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STIMULATED INDOLE ALKALOID RELEASE BY EXTRACELLULAR PH MODIFICATION OF SURFACE IMMOBILIZED *CATHARANTHUS ROSEUS* PLANT CELL CULTURES

par

Barbara A. Jardin

DÉPARTEMENT DE GÉNIE CHIMIQUE

ÉCOLE POYTECHNIQUE

MÉMOIRE EN VUE DE L'OBTENTION
DU GRADE DE MAITRE ES SCIENCES APPLIQUÉES (M.Sc.A.)
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ÉCOLE POLYTECHNIQUE

Ce mémoire intitulé:

STIMULATED INDOLE ALKALOID RELEASE BY EXTRACELLULAR PH MODIFICATION OF SURFACE IMMOBILIZED *CATHARANTHUS ROSEUS* PLANT CELL CULTURES

présenté par: Barbara Jardin

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

a été dûment accepté par le jury d'examen constitué de:

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M. Gerhardt Braunegg ....Ph. D., membre

ABSTRACT

Vacuolar entrapment of indole alkaloids remains the factor which limits the use of immobilisation technology for plant cell culture, which, otherwise, represents a more efficient system than suspension culture for this biomass. In this initial study, the release of indole alkaloids from *Catharanthus roseus* was evaluated in Alkaloid Production Medium using surface immobilised and suspension cultures.

Relative unstimulated release in immobilised cultures reached 10 to 50% of total alkaloid production which was higher than the 10 to 25% release observed in suspension cultures. Further enhancement of release in the immobilised system was attempted by altering the extracellular pH. An acidic solution (0.1N HCl) or an alkaline solution (0.1N KOH) was added (2% v/v) periodically to different cultures.

Alkalinization (from pH 5.6 to 8.6) and acidification (from pH 5.6 to 4.3) caused rapid transient variations in the extracellular pH. In both cases these variations provoked significant increases in total alkaloid (5-10$mg \cdot L^{-1}$ to 15$mg \cdot L^{-1}$), ajmalicine (0 to 0.29$mg \cdot L^{-1}$) and serpentine (0 to 0.20$mg \cdot L^{-1}$) release without apparent cell lysis.

SOMMAIRE

Le piégeage vacuolaire des métabolites secondaires demeure un facteur qui limite l'utilisation de l'immobilisation des cellules végétales à grande échelle pour la production, qui en soi-même, facilite la culture de cette biomasse par rapport à la culture en suspension. Cette étude initiale évalue le relargage des alcaloïdes indoliques produit par *Catharanthus roseus* en milieu de production Zenk (APM).

Le relargage relatif, non-stimulé des alcaloïdes observé en culture immobilisée a atteint des niveaux de 10% à 50% de la production totale ce qui surpasse les niveaux de 10% à 25% observés en suspension. Une amélioration du relargage des alcaloïdes en culture immobilisée a été tenté en modifiant le pH du milieu de culture. Des solutions acides (0.1N HCl) ou alcalines (0.1N KOH) ont été ajoutées (2%v/v) périodiquement aux différentes cultures . L'acidification (pH 5.5 à 4.3) et l'alcalinisation (pH 5.8 à 8.5) ont causé des changements rapides et temporaires de pH. Dans les deux cas, une augmentation du relargage des produits totaux (5-10$mg \cdot L^{-1}$ à 15$mg \cdot L^{-1}$) ajmalicine (de 0 à 0.29 $mg \cdot L^{-1}$) et serpentine (0 à 0.20$mg \cdot L^{-1}$) a été provoqué sans lyse apparante ou diminution de la viabilité cellulaire. Le relargage représente environ 100% des alcaloïdes produits.

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Nomenclature

a : Gas liquid interfacial area per unit volume (cm^2/cm^3).

A^- : Concentration of free complexing sites on a given substance which binds an alkaloid intracellularly.

AB5 : Gamborg's B5 medium supplemented with 5µM of Indole Acetic Acid.

APM : Zenk's alkaloid production medium.

C : Dissolved O_2 concentration in the culture medium (mmole $\cdot$ L^{-1}).

C^* : Saturated liquid concentration of O_2 (0.25 mmole $\cdot$ L^{-1}).

C_e : Extracellular alkaloid concentration.

CHO_f : Final carbohydrate concentration (g $\cdot$ L^{-1}).

C_i : Intracellular alkaloid concentration.

DAT : Enzyme used in final step of vindoline synthesis, Acetyl CoenzymeA deacetylvindoline-O-acetyltransferase.

dw : Biomass dry weight (g dw).

EtAc : Ethyl acetate.

k_L : Mass transfer coefficient (cm $\cdot$ h^{-1}).

k_v' : Association constant of an alkaloid for a complexing substance.

IAA : Auxin hormone indole acetic acid.

NMT : S-Adenosylmethionine: 16-methoxy-2,3-dihydroxy-3-hydroxy tabersonine- N-methyltransferase.

OTR : Oxygen transfer rate (mmole O_2 . h^{-1}).

OUR : Oxygen uptake rate (mmole O_2 $\cdot$ h^{-1}).

pH_e : Extracellular pH.

pH_i : Intracellular pH.

pK_a : Acidity constant.

Q_{O2} : Specific O_2 uptake rate (mmole $O_2 \cdot g\ dw^{-1} \cdot h^{-1}$).

SAM : S-adenosylmethionine.

SIPC : Surface immobilized plant cells.

SS : Strictosidine synthetase.

TDC : Tryptophan decarboxylase.

W_{m+x} : Weight of dry biomass and support structure (g).

W_s : Weight of support structure (g).

X : Plant biomass concentration ($g \cdot L^{-1}$).

X_{IPC} : Total dry weight biomass immobilized on matrix (g).

$Y_{x/s}$: Biomass yield on carbohydrate (g dw biomass / g carbohydrate consumed).

1B5 : Gamborg's B5 medium supplemented with 4.5 μM 2,4-D.

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

chapter 1

1.0 INTRODUCTION

Plants are capable of many functions. They are abundant on earth and are used for a variety of purposes. Plants are cultivated as a nutritious part of animal diets, dried herbs are used to alleviate illnesses and some extracts such as curare and hemlock have been used as poisons. Plant chemicals

are commonly obtained by cultivation in fields around the world. This type of cultivation falls victim to many factors such as environment, infestation and government controls which hinder regular, dependable supply.

In vitro cultivation of plant cells has been carried out for almost 30 years. Use of defined culture medium has lead to reproducible culture methods. Callus cultures on solid agar medium have been initiated from a variety of plant parts. These cells are generally totipotent, they are maintained in a undifferentiated state but they possess the genetic capacity of the original plant. Plant cell suspension cultures have been generated from calli and they can be cultivated under conditions where light, aeration, mixing and temperature have been controlled.

Plant cell characteristics such as low doubling times, high shear sensitivity and high viscosity at elevated cell densities have caused problems in the development of large scale suspension cultures systems. Immobilized culture systems were introduced to protect the cells from the shear sensitive environment and to improve mass transfer. However, wanted products from plant cells are generally stored intracellularly. This represents the main drawback of immobilized systems. Economic process development of this type of culture is dependent on product release by viable cultures.

The objective of this research project was to study the release of indole alkaloids from *Catharanthus roseus* immobilised cells. Indole alkaloids are believed to be stored in cells according to an ion trap mechanism whereby acidic conditions within the vacuole cause protonation of the lipophilic molecules, thus, altering their freely diffusible characteristics. Changes in extracelluar pH were evaluated as a means of decreasing intravacuolar entrapment of indole alkaloids. Both increases and decreases of extracellular pH were studied as the physio-chemical stimuli specifically aimed to establish new balance of alkaloid cellular compartmentation.

chapter 2

2.0 LITERATURE REVIEW

2.1 PRODUCTS FROM PLANTS

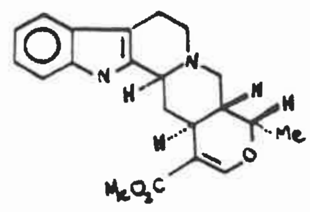
A divers number of products have been obtained from plant material. Mixtures of oils have been used to fabricate perfumes. Natural colouring agents have been extracted and used in both the cosmetic and the food industries. In 1980, 25% of the pharmaceuticals produced in the U.S. were composed of plant products or derivatives(88).

Many plant products used in the pharmaceutical industry are secondary metabolites. These substances are biosynthesized by complex pathways disassociated from primary, life-sustaining activities of the cells. Secondary metabolism involves the formation of extraneous substances unessential to normal cell function.

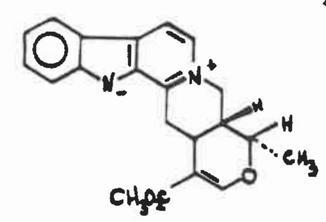
Indole alkaloids are a group of secondary metabolites which are derived from an essential amino acid called tryptophan. This essential amino acid combines with a mevalonate derivative (secologanin) to form strictosidine, the precursor of indole alkaloids. This set of bioreactions is prevalent in the *Catharanthus roseus* species. Products of interest are ajmalicine, serpentine vindoline, catharanthine, vincristine and vinblastine (FIGURE 1).

Ajmalicine is a hypotensive agent(4). In 1983, its annual market was estimated to be 3000-4000 kg. at a price of $\$1500 \cdot kg^{-1}$ (47). Vincristine and vinblastine are dimers of catharanthine and vindoline and are used in the treatment of a special form of leukaemia (1,25). The estimated market of these products was 20 kg per year at a price of $\$5$ million$\cdot kg^{-1}$(47).

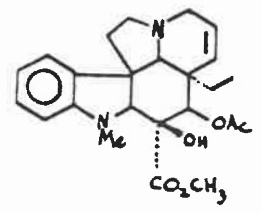
Many factors contribute to the problematic supply of these products from plants such as the following:



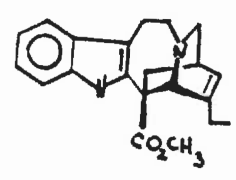
AJMALICINE



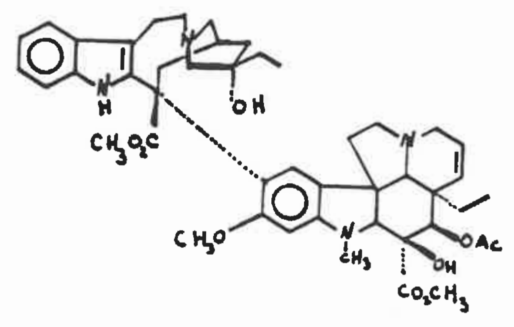
SERPENTINE



VINDOLINE



CATHARANTHINE



VINBLASTINE

FIGURE 1: Molecular structures of significant indole alkaloids produced by *Catharanthus roseus*.

1) variability in production from plant to plant;

2) environmental dependency;

3) long growth period;

4) lengthy extraction procedures;

5) low concentrations of products.

These factors prompted the evaluation of plant tissue culture as a more trustworthy source.

2.2 *IN VITRO* CULTURE OF PLANT CELLS

In vitro plant cell tissue culture began with the development of a suitable culture medium which sustained cell growth. Many types of defined media are presently available (68,69,94). A cell line can be derived from various plant organs such as leaves, roots or stems. The general procedure involves surface sterilization of the plant tissue in question and placing it on an appropriate solid nutrient medium. The cells proliferate forming a callus. Healthy callus cells can be placed on fresh agar medium monthly. If cell suspension cultures are targeted the callus can be transferred to liquid. The resulting suspension cultures allow for a greater supply of nutrients to come into direct contact with the cells, thus accelerating cell growth (69).

2.2.1 METHODS OF ALKALOID PRODUCTION IN *C. ROSEUS* CELLS

Many cell cultures are able to proliferate without producing significant levels of secondary metabolites. The failure of a culture to synthesize a given product may result from repression of enzyme synthesis and/or lack of enzyme activity (89,92). Selection and cultivation of cell lines which express high levels of desired products are essential for process development.

Callus tissue could be used to select high producing cell lines by analysing the product spectra of these cells. The biosynthetic capacity of high producing cell lines of *Catharanthus*

roseus cells has been shown to be unstable (4,3,24,30). This phenomenon could be attributed to genetic instability upon successive subcultivations (4,24). Therefore, resulting cell lines must be analyzed periodically to ensure efficient product synthesis.

2.2.2 CULTURE MEDIUM

Components of the growth medium 1B5 used for *C. roseus* cell lines were found to adversely influence the secondary metabolism. The synthetic auxin hormone, 2,4-D, was known to interfere with secondary metabolite formation (1,2,13,14,41,42,45). Indole Acetic Acid (IAA) was a suitable replacement. The use of higher sucrose concentration (5% vs. 2% in B5) stimulated secondary product synthesis. levels of inorganic phosphate were found to improve secondary metabolite production since phosphate decreased the activity of TDC (tryptophan decarboxylase) an enzyme involved in the synthesis of indole alkaloids (8,13,12,24,30,32,39,46). The presence of nitrate repressed the activity of enzymes involved in secondary metabolite synthesis (85,120).

