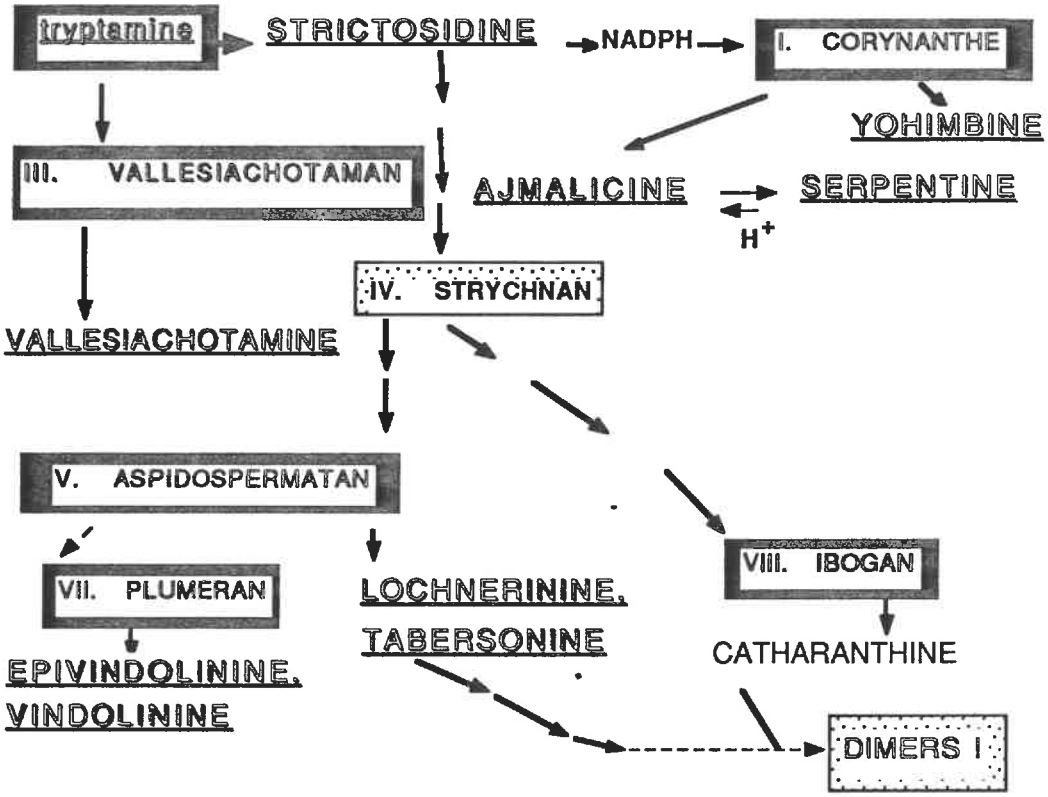
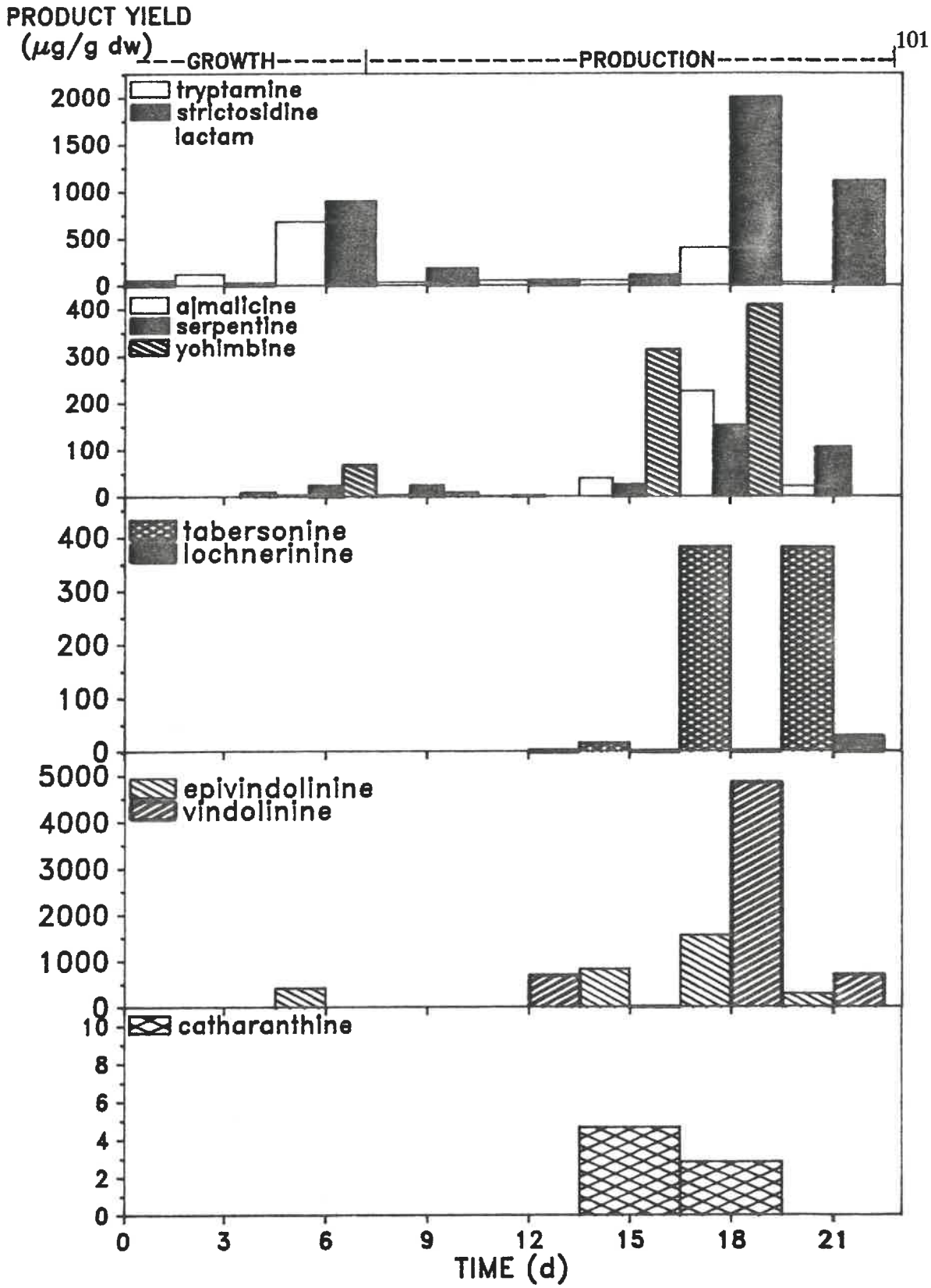


FIGURE 3.18 Alkaloid spectra detected (underlined) in 2-stage surface immobilized C.roseus cultures.