Enhanced alkaloid synthesis was accomplished by modifying the composition of the growth medium. In 1978, an Alkaloid Production Medium (APM) was formulated with the following characteristics (2);

1) 2,4-D and/or NAA were replaced with 1µM of IAA and 5µM of N^6-benzyladenine;
2) 5% sucrose replaced 2% used in 1B5;
3) inorganic phosphate was low (37);
4) nitrate levels were slightly lower (18.4mM vs. 25mM);
5) ammonium levels were 4.5 times higher.

Other methods to increase secondary metabolite production have been studied. Elicitation was developed whereby a homogenised, autoclaved preparation of a fungus was added to the cell suspension. This caused a rapid (24-72 hours) accumulation of indole alkaloids as compared to the 15 days required for increases in *APM* (2). The drawback of this protocol was the deleterious effect of the elicitor on the viability of the cells. A browning effect occurred coincidentally with alkaloid accumulation and this was correlated with loss of viability (12, 96). Rapid removal of the elicitor prevented this deleterious effect.

Another method used to stimulate alkaloid synthesis by plant cell culture was the use of hairy root cultures. These cultures were developed by the infection of plant cells with *Agrobacterium rhizogenes* bacterial cells (97). The infection caused the prolific formation of hairy roots by introducing part of the R_i plasmid into the plant cell. The transformed plant cells become differentiated thus causing an increased degree of cellular organisation which is correlated with improved biosynthetic capacity (76, 97).

2.3 LARGE SCALE CULTURE OF PLANT CELLS

The following characteristics of plant cells must be taken into account to select a suitable bioreactor for large scale culture system:

1) long cultivation times are required because of slow growth rates(0.2 to 0.5 d^{-1});

2) innate aggregation of cells causes heterogeneous cultures (13,20,111,115);

3) large cell diameter (30-100μm)and rigid cell walls render the biomass shear sensitive(20,24,25);

4) high viscosity of suspension cultures when the cell density is over 10 g·dw L^{-1} (24) ie. 20 g dw · L^{-1} resulted in an apparent viscosity of 520 cps at 50 rpm.

Catharanthus roseus cell cultures displayed uncoupled growth and alkaloid synthesis (1,5,8,13,26). This characteristic suggested the development of a 2 stage production process whereby the first phase supported cell growth with a growth specific medium while the second stage focused on product synthesis.

Large scale cultivation of suspension cultures display inherent process problems. The high viscosities achieved at high cell densities cause mass transfer limitations, foaming and dead zones. Increased mechanical mixing was not an option for this shear sensitive biomass. Some exceptions exist for specifically designed impellers. The use of gentle airlift reactors (13,24,73) was explored; however, they were unsuitable for highly viscous fluids.

The set of problems involved with suspension cultures has led to the development of immobilized cultures. Immobilization has the following advantages:

1) aggregation is induced which increases cell stability and can increase cell differentiation;

2) biomass is protected from shear stress (52,67,111,115);

3) continuous processes can be more easily set up without washout of cells since the biomass is separated from the medium (52,67);

4) simpler process control and product recovery when release can be achieved;

5) biomass is conserved for longer periods of time (7 months) which allows for reuse as biocatalyst (52,111,115);

The immobilization of plant cells also displayed limitations. Aggregation caused mass transfer problems and rendered the cell population heterogenous due to concentration gradients in the biofilm(115). This indicated that growth must be closely regulated to avoid cellular nutrient starvation. Another limitation of immobilized cultures was that targeted products were generally

stored inside the cells, as was the case for *C. roseus* cells. Large scale immobilized plant cells could only be a viable process option if release and solubilization of products without damaging the biocatalysts can be achieved.

The immobilisation technique must be gentle and cannot interfere with the secondary metabolism of the cells (35,36). Many methods of immobilisation have failed due to the harshness of the conditions. The strategies employed in some cases involved treatment with chemical substances such as gluteraldehyde and acrylamide; these have proven toxic to the cells. Gel entrapment has been the most widely used procedure (21,52,99,116) in which cellular viability was retained. The significant disadvantage of this technique was the decrease of nutrient transfer due to the intervening membrane barrier occuring between the cells and the culture medium. There were also difficulties with large scale fabrication of the beads.

In 1983, adhesion was considered as an alternative method of immobilisation, which could not be relied (35) upon, however, due to weak and unpredictable adsorption (94); yet, in 1987 surface immobilisation on a man made geotextile had been successfully developed without the problems cited above. This method was very gentle and did not inhibit cell growth or product synthesis (36,127).

2.4 SPECIAL PROPERTIES OF THE PLANT VACUOLE

Plant vacuoles are the site of most secondary metabolite storage. The usefulness of immobilized plant cell cultures depends on the release of these products. A method to stimulate release of intravacuolar alkaloids can only be elucidated once general vacuolar function is understood.

A schematic representation of the plant cell is depicted in FIGURE 2. The golgi apparatus functions as a transporter of cell wall material to the plasma membrane; the cell wall components are then exocytosed. The vacuole is formed by fusion of small provacuoles which are themselves the result of pinched-off vesicles from the rough endoplasmic reticulum. Gradually, the vacuole increases in size to form a large aqueous compartment which contains sequestered cytoplasmic components. The constituents are autodegraded within the forming vacuoles which demonstrates the lysosomal nature of the vacuole.

A fundamental function of the plant vacuole is to protect the plant cell against environmental changes (34,110). For instance, large quantities of water and nutrients are stored within the vacuole. This limited reserve can sustain the plant during periods of drought for example.

Inorganic ions, small organic compounds and large organic substances are present in the vacuoles. These osmotically active substances contribute to turgor pressure of the cell. Turgor is an internal outward pressure caused by the imbalance of solutes between the internal and external environments of the cell (33). The intracellular compartment composed mostly of the vacuole, is hypertonic compared to the outside of the cell. More specifically the concentration of salts and other osmotically active substances is greater inside the cell. Thus, water will have a tendency to enter the cell. Lysis does not occur because of the resistant cell wall. Turgor is

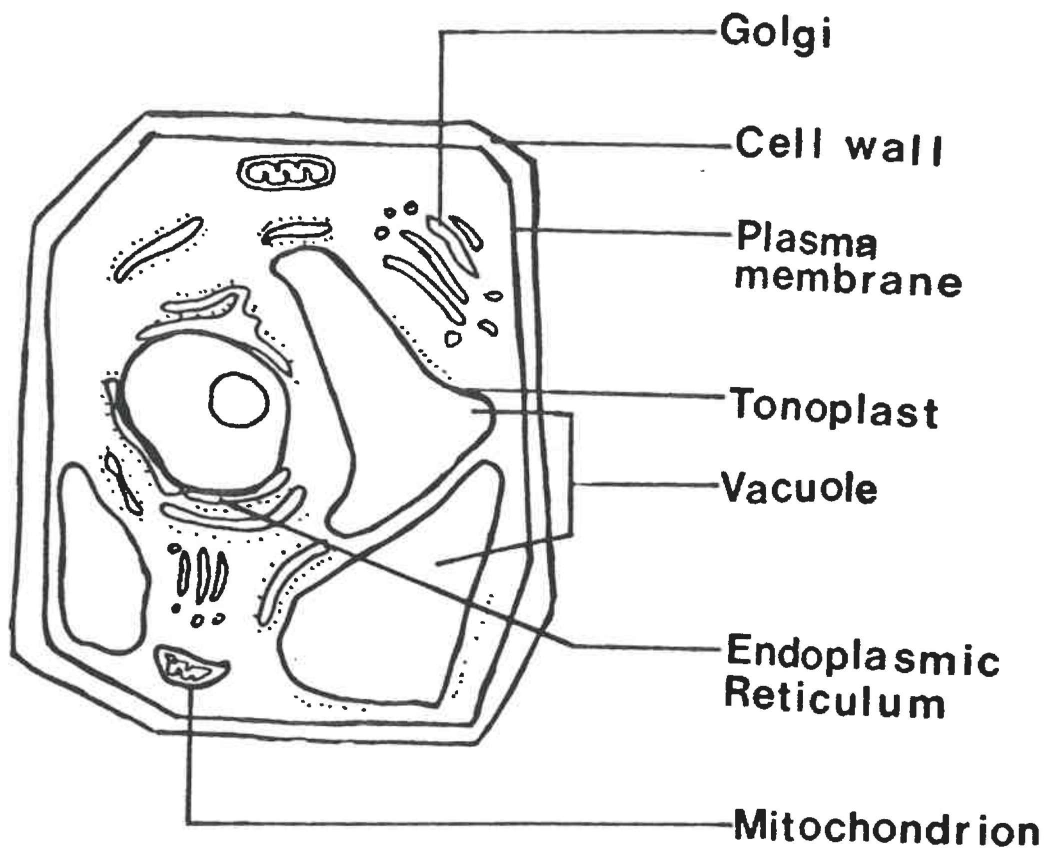


FIGURE 2: Schematic representation of the plant cell.

responsible for cell expansion, however, it does not regulate cell proliferation (110).

The following is a list of inorganic ions found in the vacuole:

1) Ca^{++},Mg^{++}, Cu^{++};

2) K^{+}, Na^{+}, H^{+};

3) Cl^{-}, PO_4^{--}, NO_3^{-}.

The flow of these ions is dynamic and the mechanisms involved in their transport into the vacuole is specific to each ion. Specific permeases are involved for nitrate and chloride accumulation. Membrane transport can also occur by cotransport systems where the transfer of one solute depends of the transport of another. The substances can either be moved in the same direction (symport) or in opposite directions (antiports). Hydrogen ions (H^{+}) are sequentially transferred in opposite directions to sodium and calcium ion transport (123).

Small organic substances include sugars, amino acids and tricarboxylic acidic cycle(TCA) intermediates such as malate and citrate (112). The TCA intermediates are compartmentalized because they would decrease the cytoplasmic pH which would interfere with normal enzyme function of the cell. The amino acids may be considered as storage forms of nitrogen for the plant cell.

Larger organic molecules found intravacuolarly include proteins and secondary metabolites. Most acid hydrolases involved in lysosomal activity are confined to the vacuole (34,106,112,113,114). The onset of senescence which occurs concomitantly with decreasing content of proteins, nucleic acids and other cellular components (110) is related to tonoplast breakdown. This process is known as autolysis. The content of the vacuole is therefore harmful to normal cellular metabolism.

It is hypothesized that the noxious nature of secondary metabolites (33) is the reason for spatial separation of these metabolites (34). Generally, a large fraction of alkaloids are localized in the vacuole (34). An exception is the root specific alkaloid berberine, which was shown to adhere to the cell wall components (100).

2.5 SECONDARY METABOLITE ACCUMULATION IN PLANT CELLS

2.5.1 ALKALOID SYNTHESIS IN PLANT CELLS

The process of secondary metabolite production by plant cells involves the biosynthesis, transport, storage, further processing and degradation (92), all of which may occur at separate locations in the cell. The spatial expression of these functions depends primarily on the location of the enzymes involved in the process. The synthesis of alkaloids is mostly restricted to cytoplasmic enzymes (92, 113) although biosynthetic enzymes have been associated with the chloroplastic and mitochondrial fractions of the cells(92).

In the case of *Catharanthus roseus* cells, for example, a key intermediate in the synthesis of vinblastine and vincristine, vindoline has been shown to have a distinct site of synthesis. For instance, the first two enzymes in the pathway (tryptophan decarboxylase and strictosidine synthetase) are located in the cytoplasm (15,81). One of the key enzymes, thereafter, is an N-methyl transferase which is believed to be associated with the chlorophyll (FIGURE 3) fraction of *C. roseus* seedlings(80,81). This enzyme could not be isolated in undifferentiated suspension cultures. The last step which involves an acetylation reaction occurred in the cytoplasm.

Transport of secondary metabolites is a complex process. In whole plants, for example, alkaloid synthesis may occur in the roots and be transported through the cell sap to extremities

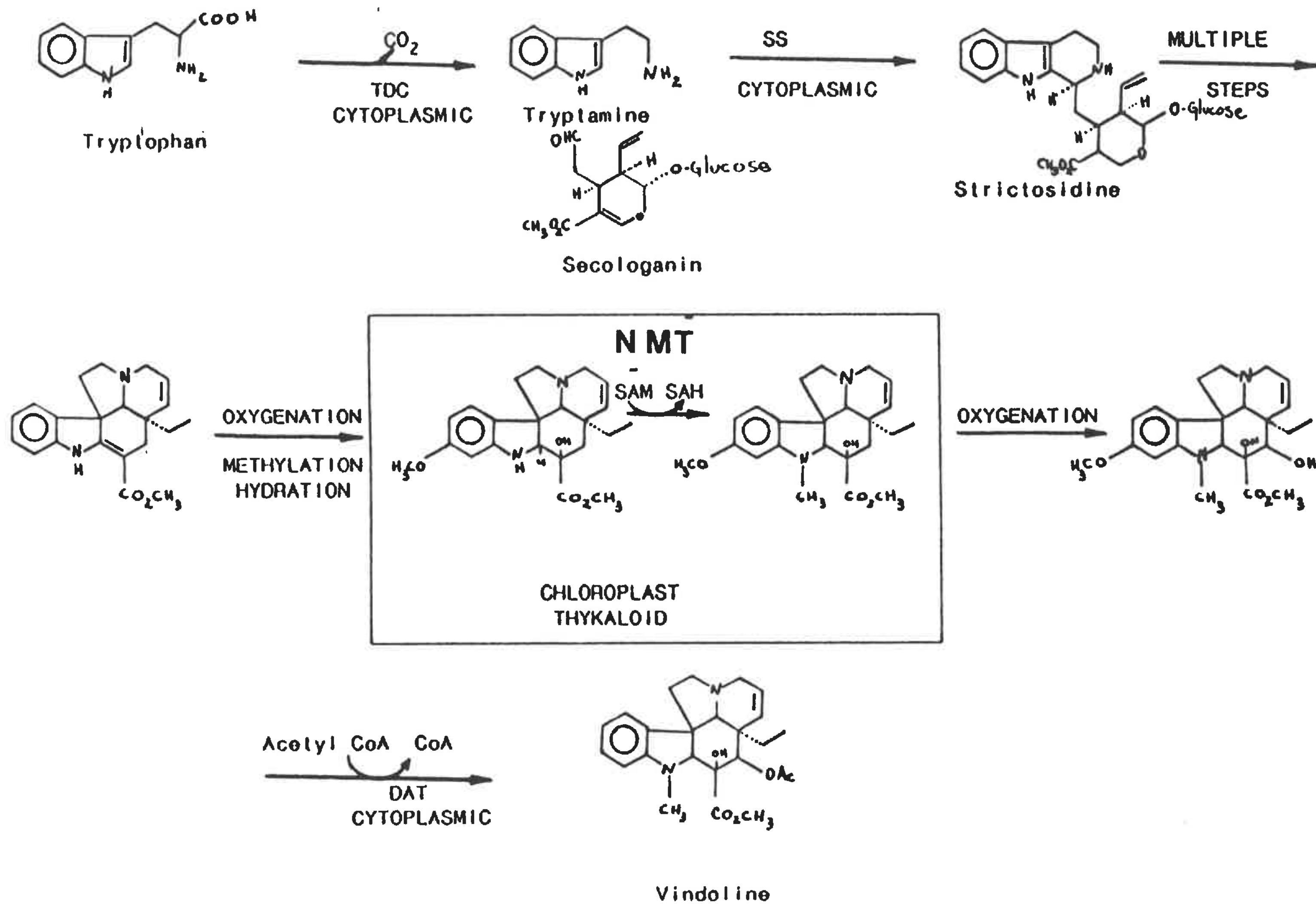


FIGURE 3: Spatial location of enzymes involved in indole alkaloid synthesis.

such as leaves (100). The alkaloids must cross many membranes to achieve this feat and it has been proposed that simple diffusion is involved (100).

Given a less complex system of *in vitro* suspension cultures for which cell organization is minimal (except for immobilized cultures), the transport is restricted to short distance translocations. In suspension cultures of *Catharanthus roseus* cells, two types of cells were distinguished according to alkaloid storage capabilities (14). It remained unresolved whether alkaloids were transported from synthesizing cells to storage cells or if synthesis and accumulation occurred in the same cell. It is worth noting that the storage cells display vacuolar pH values 1-2 units lower than non-accumulating cells. This hints at the importance of acidic pH to the entrapment of indole alkaloid.

2.5.2 PRODUCT ACCUMULATION MODEL

Conflicting theories have been postulated to explain the preferential storage of secondary metabolites in vacuoles. Originally, an "ion trap" model was postulated (FIGURE 4). This model (14) is based on the lipophilic nature of the alkaloids. There is rapid permeation of the newly synthesized neutral products from the cytosol across the tonoplast (vacuolar membrane). Once inside the vacuole, the alkaloid is protonated according to its acidity constant (pK_a). The newly formed cationic species is lipophobic and unable to cross the tonoplast. A relationship was found between the ratio of intracellular and extracellular (medium) concentrations of freely diffusible substances depending on the vacuolar pH (101):

$$C_i/C_e = \frac{1 + 10[pKa\text{-}pHi]}{1 + 10^{[pKa\text{-}pHe]}} \qquad (1)$$

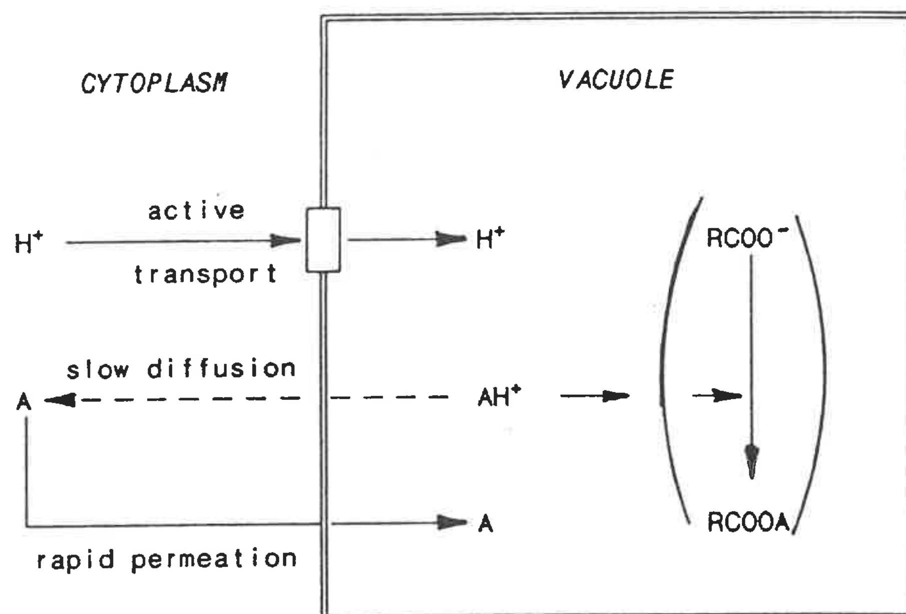


FIGURE 4: Indole alkaloid storage in the vacuole: the Ion Trap mechanism.

where:

C_i = intracellular concentration of a given alkaloid

C_e = extracellular concentration of the same alkaloid

pK_a= acidity constant of the alkaloid

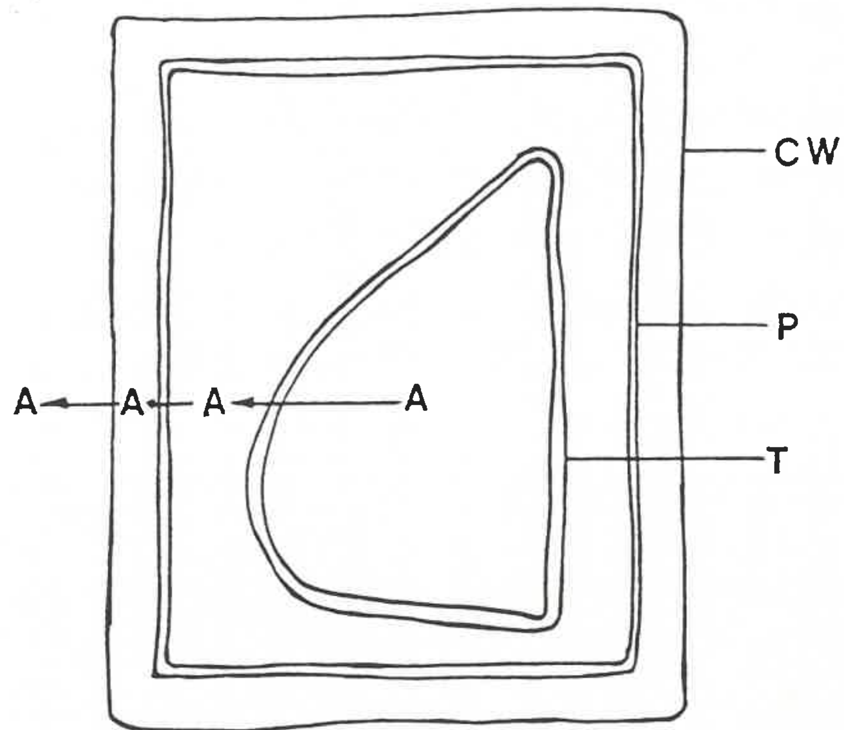
pH_i= intracellular pH

pH_e= extracellular pH.

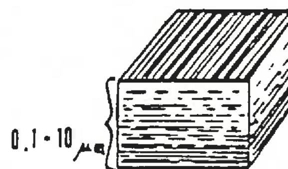
2.5.3 ALKALOID ACCUMULATION IN *CATHARANTHUS ROSEUS*

The factors governing alkaloid storage are difficult to identify. The results ensuing from studies on accumulation of secondary metabolites in the plant cell are controversial. Most of the controversy stems from the variation in biological systems used to characterize alkaloid compartmentation and special attention should be paid to these differences. Label tracing experiments have been performed on whole cell, protoplasts and vacuole preparations.

Alkaloid sequestration was generally studied by placing the labelled secondary metabolite in the medium surrounding the cell or organelle, then a time course evolution of cellular uptake was determined. The factors contributing to intracellular alkaloid (or intravacuolar) retention involved both transport and trapping of the substance. In experiments carried out with whole cells, the secondary metabolite must be transferred through the cell wall, the plasmalemma and the tonoplast (FIGURE 5) prior to entering the vacuole. The protoplasts are cells without cell walls, which was one less barrier to overcome in alkaloid uptake. Accumulation with vacuoles involved the simpler transport, only the tonoplast barrier needing to be overcome. Once the



Cell Wall (CW)



Plasmalemma (P)

Tonoplast (T)

FIGURE 5: Plant cell barriers which mut be overcome by alkaloids in oreder to released from the cell.

alkaloids were inside the vacuole they could be trapped by a variety of methods such as protonation, complexation or crystallization.

Suspensions cultures of *Catharanthus roseus* cells were used to determine the importance of pH gradient on transport of an exogenous alkaloid, tabernanthine (7). The ratio of intracellular to extracellular tabernanthine concentration reached equilibrium following 10 to 20 minutes of incubation. Acidification was carried out thereafter to obtain an extracelluar pH of 6.9. This caused a rapid efflux of the alkaloid, equilibrium being attained within 30 minutes as demonstrated by constant intracellular concentration. The extracelluar pH was increased to the initial value of 7.8 which incited alkaloid influx (7).

The indigenous alkaloid ajmalicine was also used to characterize transport(102). Whole cells were allowed to uptake radiolabelled ajmalicine. Equilibrium was established at approximately 30 minutes. Modifications of extracelluar pH led to significant changes in the intracellular (C_i) and extracellular concentrations (C_e) of ajmalicine. An acidification from 7.9 to 4.9 effected a decrease in C_i to half the original value within 10 minutes. This value increased to its original level as the external pH was buffered by the cells from 4.9 to 6.6 (60 minutes). These results demonstrate the significant effect of extracelluar acidification on alkaloid release.

A more rigorous study was undertaken with protoplasts isolated from young leaves of a *C. roseus plant* (79). The indigenous indole alkaloids displayed biphasic uptake into protoplasts: an initial rapid incorporation followed by a slow linear influx. In the case of vindoline, the rapid uptake was attributed to simple diffusion which was found to be pH dependent. The linear influx was suggested to be energy dependent and specific for vindoline. The same protoplasts were used to study two exogenous alkaloid species, morphine and nicotine. They displayed linear and

monophasic uptake respectively; thus implying the non-specific nature of transport for these exogenous alkaloids.

Vacuoles isolated from *Catharanthus roseus cells* were also used (15). The uptake against a concentration gradient was linear for ajmalicine. It required energy and was sensitive to the pH of the medium. These vacuoles only transported specific alkaloids synthesized by the species from which the vacuoles were isolated.

Different properties of alkaloid uptake (83,88) were found during other studies using vacuoles. For instance, there was rapid influx of ajmalicine which attained equilibrium within 3 minutes, there was a 5 to 10 times greater concentration in the vacuole than in the medium and ATP did not stimulate further transport. Of utmost importance was that pH modification of the surrounding medium altered ajmalicine similarly to that observed in the whole cell (7).

The retention of alkaloids within the vacuole was suggested to influence transport. As previously discussed, protonation may trap indole alkaloids in the vacuole. However, not all results supported this model. Simple diffusion and protonation could not fully explain the extent of ajmalicine accumulation in whole cells (102). It was proposed that complexation of alkaloids within the vacuole was possible and a modified relationship was suggested to better explain these findings:

$$\frac{C_i}{C_e} = \frac{1 + 10^{[pKa-pHi]}\,(1 + k_v{}' \cdot A^-)}{1 + 10^{[pKa-pHe]}} \qquad (2)$$

where:

C_i = intracellular concentration of a given alkaloid

C_e = extracellular concentration of the same alkaloid

pK_a= acidity constant of the alkaloid

pH_i= intracellular pH

pH_e= extracellular pH.

k_v'= the association constant with the complexing substance

A^- = the concentration of free complexing sites

A value of 11.6 was calculated for $k_v \cdot A^-$ suggested that 11.6 molecules could exist for every ionized molecule of ajmalicine. It has been suggested that phenolic compounds within the vacuole could be complexing with alkaloids (79,92,93,102).