PRODUCT YIELD
(μg/g dw)
GROWTH
PRODUCTION
tryptamine
strictosidine lactam
ajmalicine
serpentine
yohimbine
tabersonine
lochnerinine
epivindolinine
vindolinine
catharanthine
0
500
1000
1500
2000
100
200
300
400
3000
4000
5000
2
4
6
8
10
0
3
6
9
12
15
18
21
TIME (d)

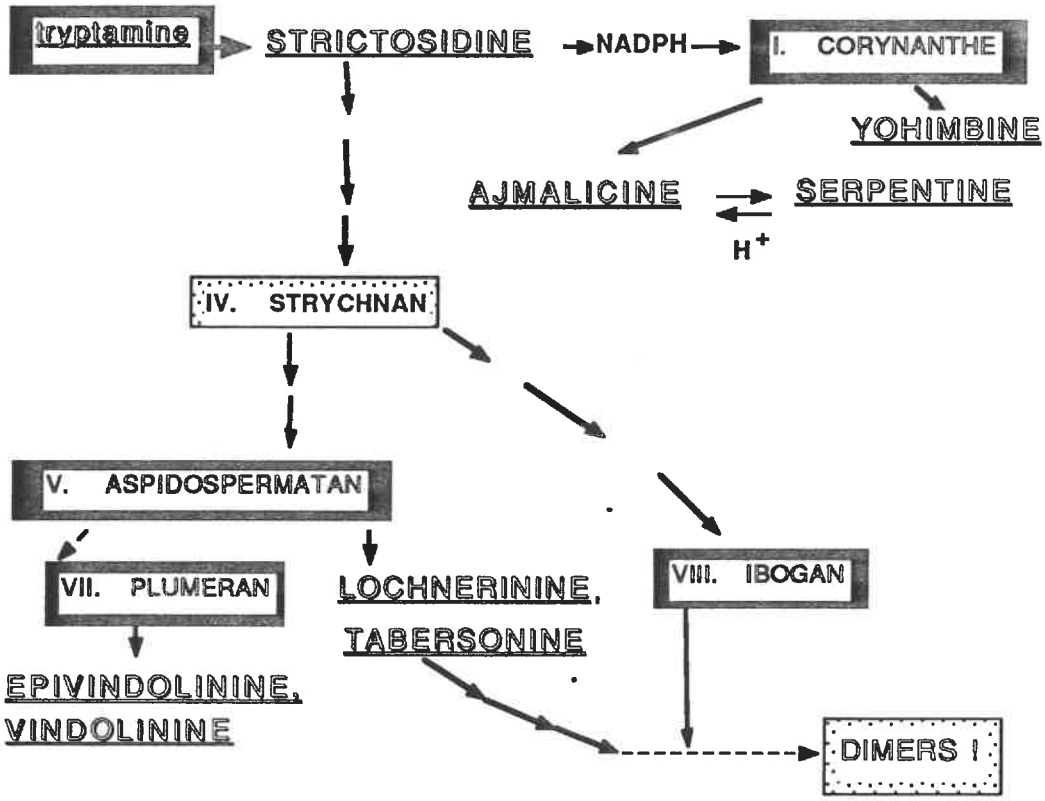


FIGURE 3.19 Indole alkaloids detected (underlined) in the medium of 2-stage surface immobilized C.roseus cultures.