In studies carried out with protoplasts it appeared that ion trapping of the alkaloid by low pH played an important role in the retention of alkaloid in the vacuole (79).

In vacuoles it was admitted that the level of ion trapping depended on the type of alkaloid and that lipophilic alkaloids were the most influenced by protonation. However up to 30 % of ajmalicine retention occurred by slowly exchangeable complexed forms of the alkaloid (83). Other authors negated the ion trap model because of the observed exchange of radioactive and non-radioactive species with vacuole preparations (15,49). True diffusion was not occurring in these vacuoles because transport was active, saturable and required a specific carrier for the alkaloid (15).

The discrepancies between results of (15) and (83) with isolated vacuoles could be attributed to organelle isolation procedure which affected vacuole properties. One group stabilized the vacuoles with a NaCl osmotically balanced medium. This procedure seemingly increased vacuole pH from 5.6 to 6.3 (83). The increase was caused by stimulation of the Na^+/H^+ antiport system (83) present in the tonoplast. In this case, the intensity of vacuolar trapping of the alkaloid was possibly diminished.

The conclusions drawn from these studies were that pH alterations cause changes in vacuolar and extracelluar fluxes of secondary metabolites when whole cells were used (7,102). However, when isolated vacuoles were studied, transport of the alkaloids in the vacuoles was specific for indigenous forms: thus, implying translocation into vacuoles by a more complex processes than simple diffusion (15,79,83,88. The retention of alkaloids could not be fully explained by the ion trap mechanism (102); thus, complexing substances in the vacuoles were suggested to be responsible for the accumulation of the alkaloids against a concentration gradient (79,92,93,102).

The studies on secondary metabolite accumulation were controversial and complex. They indicated the many facets which contribute to the sequestration of secondary metabolites in the cells. The advent and refinement of isolation and stabilization techniques of the vacuole have facilitated the study of alkaloid compartmentation which is schematically represented in FIGURE 6 (93). This model suggested a dynamic system which may be influenced by many environmental conditions.

The cultured cells could be stimulated to release sequestered alkaloids without causing cell destruction. The following modifications of the cell culture have been found to increase efflux of intracellularly compartmented products:

1) Ca^{++} (5mM $CaCl_2$) stimulated ajmalicine release (82);

2) K^+ (25mM K_2SO_4) stimulates ajmalicine efflux (82);

3) NaCl (final concentration 0.27 M) stimulated catharanthine release but this may have been caused by cell lysis (86) because the viability decreased by 30%;

4) elimination of phosphate allowed for the release of protoberberine (84);

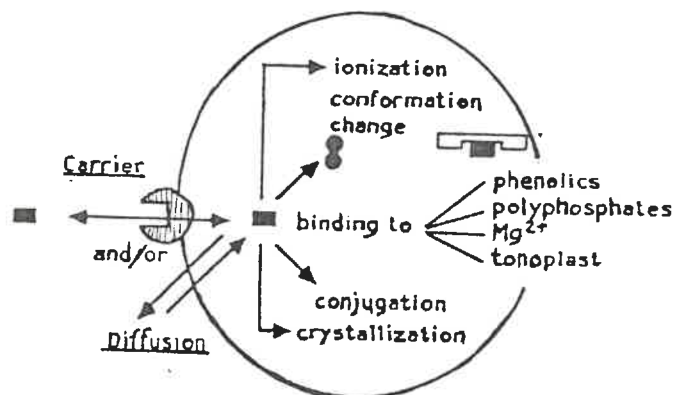


FIGURE 6: Overall schematic representation of proposed alkaloid transport into plant vacuoles and the possible methods of alkaloid entrapment.

5) elicitation of *Catharanthus roseus* with *Pythium* caused the release of indole alkaloids(12);

6) cell immobilization incites release of intracellular compounds (52,98,115);

7) alterations of ionic strength brought about release of intracellular enzymes;

8) extracelluar acidification (56,82,87,99,102) or alkalinization (36) incited release of alkaloids.

An increasing number of studies implied that a diversity of systems may have been responsible for alkaloid vacuolar accumulation. It is likely that specific carriers are responsible for overall transport of a single alkaloid into the vacuole while the trapping of the alkaloids inside the vacuole was caused by a combination of protonation and complexation. With this in mind, it was difficult to find a simple, gentle method which could be used to incite alkaloid release from immobilized cells. However, pH was acknowledged as an important element influencing alkaloid retention (7,11,14,15,26,29,31,56,59,79,82,87,99,102). Thus, it was warranted to evaluate this stimulus as a release effector for immobilized *Catharanthus roseus* cells.

2.5.4 PH CONTROL IN PLANT CELLS

Control of intracellular pH is a requisite to ensure normal cellular activity in the plant cell. Cytoplasmic pH is rigidly maintained (107) by a multitude of processes. Transport systems exist which are coupled to H^+ or OH^-. There is a fast turnover of these ions resulting in a dynamic equilibrium system to maintain cytoplasmic pH.

Vacuolar pH is more variable than the cytoplasmic pH. Values range from 3.5 to 5.5 (113). Flexible vacuolar pH is necessary to maintain a constant cytoplasmic pH(~7). The vacuole functions as a buffer and is the main site of cytosol pH regulation.

Tonoplast ATP'ase/H^+ transport systems exist (113). There are other substrate specific transport systems which cause release of H^+ from the vacuole. They may be symports where H^+ and the substrate move in the same direction or antiports where H^+ and substrate move un opposite directions(113).

A specific type of pH control is depicted in the following example. CAM (Crassulacean Acid Metabolism) is a process whereby CO_2 is stored in the form of malate (63,106). This mechanism is stimulated in darkness. Malate formation consumes OH^- ions which would cause acidification of the cytoplasm. This does not, however, occur because malate is transported along with one H^+ into the vacuole. The vacuole is normally 1-2 pH units more acidic during obscurity than under normal photosynthetic conditions. This process occurs in both whole plants (106) and *in vitro* plant cell cultures.

Proton associated transport also occurs at the plasmalemma whereby H^+ ions are secreted into the medium. There are H^+/ATP'ases on the plasmalemma. When extracellular pH is less than 9-10 there is an active efflux of H^+ (63). Proton extrusion is stimulated by the auxin IAA; it acts as a redox driven H^+ pump at that plasmalemma (63,118). Increases in extracellular osmolarity can also induce release of H^+ so as to acidify the culture medium.

Modification of extracellular medium has been carried out and rapid pH autoregulation was observed repeatedly (87,102,119). With the wide range of pH regulators mentioned previously, it seems that gentle momentary pH alterations of the medium should not pose a threat to normal cell activity.

chapter 3

3.0 OBJECTIVES

The main objective of this project was to study the release of indole alkaloids from *Catharanthus roseus* immobilized plant cells and to evaluate the effect of changing extracellular pH on this release process. This was broken down as follows:

1) briefly review the growth characteristics of three systems namely the 200mL suspensions, the 2L and the 6L immobilized plant cell bioreactors;
2) determine of natural release in suspension cultures and immobilized cultures of *Catharanthus roseus* cells;
3) determine of the effect of changing the medium pH on normal cell activity;
4) determine of the effectiveness of acid and base on the release of indole alkaloids.

chapter 4

4.0 MATERIALS AND METHODS

4.1 SUSPENSION CULTURES

4.1.1 SUSPENSION CULTURE MAINTENANCE

Suspension cultures of *Catharanthus roseus* cells (line MCR17) were generated from the leaf of the Periwinkle plant. The cell line has been maintained since 1984 in Gamborg's B5 (68) medium supplemented with 4.5 μM of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2% sucrose (1B5). The media were steam sterilized (121°C,15psi,60minutes). Rigid sterilization was carried out to ensure sterility. Weekly subcultures were carried out by inoculating 20 mL into 500mL Delong flasks containing 200 mL (10% v/v inoculation ratio) of sterile medium. These were kept at 28±1°C on a rotatory shaker at 150 R.P.M. under continuous diffuse light.

4.1.2 GROWTH KINETICS IN SUSPENSION

The growth of the cell culture was evaluated by sampling 10 mL every 2-3 days. The samples were filtered, the liquid was collected for nutrient analysis. The biomass was rinsed twice with distilled water; the fresh and dry (dried at 80°C until constant weight) weights were determined.

4.2 SURFACE IMMOBILIZED PLANT CELL BIOREACTORS

4.2.1 THE 2 L BIOREACTOR

The 2L Surface Immobilized Plant Cell (SIPC) bioreactor consisted of a 2 L conical vessel as per FIGURE 7A. The upper cover contained 4 ports. A sintered glass air sparger was placed in the middle hole and penetrated the spiral matrix (FIGURE 7B). One port was used for sampling, one port was used for a glass condenser and the final port was used to transfer

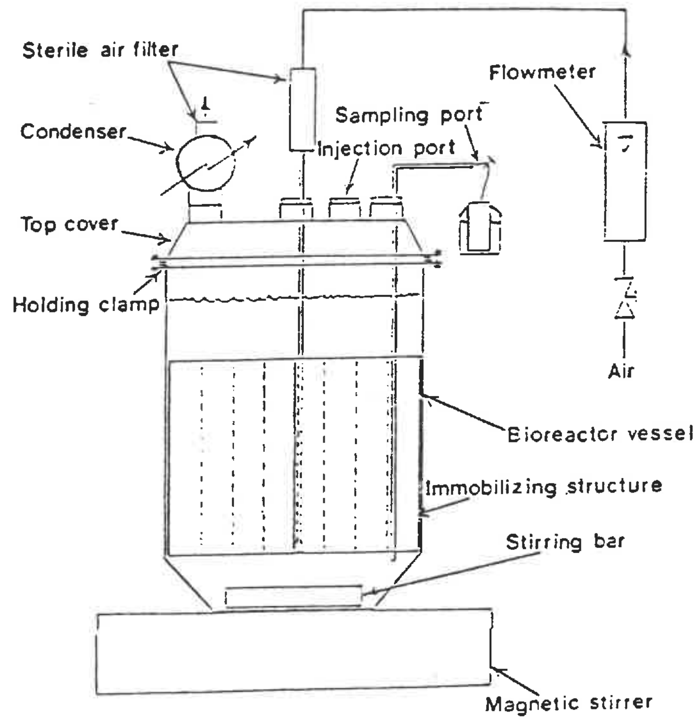


FIGURE 7A: Configuration of the 2L SIPC

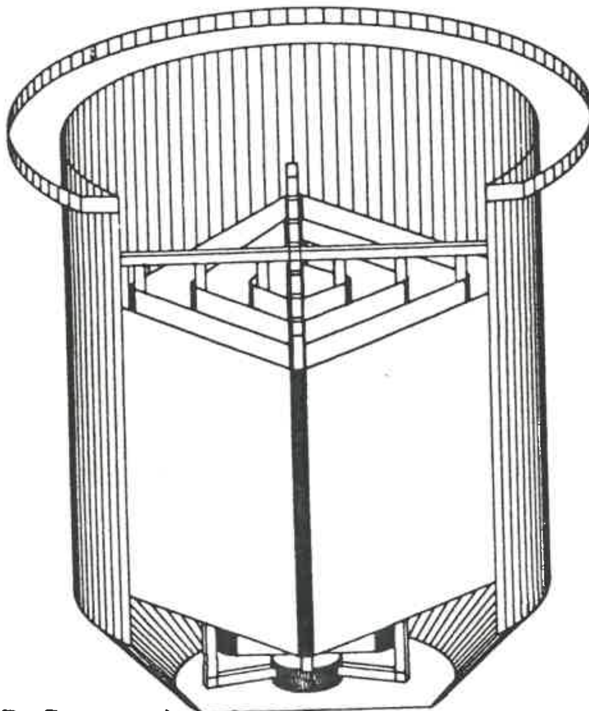


FIGURE 7B: Cross-section of spiral matrix structure

medium. A Viton O-ring was placed between the vessel and the cover which were clamped to ensure sterility.

The matrix material used was a man-made geotextile (A07 from Texel Inc.) with an average thickness of 1.6mm. The material was washed prior to use by soaking in hot MeOH, then rinsed with distilled water and dried. The material was cut into rectangular strips of ~90 x 10 cm which resulted in an average surface area of 1845±135cm^2. The material was wrapped around a stainless cage to form a spiral configuration as per FIGURE 1B. The spacing between the each layer was 1.3 cm. The time required for cells to completely adhere to the matrix surface is 24 hours.

The temperature of the bioreactor was maintained at 28±1°C using the following system. A surface resistance temperature probe attached to the exterior of the glass vessel was connected to a PID Viconics (TÇ300) regulator which controlled the temperature through a heating/magnetic stirring plate (Thermolyne Sybron Inc.). This system was developed in 1988 (72).