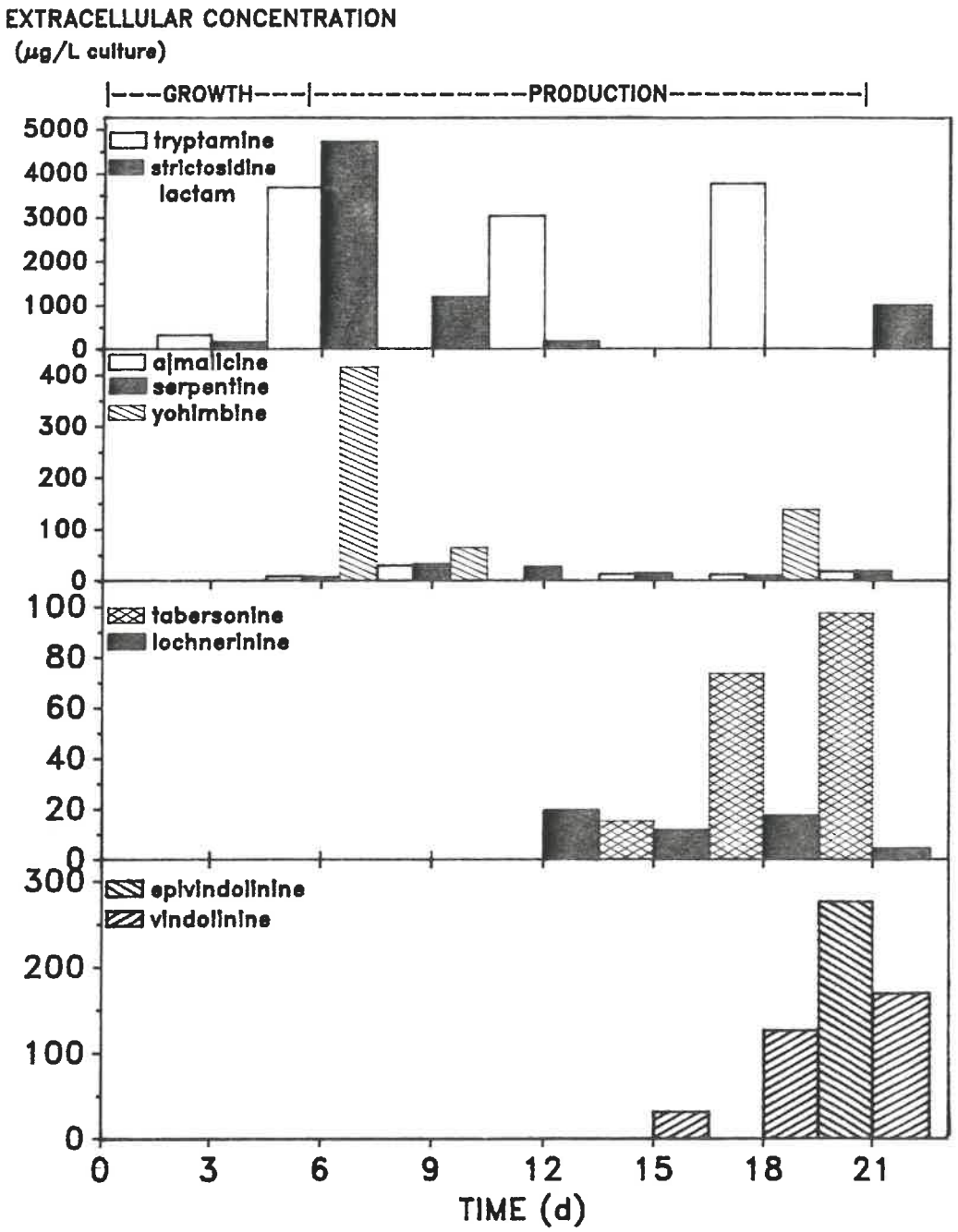

EXTRACELLULAR CONCENTRATION
(µg/L culture)
|---GROWTH---|----------PRODUCTION----------|
tryptamine
strictosidine lactam
ajmalicine
serpentine
yohimbine
tabersonine
lochnerinine
epivindolinine
vindolinine
5000
4000
3000
2000
1000
0
400
300
200
100
0
100
80
60
40
20
0
300
200
100
0
0
3
6
9
12
15
18
21
TIME (d)

TABLE 3.8 MAXIMUM ALKALOID YIELDS BY *CATHARANTHUS ROSEUS* CELL LINE MCR17.

YIELD (ug/g dw) (day) / ALKALOID	200 mL SUSPENSION CULTURES		2 L IMMOBILIZED CULTURES	
	1-STAGE	2-STAGE	1-STAGE	2-STAGE
TRYPTAMINE	150 (3)	220 (18)	25 (3)	680 (6)
STRICTOSIDINE LACTAM	80 (15)	3960 (9)	315 (12)	2000 (18)
AJMALICINE	5 (9)	340 (9)	80 (15)	230 (18)
SERPENTINE	25 (15)	680 (18)	20 (12)	150 (18)
YOHIMBINE	-	350 (21)	-	400 (18)
TABERSONINE	-	150 (21)	40 (12)	380 (18)
LOCHNERININE	-	50 (9)	-	30 (21)
EPIVINDOLININE	35 (3)	560 (21)	20 (15)	1500 (18)
VINDOLININE	-	-	1670 (12)	4800 (18)
CATHARANTHINE	-	35 (21)	-	5 (15)
TIA YIELD (mg/g dw)	6 (3)	40 (15)	4.5 (6)	18 (18)
TIA CONC. (mg/L)	50 (3)	700 (15)	53 (15)	185 (18)

TABLE 3.9 MAXIMUM EXTRACELLULAR ALKALOID CONCENTRATIONS BY *CATHARANTHUS ROSEUS* CELL LINE MCR17.

CONCENTRATION (ug/L) (day) / ALKALOID	200 mL SUSPENSION CULTURES		2 L IMMOBILIZED CULTURES	
	1-STAGE	2-STAGE	1-STAGE	2-STAGE
TRYPTAMINE	90 (3)	1050 (12)	45 (12)	3800 (18)
STRICTOSIDINE LACTAM	-	400 (18)	460 (15)	4700 (6)
AJMALICINE	10 (15)	50 (9)	15 (15)	30 (9)
SERPENTINE	5 (15)	730 (12)	30 (15)	30 (9)
YOHIMBINE	350 (21)	4600 (21)	-	410 (6)
TABERSONINE	-	-	20 (15)	100 (21)
LOCHNERININE	-	-	-	20 (12)
EPIVINDOLININE	-	400 (21)	-	280 (21)
VINDOLININE	280 (3)	-	380 (15)	150 (21)
CATHARANTHINE	-	-	-	-
TIA CONCENTRATION (mg/L)	30 (15)	65 (18)	20 (6)	60 (3)

Like the 2-stage suspension cultures, no catharanthine was detected in the medium. The 2-stage suspension and immobilized cultures both contained corynanthean alkaloids (ajmalicine, serpentine, yohimbine) and epivindolinine in the medium, however the latter cultures had also contained extracelluar quantities of tabersonine (100 μg/L), lochnerinine (20 μg/L) and vindolinine (150 μg/L, Fig. 3.19). Tryptamine, strictosidine lactam and tabersonine were found in the medium at 5 to 10-fold higher concentrations in the 2-stage immobilized cultures than in the 1-stage immobilized cultures. Similar quantities of ajmalicine and serpentine (15 - 30 μg/L) were found in the media of both production processes.

3.5.6. PRODUCTIVITY OF 2-STAGE IMMOBILIZED CULTURES

The TIA estimates for the 2-stage immobilized cultures were about 3.5 times greater than for the 1-stage immobilized cultures (Fig. 3.20). Maximal alkaloid concentrations (185 mg/L) and content (17.2 mg/g dw) were observed on day 18 when 7 out of 10 identified alkaloids had peaked. A peak was observed on day 3 during the growth stage which was 13% of the maximum peak on day 18 although not many alkaloids were identified then. While nitrate was present in the medium, the TIA concentration remained constant around 50 mg/L while the total alkaloid content was about 6 mg/g dw between day 6 and 15. After complete consumption of the inorganic nutrients on day 15, the TIA concentration and content increased by 3.5 and 3-fold, respectively, on day 18. The identified alkaloids accounted for 37% of the TIA value. The TIA pattern for immobilized cultures was reproduced twice to within 25% for the production peak on day 18 (Fig. 3.21). The peak on day 3 of the 2-stage process was not observed in the first experimental series because the point was not measured then.

The cells which entered stationary phase concerted their energies toward alkaloid synthesis. With no nitrate available in the medium they probably utilized their nitrogen stores to produce indole alkaloids which appeared to be maximal 3 days after nitrate depletion from the medium. It appeared that the production peak was followed by a 3-fold decline in the following 3 days.