Aeration was maintained at 0.07±0.02VVM; mixing was achieved using a magnetic stirring bar rotating at 300 r.p.m. (impeller tip speed of 121$cm \cdot s^{-1}$; impeller Re=29000). These operating conditions allowed for an oxygen transfer coefficient of (k_La)~ 6h^{-1} (APPENDIX 1). This plot encorporates the O2 transfer attributed to surface aeration which explains the k_La ~4h^{-1} at 0 V.V.M. One could flush the head space in the bioreactor to decrease this effect so as to measure kLa strictly as a function of sparging.

The reactors were sterilized with 1.2 L of medium at 121°C and 15 psi for 60 minutes. A 10% inoculation was achieved by adding 180-190 mL of a 6 day suspension culture

($\sim$10gdw$\cdot L^{-1}$) into the bioreactor. Thereafter, sterile medium was added to attain a total initial volume of 1.8L.

Liquid samples were taken every 2-3 days for nutrient and/or alkaloid determination. Biomass concentration could only be measured when the reactor was dismantled. Fresh and dry weights were determined. The following equation was employed to calculate the true dry weight biomass (36) which took into account the residual sugars retained in the matrix material:

$$X_{IPC} = W_{M+X} - W_{structure} - 0.92 * V_{matrix} * CHO_f \quad (3)$$

X_{IPC} = total dry weight biomass immobilized on the matrix (g);

W_{M+X} = weight of dry biomass and weight of structure (g);

$W_{structure}$ = weight of the original,dry structure and matrix prior to culture(g);

0.92 = void fraction of the matrix (cm^3 H_2O/cm^3);

V_{matrix} = volume of matrix (cm^3);

CHO_f = final total alkaloid concentration (g/cm^3).

4.2.2 METHODS OF PRODUCTION: THE 1-STAGE AND 2-STAGE PROCESSES

Indole alkaloids biosynthesis was induced in 2 manners. The 1-Stage process consisted of a 15 day culture period in Zenk's Alkaloid Production Medium (APM) (2). Refer to APPENDIX 2 for a comparison between B5 and APM media composition.

The 2-Stage process was carried out with a 6 day growth period in Gamborg's (B5) medium supplemented with 5μM of IAA, (medium = **AB5**). Up to 90% of the medium was removed at day 6 and fresh **APM** was added . The production period lasted 15 days, similar to the 1-Stage

process. In both processes, samples were taken every 2 to 3 days to determine culture parameters (pH, NO_3, conductivity and carbohydrates) and to quantify extracellular alkaloid content.

4.2.3 THE 6 L SURFACE IMMOBILIZED BIOREACTOR

A glass cylinder 15.3 cm in diameter and 40 cm in height with a sampling port was used in conjunction with upper and lower stainless steel flanges to form the shell of the 6 L modified airlift reactor with an effective volume of ~5.2 L. The centrally placed riser tube within the stainless immobilizing structure allowed the passage of air sparged from the bottom of the reactor. Approximately 2.5 cm above the tube was a diverting plate to allow for better mixing. A teflon profile was inserted at the bottom of the reactor to prevent the formation of dead zones (FIGURE 8).

The inert immobilizing material A07 was cut into strips of 24 x 130 (immobilizing area=6270±150 cm^2) and wound around the stainless steel cage in a spiral configuration. The matrix covered cage (15 x 15 x 24 cm) was placed vertically into the 6L glass vessel of the modified airlift bioreactor (IA). The upper plate was fitted with the appropriate accessories (Freidrich condenser and calibrated pH probe) and the reactor was steam sterilized (121°C, 15psi, 1h). The media were filtered sterilized with a 0.22μm Millipore (Millipak 20) system. The media containers were connected to the bioreactor following bioreactor sterilization in a laminar flow hood. Prior to the addition of the inoculum, 1 L of the starting medium was added to prevent the clogging of cells. The bioreactor was inoculated with 1 L of a early exponential phase culture (6-7 d, 10 g d.w.$\cdot L^{-1}$). The medium was initially filled just above the height of the matrix structure (~4L) to ensure rapid immobilization of the cells to the matrix material

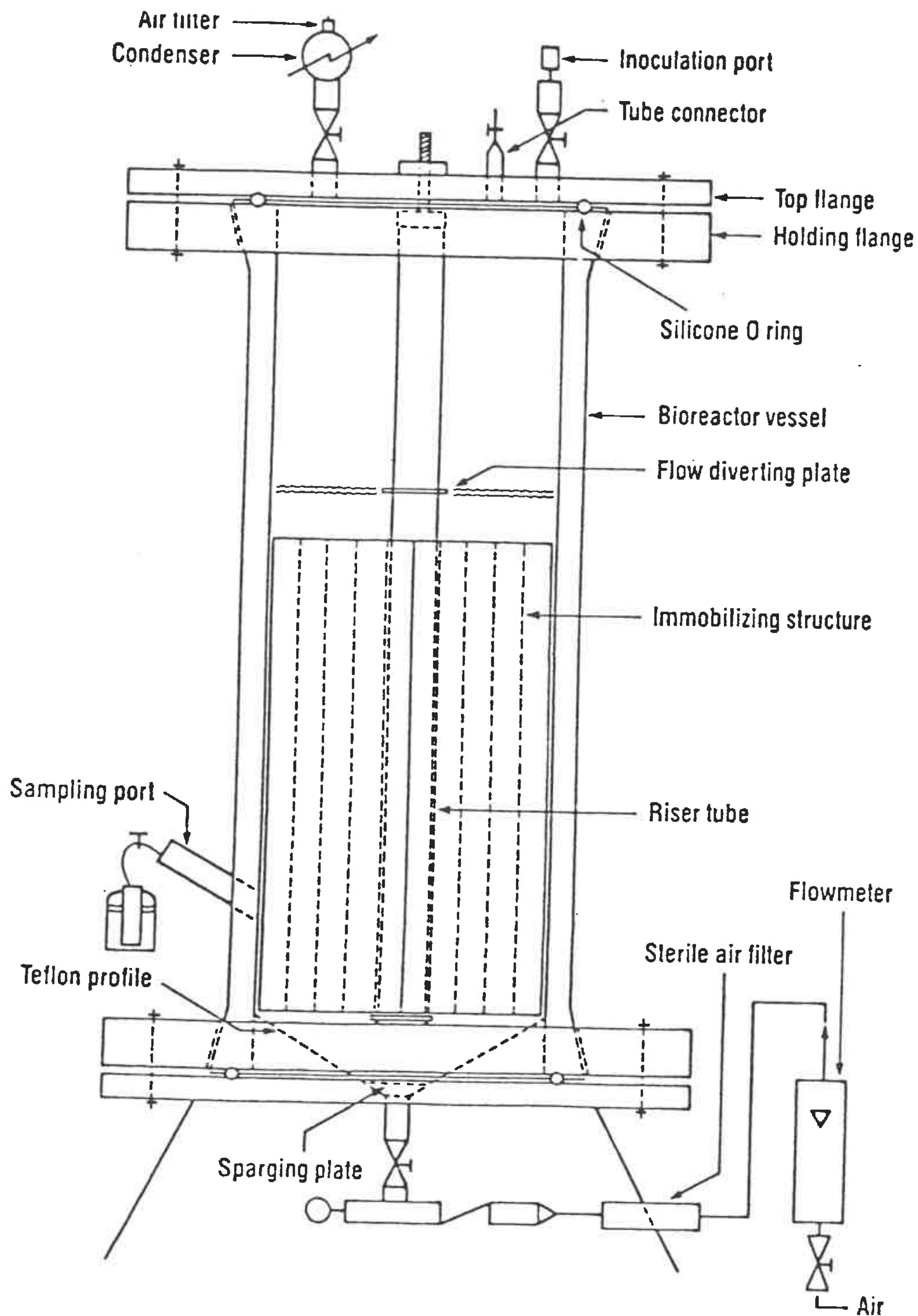


FIGURE 8: Configuration of the 6 L surface immobilized plant cell bioreactor.

which required 24 hours. The following day the medium level was increased to ~5.5 L.

The modified airlift was aerated at 0.3-0.5 VVM which fulfilled both the requirements for aeration and mixing (k_La=6-8 h^{-1}, APPENDIX 3). The temperature was maintained at 28±1°C by a modified version of the 2L control system. All 6L bioreactors were shielded from light with aluminum foil.

4.2.4 PH MODIFICATION EXPERIMENTS

The 2 STAGE process which was used, consisted of a 6 day growth period in B5 medium supplemented with 1 μM IAA . At day 6, 90 % of the medium was removed and fresh APM was added to initiate the production phase. For reactors IA22(base) and IA23(acid), release was stimulated by adding 2% of 0.1N KOH or HCl starting at day 9. During the following 3 hours, ~100 mL samples were acquired at close intervals. This sampling schedule was deemed necessary to pinpoint maximal product release which had been reported to occur shortly after stimulation (7,36). Two(2) days following each experiment, fresh APM was added(~20% v/v) to the reactor to replenish the system for subsequent product release experiments which were performed at 3 day intervals up to and including day 21.

Other cultures IA12(base), IA19(base) were treated similarly except that pH modifications were only carried out 2 times; at day 9 and 12 for IA12 and day 15 and 18 for IA19. IA20 did not undergo any such pH modifications and was dismantled at day 12 of culture.

4.3 ANALYTICAL METHODS

4.3.1 CULTURE PARAMETERS.

Nutrient consumption was monitored for all cultures. Carbohydrate levels were evaluated using high pressure liquid chromatography (HPLC). The system consisted of a Waters model 1590 pump, a Waters U6K injector, a temperature control unit, a Waters 410 refractometer connected to a Spectra Physics SP4270 integrator. Sucrose, glucose and fructose were separated using an Aminex carbohydrate HPX-87C column from Bio Rad. Water was the eluent and it was filtered at 0.45µm prior to use. The isocratic flow was maintained at 1 $mL\cdot min^{-1}$ which gave rise to a back pressure of 1000psi . The temperature was maintained at 80°C.

Nitrate concentrations were determined using an Orion nitrate electrode (model 93-07) (5). The ionic strength of the samples was adjusted by adding 2M $(NH_4)_2SO_4$ (2%v/v) prior to voltage reading. Standards ranging from 10^{-1} to 10^{-4}M were used for the standard curve as demonstrated in FIGURE 9. There was a logarithmic relationship between voltage and nitrate concentration. When the solutions were alkaline, as for the alkalinized cultures IA12, IA19, and IA22, a significant interference was observed (FIGURE 9).

Conductivity of the culture was also evaluated. A YSI model 3 conductance meter was used. The volume required for conductance measurement was ~27mL.

4.3.2 ALKALOID ANALYSIS AND QUANTIFICATION

Culture medium was extracted by a well known liquid-liquid extraction procedure (125) used regularly for *C. roseus* alkaloid extraction. It was beyond the scope of this project to futher explore this procedure.

Base (10N NaOH) was added to the sample of medium to increase the pH to 10. Thereafter

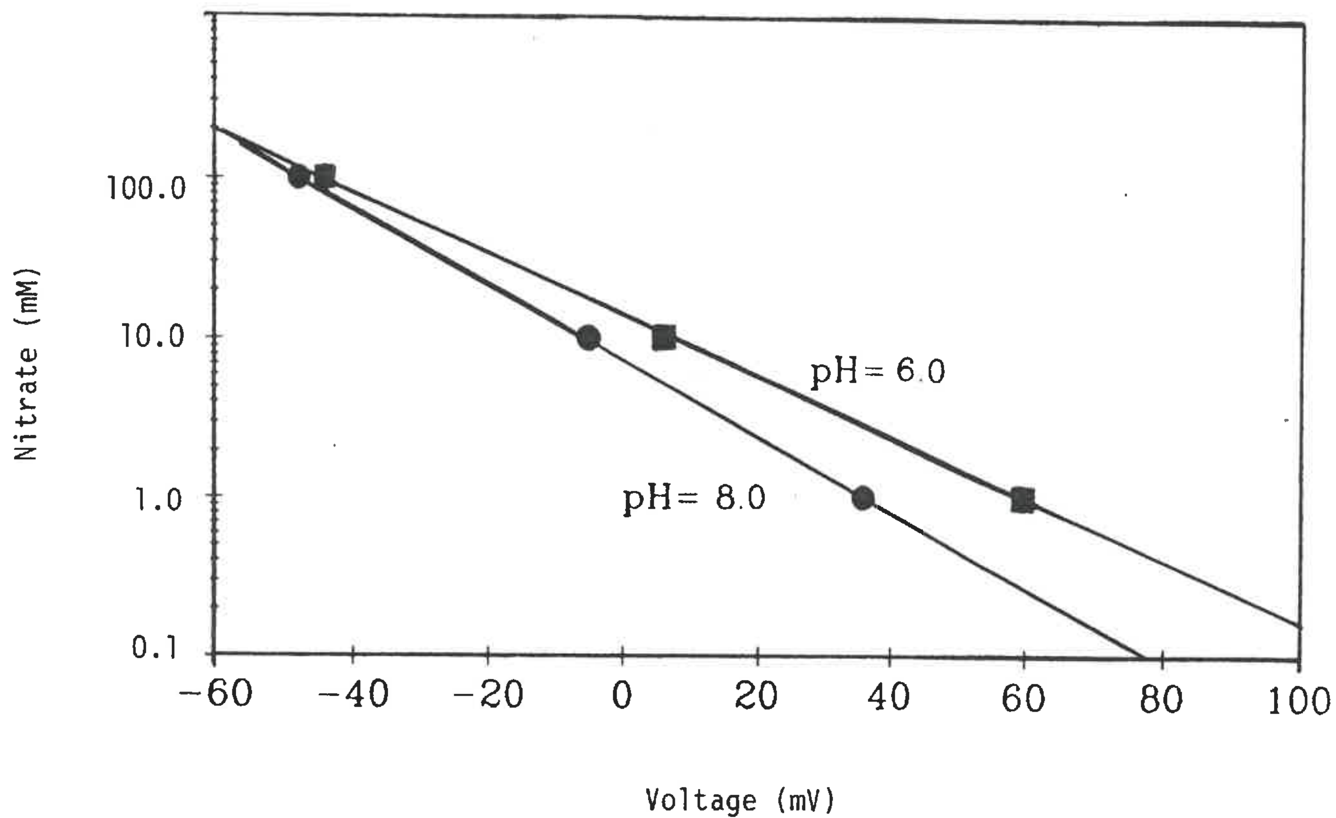


FIGURE 9: Standard curve used for nitrate determination and the effect of high pH on the orion Nitrate probe.

it was extracted 3 times with an equal volume of ethyl acetate. The organic phase was recuperated and evaporated to dryness with a rotoevaporator. The resulting alkaloid crystals were dissolved in 4 mL of a 50% mixture of ethyl acetate/methanol.