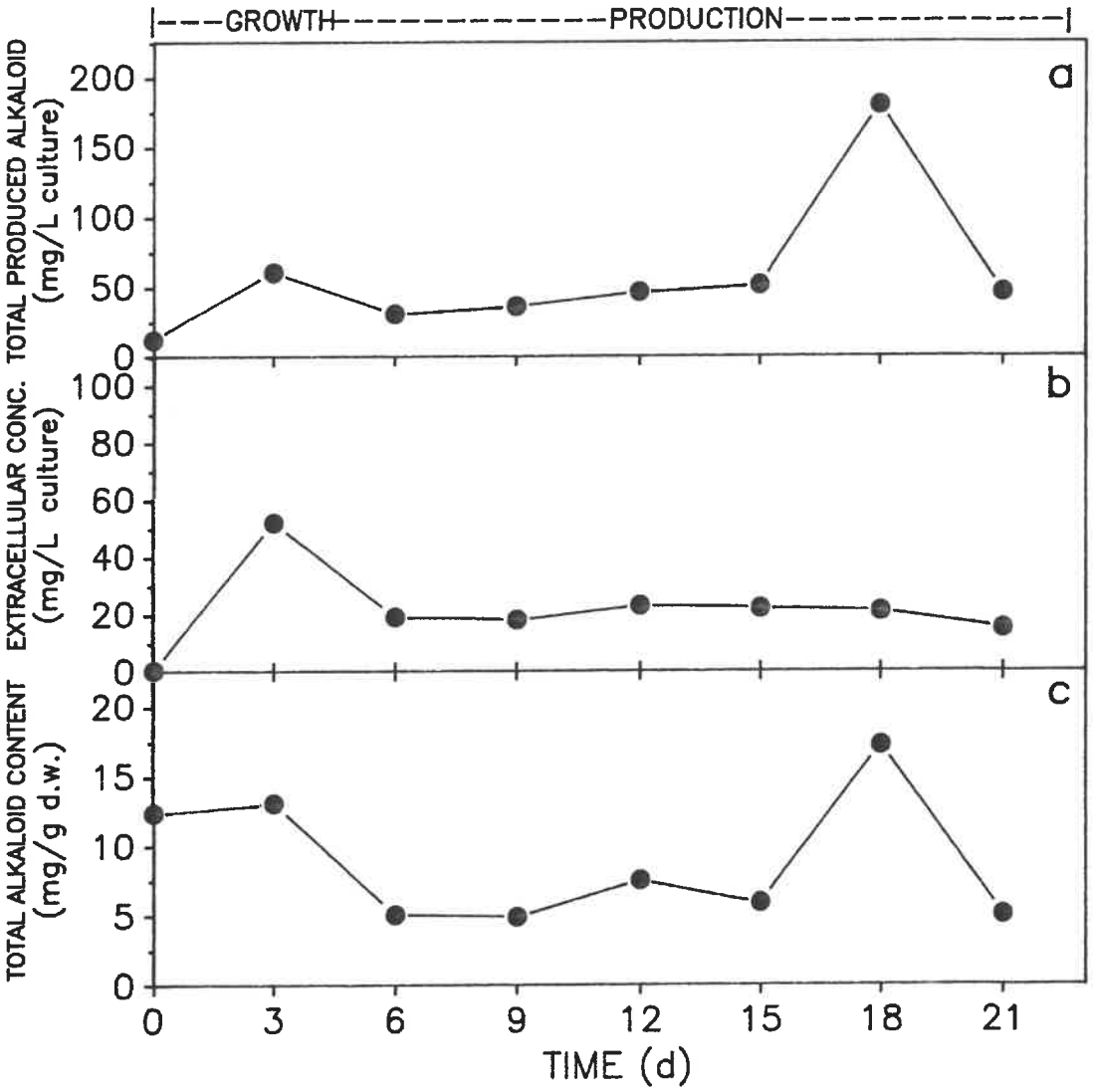


FIGURE 3.20 TOTAL INDOLE ALKALOID (TIA) QUANTIFICATION OF 2-L IMMOBILIZED MCR17 CULTURES IN THE 2-STAGE PROCESS. a. TOTAL PRODUCED ALKALOIDS REPRESENT INTRA-AND EXTRACELLULAR QUANTITIES PRODUCED BY THE CELLS. b. EXTRACELLULAR CONC. IS EXPRESSED ON A PER LITRE CULTURE BASIS. c. TOTAL ALKALOID CONTENT IS THE PRODUCT YIELD OF THE INTRA-AND EXTRACELLULAR QUANTITIES PER GRAM DRY WEIGHT.

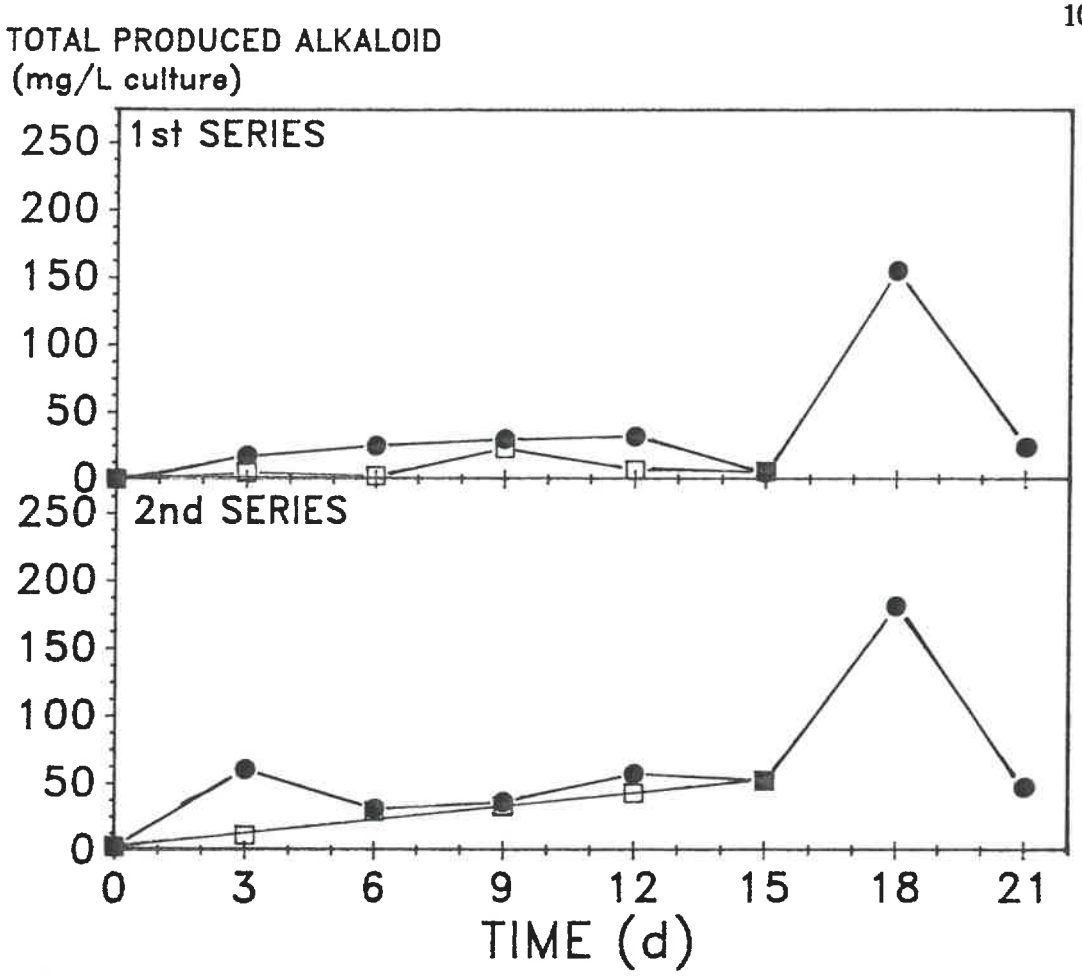


FIGURE 3.21 TWO EXPERIMENTAL SERIES ON ALKALOID PRODUCTION BY 2 L IMMOBILIZED MCR17 CULTURES BASED ON TOTAL INDOLE ALKALOID (TIA) ESTIMATIONS OF INTRACELLULAR AND EXTRACELLULAR CONTENTS.

(□) 1-STAGE PROCESS; (●) 2-STAGE PROCESS.

The cells' productivity may have been hampered by the presence of the potentially toxic metabolites and/or the alkaloids may have been degraded by the cells in order to furnish a source of reduced carbon and nitrogen substrates.

Table 3.7 shows that a 3-fold increase in volumetric (10 mg/L·d) and specific productivities (0.96 mg/g dw·d) were indicative of biosynthetically active cells. For the 1-stage cells, the productivities remained constant between day 9 and 15. A 4-fold decline in volumetric (2.2 mg/L·d) and specific productivity (0.24 mg/g dw·d) on day 21 may have indicated the occurrence of product turnover and/or degradation by the cells. The maximal volumetric (57 mg/L·d) and specific productivity (2.7 mg/g dw·d) of 2-stage suspension cultures occurred on day 12 which were 14 and 4.5-fold higher than for the immobilized cultures respectively. However on day 18, where the latter system had maximal productivities, the specific productivity was only 25% less than the suspension culture value.

The extracellular TIA concentrations remained constant around 20 mg/L between day 6 and 18 despite having found richer product spectra as compared to the 1-stage cultures. Extracellular TIA estimates represented 60% of TIA produced between day 6 and 15, and 40% of TIA produced on day 21. On day 18 the extracellular TIA concentration was 11% of the TIA produced. Extracellular TIA concentrations in both 1 and 2-stage immobilized cultures were similar (10 -20 mg/L) and represented between 10 and 60% of total TIA produced whereas the extracellular TIA of the suspension cultures represented about 10% of the total TIA produced. Identified alkaloids (ca. 1567 μg/L) detected in the media of the 2-stage immobilized cultures represented about 12% of the TIA value (ca. 13 mg/L) on day 21.

CHAPTER IV

4.0. DISCUSSION

Inorganic phosphate is a growth limiting nutrient for it furnishes a source of energy (ATP) and a nucleotide substrate for DNA synthesis[7,94]. In these experiments it was not obvious that it was the growth limiting substrate since it was assimilated so rapidly by the cells. However it is one of parameters that is varied in discerning basal growth media and alkaloid production media as shown in the Materials and Methods section (Table 2.0). The APM formulation was composed of more ammonium, less phosphate and nitrate than the B5 to promote slower growth rates (specific, absolute). Phosphate and ammonium monitoring did not delineate the growth and alkaloid production cycles which was clearly discernable in the nitrate monitoring. Nitrate depletion demarcated the onset of alkaloid production unlike the other media constituents. In combination with higher initial sucrose concentration (as an osmotic stressor) nitrate depletion contributed to the nutritional stress to induce the cells to cease growth and commence alkaloid production at least 3 days later.

4.1. SUSPENSION CULTURE GROWTH

In both production processes, the suspension cultures attained virtually the same biomass concentrations despite the higher inoculation ratio for the 1-stage process and the added nutrients in the 2-stage process. Carbohydrates appeared to be the growth-limiting nutrient which was observed for *C.roseus* cells growing in B5 medium (1B5 or AB5). Fowler[135] had reported similar observations for *C.roseus* cells cultured in growth media with sucrose

concentrations less than 50 g/L. Cells that had grown in APM only reflected higher biomass yields than the cells grown in AB5 and APM together due to the higher initial sucrose concentration in APM (50 g/L).

However, from the alkaloid production experiments (suspension and immobilized cultures), nitrate also appeared to be the growth-limiting nutrient; upon depletion the growth rate slowed down as the cells entered stationary phase. The standard curve in Figure 4.1 shows that there is a linear correlation between conductivity and decreasing ammonium nitrate concentrations, at room temperature. It can be inferred from this curve that nitrate contributes largely to the conductivity of the medium in a linear fashion. Conductivity and nitrate readings have been used as markers of the cell viability[136,137,138]. The re-appearance of ammonia at the end of the cultures was likely indicative of the onset of cell lysis[138] which occurred in the 1-stage suspension cultures. Continuous readings of conductivity changes of the plant cell cultures could approximate the culture status with regard to the nutritional elements and cell viability thus reducing the frequency of sampling.