The biomass was extracted in hot MeOH for 1 hour. The resulting methanol mixture was evaporated to dryness under vacuum. The crude mixture was redissolved in ~100 mL of a 1N HCl solution (fraction 1) and in ~100 mL of ethyl acetate (fraction 2). The ethyl acetate Fraction 2 was extracted 2 times with an equal volume of a solution of 1N HCl (fraction 3). The aqueous phases (fractions 1 and 3) were combined and 10N NaOH was added to reach a pH of 10. Further extraction was carried out with 3 times with an equal volume of EtAc. The resulting organic fraction was evaporated to dryness. This alkaloid fraction was dissolved in a 50:50 MeOH:ethyl acetate mixture. The resulting alkaloid concentrates were analyzed by two types of chromatography : High pressure Liquid Chromatography and Thin layer chromatography.

Thin Layer Chromatography was used to separate components of a mixture. The alkaloid extract was eluted on glass plate covered with silicone. The Baker plates (Si 250f (4c)) consisted of 4 channels of treated silicone which allowed for component detection under UV light at 254 nm. Approximately 100µL of the alkaloid extract was placed at 2 cm from the bottom of the plate. The separation occurred by elution against gravity with a mixture of 90% EtAc in MeOH. The front of the eluent climbed upward on the plate until it reached 12 cm which required ~25 minutes. The plates were dried and observed under UV_{254}. The distance travelled by each component was recorded and compared to the eluent front. Thereafter, R_f values were calculated in the following manner:

$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by the eluent front}} \qquad (4)$$

The plates were treated with a 1% (w/v) of ceric ammonium sulphate in phosphoric acid. The chromogenic responses were determined and R_f values were calculated as per equation 4. The R_f values and colour responses were compared to standards and alkaloids studied by Farnsworth *et. al.*(107) for identification.

Individual alkaloids were quantified using reversed phase HPLC. A Brownlee Labs OS-5A C^8 column was maintained at 48°C and the eluent was pumped at 2.0 mL/min. A gradient was developed with a starting concentration of 50:50 MeOH:H_2O. The MeOH was increased at 1%/min to a final concentration of 85% MeOH in H_2O. The mixture had a constant level of tetra butyl ammonium phosphate (1.87mM). The alkaloids were detected at 254 nm using a Multiwavelength detector (Waters 490E) connected to a Spectraphysics Integrator. The retention times of the alkaloids originating from the samples were compared to the available standards and their concentrations were calculated thereafter.

Following extraction, many compounds were detected in the JPLC chromatograms; they however, remained unidentified due to lack of standards. It has been well documented that a great number of alkaloids are produced by the *C. roseus* species, and it was no suprise to observe this multitude of unknown products. Some of the substances displayed interesting behaviours during alkaloid release experiments. The absorbance was used to estimate a relative extracellular concentration.

The total alkaloid concentration is determined as per Lee *et. al.* (108). A mixture of 13 alkaloids was used as a standard solution (TABLE 1). A series of dilutions were carried out and the absorbance at 280 nm was measured. Results were correlated by the following equation which was utilised to determine the total alkaloid concentration of the alkaloid extracts:

TABLE 1: Alkaloid mixture used for total alkaloid determination

ALKALOID	[Alkaloid] (mg/L)
Tryptamine	51.6
Strictosidine lactam	54.7
Yohimbine	59.4
19-epi-vindolinine	34.4
Vindolinine	37.3
Vindoline	35.1
Catharanthine	63.6
Vinblastine	34.3
Tabersonine	72.2
Vincadifformine	53.2
Vincristine	33.1
Ajmalicine	52.2
Serpentine	3.3
Lochnerinine	19.3
Total alkaloid concentration	603

Standard curve for alkaloid determination

Dilution	ABSORBANCE 280nm	Total alkaloid Concentration (mg/g)
5	1.79	0.120
10	0.92	0.060
16.25	0.56	0.037
20	0.45	0.030
40	0.13	0.015

$$\text{Concentration} = [64.4 * \text{Abs}] + 2.8 \qquad (5)$$

where concentration was in mg/kg of extract and Abs = absorbance at 280nm.

4.4 DETERMINATION OF NATURAL PRODUCT RELEASE IN DIFFERENT CULTURE SYSTEMS

The relative basal release of alkaloids in the 200mL suspensions was determined by removing ~ 40mL from a shake flask. The cells were filtered and the medium was collated. The biomass and the medium were extracted and analyzed for alkaloid content as explained previously. The relative or percent release was calculated in the following manner:

$$\text{RELATIVE release} = \frac{\text{content in medium}}{\text{content in medium} + \text{content in biomass}} \qquad (6)$$

The relative natural release in the 2 L and 6 L immobilized cultures was evaluated differetnly due to the inaccessability of the biomass. The bioreactor was dismantled while the medium was recovered for extraction and alkaloid analysis. The biomass was extracted and similarly analyzed. The percent release was evaluated as per equation (6). The absolute release was also used to compare alkaloid concentrations in the culture medium. During the induced alkaloid release experiments the absolute concentrations were used more often because the biomass could not be evaluated for alkaloid content.

chapter 5

5.0 GROWTH AND NATURAL PRODUCT RELEASE

5.1 GROWTH KINETICS OF CULTURED *CATHARANTHUS ROSEUS* CELLS

Results presented in sections 5.1 to 5.3 summarize experimental work performed in cooperation with R. Tom (120). They were essential to the understanding of subsequent experimentation performed to induce product release.

5.1.1 SUSPENSION CULTURES GROWN IN 1B5 MEDIUM

Inocula for surface immobilized plant cell bioreactors were grown as suspension cultures in 1B5 medium. A characteristic growth pattern of the 200mL suspensions cultured in 500mL shake flasks is presented in FIGURE 10. The biomass dry weight demonstrated a decline of rapid cell growth (exponential) following day 4; this coincided with a slower consumption of carbohydrates. The stationary phase occurred at day 6 with a concomitant depletion of carbohydrates. The decrease of dry weight biomass at day 8 probably resulted in cell deterioration and/or consumption of stored starch. Viability studies were not performed and the cause of declining dry weight could not be pinpointed. The specific growth rate and biomass yield relative to carbohydrates of these triplicate cultures were 0.50 d^{-1} and 0.59 g dw/ g carbohydrate consumed respectively.

5.1.2 SUSPENSION CULTURES GROWN IN ALKALOID PRODUCTION MEDIUM

Shake flask suspension cultures (200mL) were grown in Alkaloid Production Medium (APM). The growth characteristics of triplicate cultures are illustrated in FIGURE 11. The initial sucrose level was 50 $g \cdot L^{-1}$. Biomass concentration reached a maximum of 24.2 g $dw \cdot L^{-1}$ at day 15 and carbohydrates were exhausted by day 22. The specific growth rate was $0.34 d^{-1}$ and an

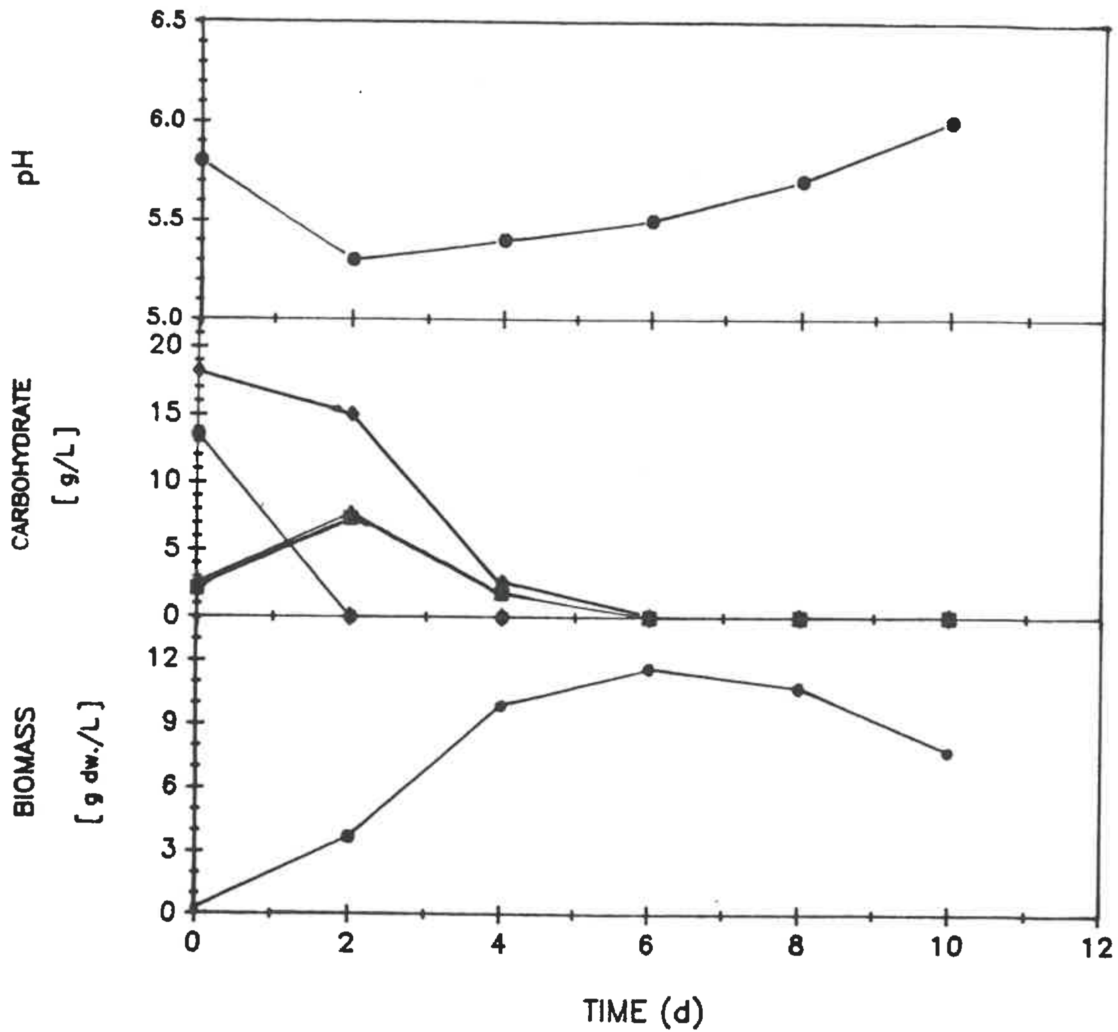


FIGURE 10: Growth of C. roseus suspension cultures (200mL). Cultures were maintained in 1B5 medium at a temperature of 28±1°C on a rotatory shaker (150 RPM).
● = Sucrose, ▲ = Glucose,
■ = Fructose, ◆ =Total carbohydrate.