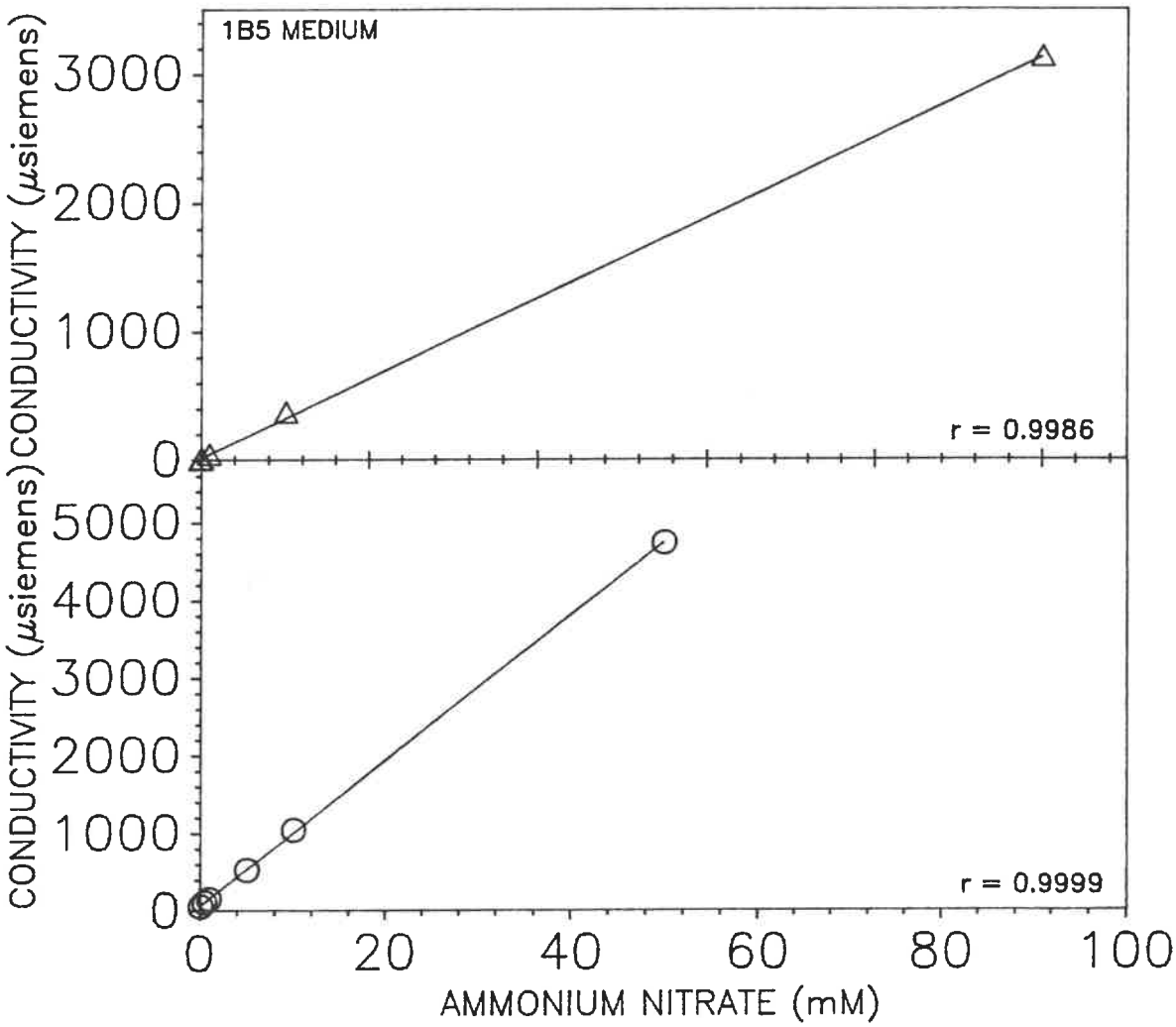


FIGURE 4.1 STANDARD CURVES OF CONDUCTIVITY VERSUS NITRATE CONCENTRATION. TOP GRAPH REPRESENTS CONDUCTIVITY READINGS OF SERIALLY DILUTED 1B5 MEDIUM.

BOTTOM GRAPH REPRESENTS CONDUCTIVITY READINGS IN RELATION TO VARIOUS AMMONIUM NITRATE CONCENTRATIONS.

4.2 IMMOBILIZED CULTURE GROWTH

The immobilized *C.roseus* cells yielded biomass concentrations that were about 50% of the maximum values in suspension cultures. Although the 2-stage process allowed the cells to be 50 to 570% richer in nutrients, relative to the 1-stage process, the maximal biomass concentrations were nevertheless similar. Compared to the suspension cultures, the immobilized cells exhibited smaller specific growth and average carbohydrate consumption rates in both production processes. These differences show that suspension and immobilized cultures did not necessarily utilize their substrates similarly. The immobilized cells were limited by a factor that was not subjected to the suspension cultured cells. Factors that contributed to the immobilized cells' lower growth, compared to suspensions, were manifold. The immobilized cells were sparged and the may have experienced CO_2 stripping[70], which could have compromised the overall productiveness of the immobilized cultures, as compared to the surface-aerated suspension cultures (Table 4.0). Because plant cells are 95% water by volume their growth is spatially limited to within the layers of the matrix[139] while the cells at the outer surface have no limitation except for the reactor walls. The immobilized and suspended cells easily depleted the inorganic nutrients from the media. Residual carbohydrates were always found in the immobilized cultures whereas in the 2-stage suspension cultures they were consumed.

TABLE 4.0 SUMMARY **200 mL SUSPENSION CULTURES VS 2 L IMMOBILIZED CULTURES**

PARAMETER	1-STAGE	2-STAGE	1-STAGE	2-STAGE
Xmax. (g_{dw}/L)	22	21	13	10
μ (d^{-1})	0.25	0.30	0.15	0.13
$Y_{x/s}$ (g_{dw}/g sugar)	0.59	0.42	0.41	0.28
max. TIA (mg/L cult.)	50 (3)	700 (15)	53 (15)	185 (18)
# known pdts detected	4	10^+	6	10^+
$\Sigma Y_{P/X}$ ($\mu g/g_{dw}$) of known products	105 (15)	4350 (9)	2045 (12)	9460 (18)
# extracellular pdts detected	4	5	5	8
AERATION MODE	surface	surface	sparging	sparging
k_La (h^{-1})	~10	~10	~6	~6

+: TLC analysis actually showed the presence of at least 20 product components.

The dissolved oxygen could not be systematically measured at the time but oxygen was the likely a limiting factor in the immobilized cultures, across the biofilm layers, while it was not for the suspension cultures. With the growth of the biofilm the transfer capacity (k_La) across the thickening plant cell biolayer decreases. The suspended cells probably received sufficient oxygen, in the absence of CO_2 stripping, so that they could oxidize most of their carbon sources for growth, and eventually alkaloid production. The immobilized cultures thus did not completely consume their carbohydrates, nor attain biomass concentrations comparable to the suspensions.

4.3. ALKALOID PRODUCTION IN SUSPENSION CULTURES

It was observed that the doubled inoculation ratio (20% V/V) for the 1-stage cultures (suspension or immobilized) did not lead to better growth than the 2-stage cultures although the latter process period was 40% longer. A doubled inoculation would have carried over 2 times more 2,4D to the 1-stage cultures, than the 2-stage cultures, thus impairing secondary metabolite synthesis[21,113,116,134]. The difference in 2,4D however, between the 2 cultures was only 0.1 ppm and it is unlikely that it could have impaired production in the 1-stage cultures. The changeover of AB5 to APM further diluted the residual 2,4D which probably led to higher production, specific and volumetric activities in the 2-stage cultures, when compared to the 1-stage cultures.

Rokem et al.[141], as well as others[21,32,114] stressed the importance of having an active plant biomass in order to obtain substantial secondary metabolite synthesis. The 2-stage

suspension cultures were richer in nitrate by 376%, relative to the 1-stage suspension cultures, while the nitrate content was 174% greater in the 2-stage immobilized cultures than in the 1-stage immobilized cultures. The ammonium content was 18% higher in the 2-stage than in the 1-stage suspension cultures while the 2-stage immobilized cells received 30% more than the 1-stage immobilized cultures. The higher nitrogen content probably contributed to the larger alkaloid yields and spectra in the 2-stage cultures overall. The 2-stage cultures achieved biosynthetically active cells which was indicated by their lower biomass yields as compared to the 1-stage process. Less carbohydrates contributed to the biomass for they may have been consumed by active cells for energy, and/or they may have been utilized[77] for secondary metabolite synthesis. The 2-stage cultures exhibited a more biosynthetically active biomass as compared to the 1-stage cultures since total indole alkaloid production was shown to be 10 times greater in the former cultures. Nutrient consumption was complete in the 2-stage cultures while 50% of the carbohydrates remained in the 1-stage cultures. Marked alkaloid production generally occurred 3 days after nitrate depletion, in both regimes.

For both suspension and immobilized cultures, indole alkaloid quantification was not a simple task due to the limited availabilty of alkaloid standards, whereas about 100 alkaloids actually produced by *C.roseus*[86,130]. TIA measurements have been used by other researchers[30,73,77] to quantitate global alkaloid production, as was done in these experiments. Lee et al[30] had reported TIA yields ranging from 9 to 13 mg/g dw while the yields in these alkaloid production experiments were lower but within range (avg. 6 mg/g dw). Their individual alkaloid yields (catharanthine, ajmalicine) only corresponded to within 6 to 8%

of TIA estimations. The highest correlation between identifiable alkaloid and TIA estimates, in these experiments, was 60 - 70% for the 2-stage immobilized cultures.

4.4. ALKALOID PRODUCTION IN IMMOBILIZED CULTURES

The immobilized cultures generally had lower biomass yields, growth and nutrient consumption rates than the suspension cultures. According to the TIA evaluation of the 1 and 2-stage production processes, the suspension cultures had higher volumetric and specific productivities in the 2-stage process while the productivities were similar for 1-stage suspension and immobilized cultures. The 2-stage immobilized cultures yielded about 4-fold less alkaloids than the suspension cultures.

It is not unprecedented that the suspension cultures had been more productive than their large-scale counterparts[12,20,66,73]. A study of bioreactor configurations and their comparison to shake flask suspension cultures demonstrated that the latter were more productive than the former[25]. Recent experiments, done by Archambault et al.[139], showed that the k_La of shake flask-grown cultures was in the order of 10 h^{-1}. Smart et al[140] reported that non-oxygen limited suspension cultures attained 14 g dw/L, with a specific growth rate of 0.38 d^{-1}, at a k_La of 15 h^{-1}. The k_La value of 6 h^{-1} for the SIPC cultures was well below the shake flask values which likely manifested lower biomass yields, specific growth rates, and productivities, when compared to the suspension cultures.

Different metabolic activities, such as growth or production, do not exert the same oxygen demands[25,141-143]. Wagner et al.[25] showed that anthraquinone production, by *Morinda*

citrifolia cells, was reduced by 60% when the aeration rate was reduced from 0.5 to 0.17 VVM, despite no change in the biomass yields. Kobayashi (1989)[144] also observed reduced berberine production by immobilized *Thalictrum minus* cells when the aeration rate was reduced. The oxygenation of the suspension was probably more efficient due to the continual transient biofilm formation on the inside flask walls[40] thus increasing the area-to-volume ratio of this culture system (the interfacial surface of the liquid biofilm inside the shake flask walls could not be measured in this project).

The k_La of the immobilized system could be improved by increasing the oxygen transfer rate (OTR) in order to augment the productivity of this culture system.

Compared to the suspension cultures, the surface immobilized *C.roseus* cultures were compromised by oxygen limitation at the interface between the bulk liquid and the biofilm surface. As compared to the suspension cultures, the immobilized cells exerted higher oxygen demands (pers. comm. J. Archambault), during the alkaloid production experiments, which could not be met with the existing impedance across the biofilm layer; furthermore, only air was supplied to the cells. No oxygen was supplemented to the air supply, which was done in C.M. Pouyez's master's[145] project. When the bulk oxygen concentration was increased from 10 to 90%, the immobilized cells had higher specific growth rates and complete carbohydrate consumption[145]. Had the bulk oxygen concentration been increased in these alkaloid production experiments (by oxygen-suplemmented air), the total indole alkaloid production (TIA estimates) might have been equivalent or perhaps greater than the yields of the 200 mL suspension cultures, due to the increased oxygen availability to the cells.

A biosynthetic progression of indole alkaloid synthesis was observed in suspension and immobilized cultures. Generally, a tendency for decreasing tryptamine yields was observed while increasing quantities of the other alkaloids appeared in the cells as well as in the medium. From day 18 to 21 the product yield declined as simple alkaloid products were degraded and/or converted to more complex alkaloids (ie. tabersonine, lochnerinine, catharanthine) in the 2-stage process.

The presence of alkaloids in the medium may have been influenced by proximity of their pK_a values (Appendix III) and the pH of the culture medium. In both production processes the corynanthé (ajmalicine, serpentine, yohimbine) and the plumeran (epivindolinine, vindolinine) alkaloid classes were frequently detected in the medium. Only the pK_a of serpentine (pK_a=10) was not close to the culture medium pH (ca. pH 6.0). Its presence in the medium could be explained by the oxidation of ajmalicine[10], or possibly by the existence of alkaloid-specific transporters[41,42]. The presence of extracellular tryptamine (pK_a=10) could be explained by the latter reason also. Only the 1 and 2-stage immobilized cultures were shown to release the aspidospermatan alkaloids (tabersonine, lochnerinine) to the medium while none of them were detected in the suspension cultures. Perhaps the tissue-like organization of the immobilized *C.roseus* cells favored the release of a broader alkaloid spectrum to the medium, which was not endowed to the suspension cells.

In spite of the limited alkaloid standards availability, TLC analysis revealed that at least 20 compounds were produced by the 2-stage regime while only about 1 dozen compounds were produced by the 1-stage regimes. Only in the 2-stage immobilized cultures an intermediate compound between tabersonine and vindoline could be tentatively identified

by its chromagenic reaction: vindorosine. No traces of vindoline, vinblastine nor vincristine could be detected in the suspension-and immobilized cultures since tabersonine could not be completely converted to vindoline. In the absence of the chloroplast-associated enzyme, N-methyl transferase, products before vindoline accumulated, such as tabersonine and vindorosine. Without vindoline formation catharanthine could not be coupled to the former (via cytoplasmic peroxidases) to produce vinblastine and vincristine. Spots on the TLC plates, that were in the proximity of the 2 latter alkaloids, were scraped off, concentrated and redissolved in methanol to be subsequently analyzed by FAB (Fast Atom Bombardment) mass spectrometry in Dr. Michael Mancini's laboratory at BRI. Unfortunately the masses corresponding to the dimeric alkaloids (ca. 812 M.W.) could not substantiate the presence of these highly-valued products isolated from the TLC plates.