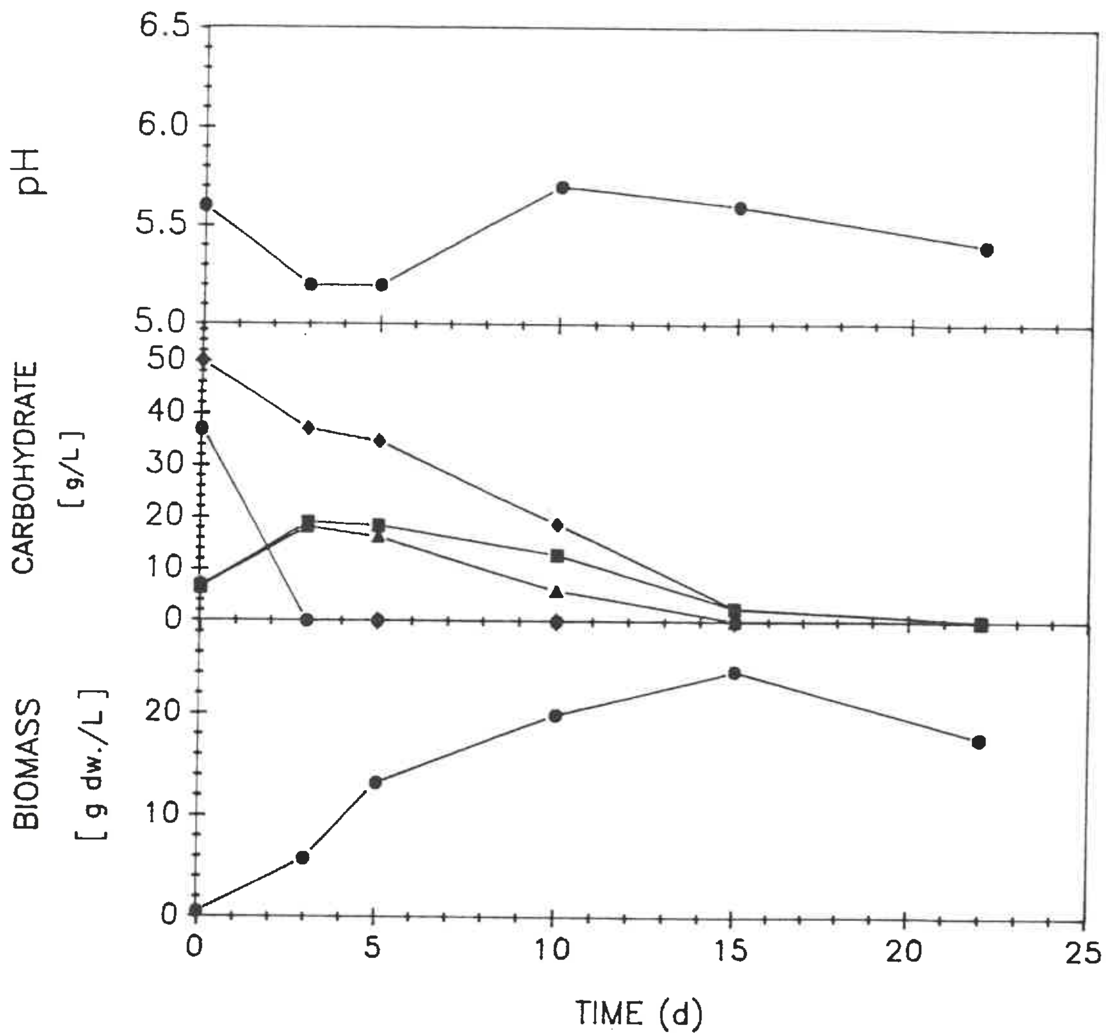


FIGURE 11: Growth of C. roseus suspension cultures (200mL) Cultures were maintained in APM at a temperature of 28±1°C on a rotatory shaker (150 RPM).
● = Sucrose, ▲ = Glucose,
■ = Fructose, ◆ =Total carbohydrate.

average yield ($Y_{x/s}$) was 0.50 g dw/ g carbohydrate consumed.

Slow growth rate of these cultures compared to the 1B5 cultures was attributed to the lower initial concentrations of nitrate (APM=18.4mM, 1B5=24mM) and inorganic phosphate(APM=0.54M, 1B5=1.09M). High sucrose concentration was inhibitory to growth and the phytohormone IAA used in APM was less potent a growth hormone compared to 2,4-D in the growth medium 1B5. The high dry weight was due to starch accumulation.

5.1.3 TWO LITER IMMOBILIZED CULTURES GROWN IN 1B5

The growth characteristics of *C. roseus* cells in 2 L immobilized bioreactors cultivated in 1B5 medium is presented in FIGURE 12. Each point represents an average of two bioreator cultures. A concomitant levelling off of biomass proliferation and carbohydrate consumption between day 4 and day 6 was observed. The carbohydrates were not completely consumed. A maximum dry weight concentration of 7 g dw·L-1 was obtained. The specific growth rate (μ=0.48d-1) was similar to that of the 200mL suspension cultures. The biomass yield (Yx/s) was somewhat lower (0.40 vs. 0.59 g dw/ g carbohydrate consumed).

5.1.4 TWO LITER IMMOBILIZED CULTURES GROWN IN APM:THE 1-STAGE PROCESS

The growth characteristics of these cultures are presented in FIGURE 13. The cultures were monitored until day 15. Incomplete consumption of carbohydrate was observed. The specific growth rate was $0.19d^{-1}$ which was low compared to APM suspension cultures. The biomass yield during linear growth phase was 0.53 g dw/ g carbohydrate consumed. The Yx/s for the whole culture period was 0.40 g dw/ g carbohydrate consumed. Each point represents two bioreactors.

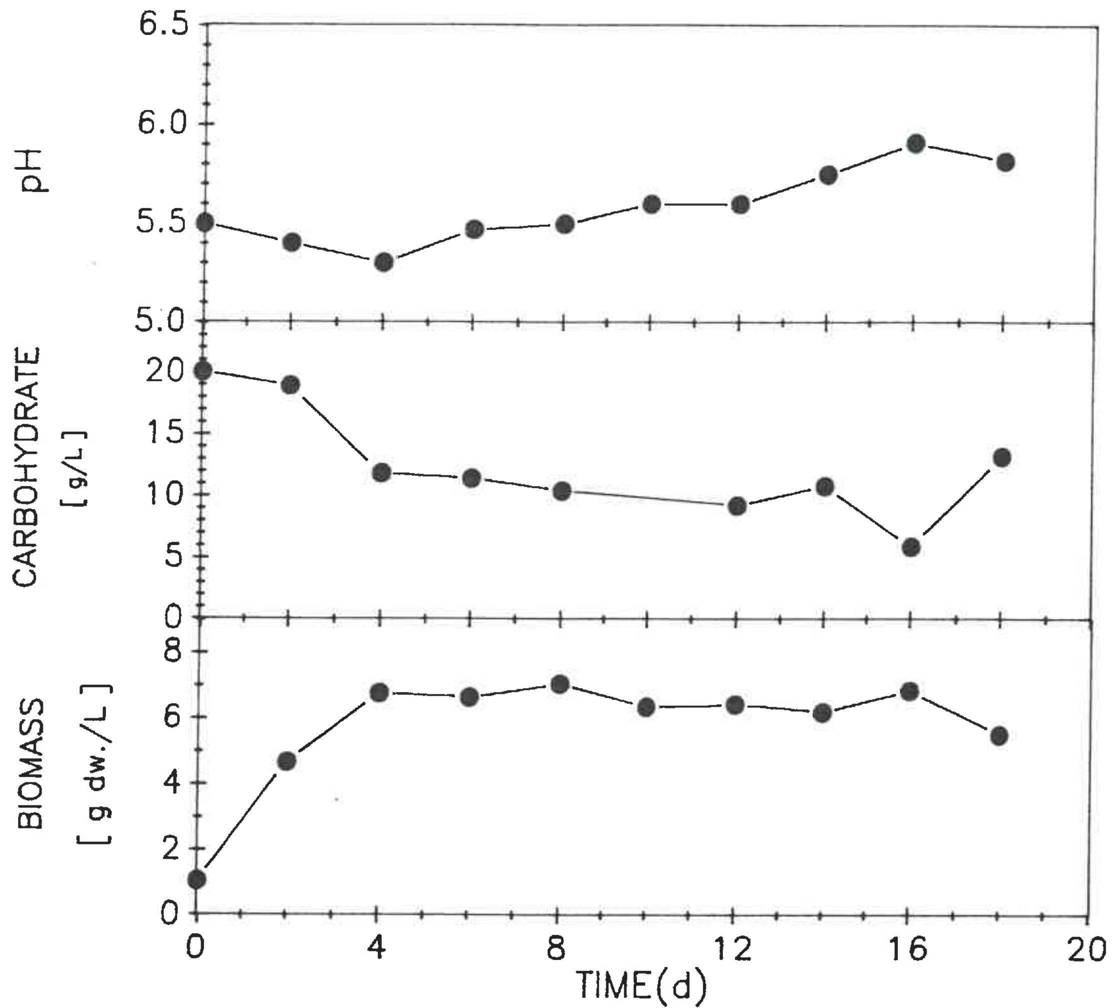


FIGURE 12: Growth of C. roseus immobilized cultures (2L). Cultures were maintained in 1B5 at a temperature of 28±1°C, the aeration was constant at 0.07VVM (K_la ~$6h^{-1}$) and stirring was kept at 300RPM.

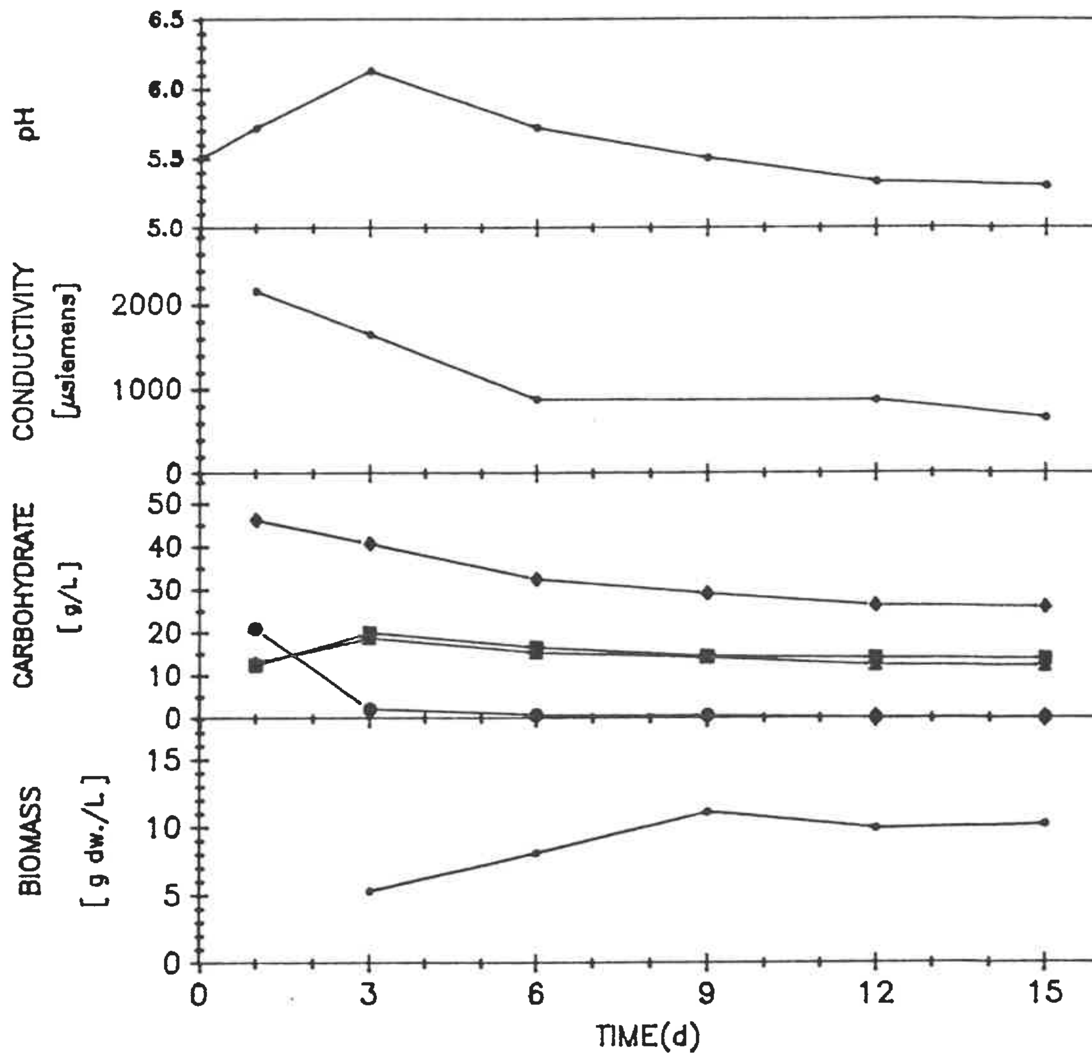


FIGURE 13: Growth of C. roseus immobilized cultures (2L). Cultures were maintained in APM at a temperature of 28±1°C, the aeration was constant at 0.07VVM (K_la ~6h^{-1}) and stirring was kept at 300RPM. • = Sucrose, ▴ =Glucose, ▪ =Fructose, ♦ = Total carbohydrates.

The maximum biomass dry weight occurred at day 9 (11 g dw·L^{-1}). This biomass concentration was higher than observed in immobilized cultures grown in 1B5 medium due to high levels of carbohydrates in APM.