CHAPTER V

5.0. CONCLUSIONS

5.1. CONCLUDING REMARKS

The 2-stage production process produced the same biomass as the 1-stage process in either suspended or immobilized cultures. The 2-stage process favoured higher alkaloid production (at least 20 compounds detected) than the 1-stage production process (less than 20 compounds) in suspension and immobilized cultures. The added growth stage nutritionally primed the cells so that they were more productive than the 1-stage cultures.

Nitrate concentrations, as well as the conductivity changes in the medium can delineate the growth and secondary metabolic activities of suspension or immobilized cells. Since biomass sampling is limited to harvested cultures for immobilized systems, the conductivity and nitrate readings can be correlated to the growth status of the immobilized cells.

The oxygen availability to the surface immobilized *C.roseus* cells was probably limiting and consequently their productivity, with respect to total indole alkaloid (TIA) estimates, was compromised as compared to suspension cultures. The suspension cultures produced 4 times more TIA than the immobilized cultures. However the immobilized cultures had higher specific productivities than the suspension cultures, in both production processes, with respect to the known alkaloid products (Table 4.0)

Since the suspension cultures were not sparged the immobilized plant cells could have experienced stripping of carbon dioxide[70] from their cell surfaces. Without risking CO_2 stripping, the surface immobilized cells should have been aerated at greater than 0.07 VVM (k_La = $6h^{-1}$) to match at least the k_La ($10h^{-1}$) of shake flask systems, in order to attain

equivalent productivities. An oxygen-supplemented air supply[145] with ca. 2% (V/V) CO_2 might meet the respiratory demands of the immobilized plant cells during secondary metabolite production, as long as the dissolved oxygen concentration does not fall below 30%[145].

Catharanthus roseus cell line MCR17 could be characterized by its propensity to produce large quantities of the vincosan (strictosidine lactam) and plumeran family (epivindolinine and vindolinine) alkaloids. Unfortunately no dimeric alkaloids such as vinblastine nor vincristine were detected. The monomeric half, catharanthine, was quantitated only in 2-stage suspension (0.004% dw) and immobilized cultures (0.0005% dw), whereas 0.0017% dw is usually found in intact plants[18]. Tabersonine, a biogenetic precursor of vindoline, accumulated in the late stages of the 2-stage process since it could not be converted into the latter alkaloid.

5.2. RECOMMENDATIONS

Future alkaloid production experiments should involve the growth of inoculum cells in AB5 medium (devoid of 2,4D), or rinsing 1B5-grown cells in 2,4D-free media prior to inoculation in order to minimize any interfering effects of residual 2,4D on production.

Analytical techniques have to be improved with respect to sensitivity and specificity; the presently used extraction procedures are time-consuming and have low efficiencies which lead to product losses[101]. Because many chemically-related products can be produced by any given plant cell strain, individual product concentrations are often found in the nano to

microgram range. It was shown that solid-liquid partitioning[133] of medium samples yielded improved alkaloid purification as compared to the widely used liquid-liquid partitioning technique[37].

The 2-stage immobilized cultures were shown to release broader alkaloid spectra to the medium than the 2-stage suspension cultures. Product-specific adsorbents could be added to the immobilized culture medium in order to recuperate released alkaloids during peak production periods. Alternatively, the medium could be exchanged for fresh production medium as a fed-batch process. These factors could conceivably relieve feedback inhibition (by potentially toxic metabolites), product re-uptake and/or degradation, and subsequently increase the productivity of the culture system.

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APPENDIX I

GAMBORG'S (50) B5 GROWTH MEDIUM

MACRONUTRIENTS	mg/L
NH_4NO_3	0
KNO_3	2500
$CaCl_2$-$2H_2O$	150
$MgSO_4$-$7H_2O$	250
KH_2PO_4	0
$(NH_4)_22SO_4$	134
NaH_2PO_4-H_2O	150

MICRONUTRIENTS	mg/L
KI	0.75
H_3BO_3	3.0
$MnSO_4$-$4H_2O$	0
$MnSO_4$-H_20	10
$ZnSO_4$-$7H_2O$	2.0
Na_2MoO_4-$2H_2O$	0.25
$CuSO_4$-$5H_2O$	0.025
$CoCl_2$-$6H_2O$	0.025
EDTA-FERRIC SALT	43.0
SUCROSE	20000

VITAMINS AND HORMONES	mg/L
Inositol	100
Nicotinic Acid	1.0
Pyridoxine hydrochloride	1.0
Thiamine hydrochloride	10.0
Indole-Acetic Acid	0.1752 (AB5)
2,4 dichlorophenoxyacetic acid	1.0 (1B5)

APPENDIX II

ZENK`S (20) ALKALOID PRODUCTION MEDIUM

MACRONUTRIENTS	mg/L
NH_4NO_3	720
KNO_3	950
$CaCl_2$-$2H_2O$	220
$MgSO_4$-$7H_2O$	185
KH_2PO_4	68
SUCROSE	50000
SEQUESTRENE (330 Fe)	55.9

MICRONUTRIENTS	mg/L
KI	0.375
H_3BO_3	2.4
$MnSO_4$-H_20	7.0
$ZnSO_4$-$7H_2O$	4.05
$(NH_4)_6MoO_7O_{24}$-$4H_2O$	0.0925
$CuSO_4$-$5H_2O$	0.01
GLYCINE	2.0
FOLIC ACID	0.5
BIOTIN	0.05

VITAMINS AND HORMONES	mg/L
Myo-Inositol	100
Nicotinic Acid	5.0
Pyridoxine hydrochloride	0.5
Thiamine hydrochloride	0.5
Indole-Acetic Acid	0.1752
6-Benzylaminopurine	1.125

APPENDIX III

pKa VALUES OF THE INDOLE ALKALOIDS (133)

ALKALOID	pKa
Tryptamine	10.2
Strictosidine lactam	?
Ajmalicine	6.3
Serpentine	10.0
Yohimbine	6.34
Tabersonine	5.5 - 6.0
Lochnerinine	5.5
19-Epivindolinine	?
Vindolinine	?
Catharanthine	6.8
Vindoline	5.5
Vinblastine	5.4, 7.4
Vincristine	4.68

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