5.1.5 GROWTH IN THE 2L IMMOBILIZED CULTURES USING THE 2-STAGE PROCESS

The characteristic growth parameters of the 2L, 2-stage immobilized cultures are summarized in FIGURE 14 and TABLE 2. The growth of *C. roseus* cells was faster in 2,4-D than in IAA, as demonstrated by doubling times of 2.1d and 3.2d in 1B5 and AB5 respectively. The maximal biomass is 12.3 g dw L^{-1} which occurred at day 18 of the culture. This was higher than in 1B5 medium because of increased level of carbohydrate available for growth following APM addition. The biomass yield for the whole culture was lower in this system than in the 200mL suspension cultures grown for 22 days in APM (TABLE 2).

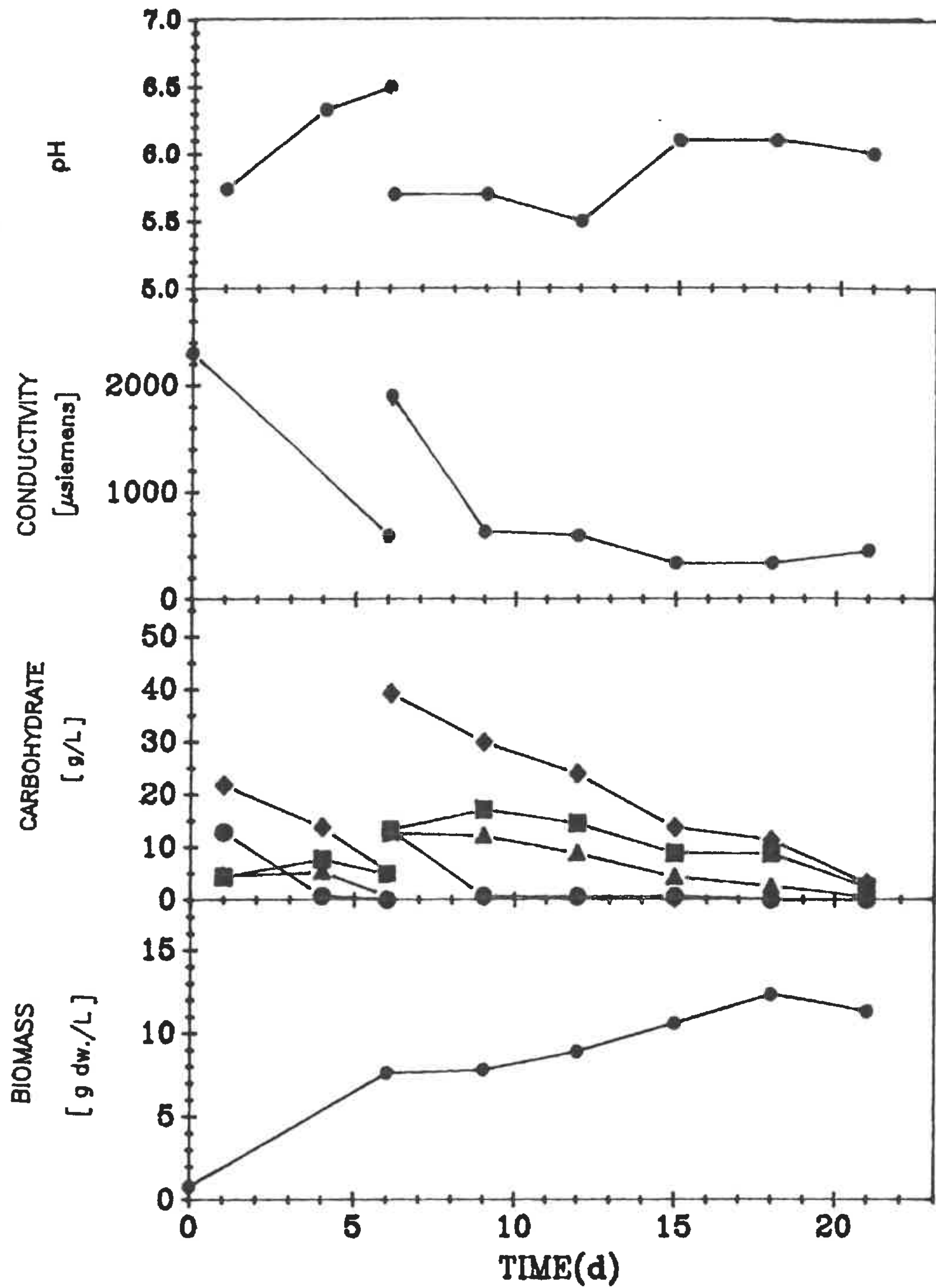


FIGURE 14: Growth in 2L SIPC bioreactors using the 2-Stage process

● Sucrose, ▲ Glucose, ■ Fructose, ◆ Total carbohydrates.

TABLE 2

Comparison of growth parameters in the 200mL suspension cultures and in the 2L immobilized bioreactors

Culture	Total Culture Period (d)	Medium	Maximal Biomass (g dw·L^{-1})	Biomass yield ($Y_{x/s}$) (g dw/ g carbohydrate)	Specific Growth Rate (d^{-1})
200mL	10	1B5	10.3	0.59	0.50
200mL	22	APM	24.2	0.50	0.34
2L SIPC	18	1B5	7.4	0.40	0.48
2L SIPC	15	APM	11.1	0.40	0.19
2L SIPC	21	2-STAGE [1]	12.3	0.14 [2] 0.23 [3]	0.31 [4]

[1] 2-STAGE= 2 stage culture with a 6 day growth period in AB5 an a production period of 15 days in APM.

[2] Biomass yield after 6 days in AB5.

[3] Biomass yield during whole culture period (21 days).

[4] Specific growth rate calculated after 6 days in AB5.

5.2 NATURAL PRODUCT RELEASE

NATURAL or CONSTITUTIVE release of indole alkaloids by cultured *Catharanthus roseus* cells into the medium was defined as the amount of product detected in the culture medium during a typical culture. RELATIVE product release was calculated as the fraction of alkaloids in the medium compared to the total alkaloids produced by the culture (Equation 6). The ABSOLUTE release referred to the concentration of a given alkaloid detected extracelluarly.

5.2.1 NATURAL PRODUCT RELEASE IN 200ML SUSPENSION CULTURES

The 1-STAGE PROCESS 200mL suspensions were cultured for 15 days in **APM.** The relative extracelluar total alkaloid content was initially detected at 13% on day 3. It increased until the end of the culture where it reached 32%. The pH remained constant during cultivation and did not seem to be responsible for the observed increases in release. The significant excretion (21.6%)observed from day 9, may have resulted from cell lysis as indicated by the ammonium ions detected in the medium from that point(102).

RELATIVE alkaloid release results obtained in suspension cultures performed according to the 2-STAGE process are illustrated in FIGURE 15. The fractional release of total alkaloids was high at day 3 during maximal growth and dropped rapidly at day 6. The medium exchange at day 6 stimulated high release from 2% to 37%. Total alkaloid release dropped to a low level of ~6% at day 9 which stayed mostly constant for the remainder of the culture.

The increase of alkaloids present in the medium following medium exchange was not the result of cellular lysis because complete spectrum of alkaloids found intracellularly was not detected in the medium.

TABLE 3

Natural release of total alkaloids in *C. roseus* cell cultures

Culture Time (d)	Suspension cultures Alkaloid concentration			Immobilized cultures Alkaloid content		
	Medium [mg/L]	Cells [mg/L]	% Product release	Medium [mg/L]	Cells [mg/L]	% Product release
1-Stage[1]						
0	0	0	0	0	3.9	0
3	6.2	46.0	11.9	1.6	9.7	14.1
6	3.0	46.0	6.1	16.0	13.0	55.2
9	7.6	29.0	20.8	12.0	21.0	36.0
12[3]	12.0	68.0	15.0	3.1	40.0	7.2
15[3]	19.0	45.0	29.7	6.2	46.0	12.0
2-Stage[2]						
0	0	0	0	0.5	1.9	20.8
3	17.0	37.0	31.5	52.0	8.4	86.1
6	4.5	170.0	2.6	19.0	12.0	61.3
6[4]	26.0	40.0	39.4	-	-	-
9	9.0	190.0	4.5	18.0	18.0	50.0
12	N/A	680.0	N/A	23.0	24.0	48.9
15	65.0	670.0	8.8	22.0	30.0	42.3
18	25.0	430.3	5.5	21.0	160.0	11.6
21	39.0	380.0	9.3	15.0	32.0	31.9

[1] 1-Stage: one stage cultivation in APM.
[2] 2-Stage: two stage cultivation first for 6d in AB5 then in APM.
[3] Suspected partial lysis as indicated by the release of 0.3 mM NH_4^+ into the medium of suspension cultures.
[4] After medium exchnage from AB5 to APM.

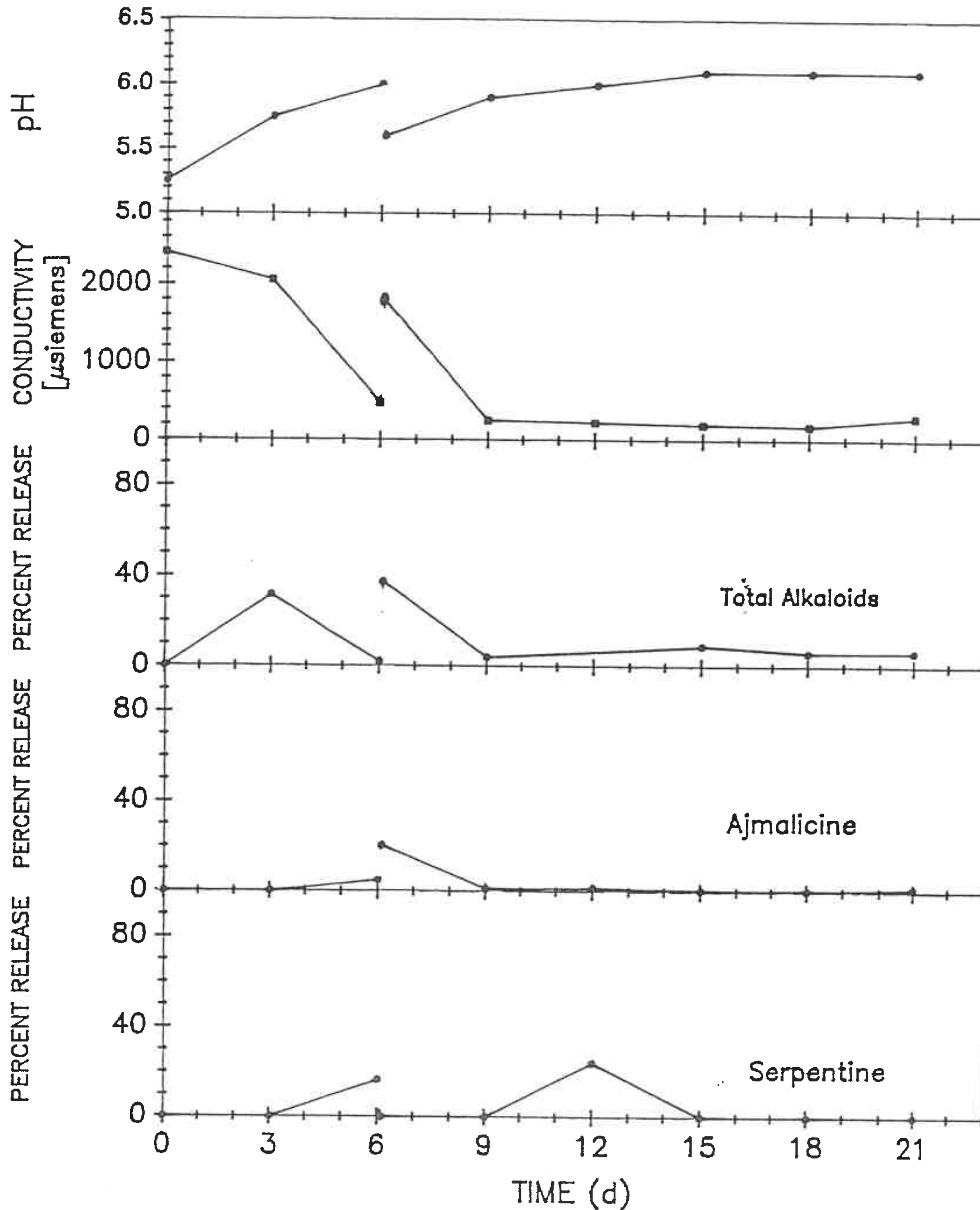


FIGURE 15: RELATIVE alkaloid release; total alkaloids, ajmalicine and serpentine in suspension cultures (200mL). Cells were cultured according to the 2 STAGE process (6 days in AB5, 15 days in APM). Cultures were maintained at 28±1°C under diffuse light on a rotatory shaker (150RPM).

The fraction of ajmalicine released into the medium began to increase at day 6. The medium transfer incited a significant increase in release which coincided with a drop in pH from 6.0 to 5.6. Thereafter, the range of values obtained for fractional release were low. Ajmalicine displayed similar percent release as observed for total alkaloids.

Serpentine was present extracelluarly prior to medium exchange at day 6. However, it could not be detected immediately following APM addition. At day 6, 16% of the total serpentine in culture was found extracelluarly compared to 5% for ajmalicine. Maximal percent release for serpentine was attained at day 12 with 24%, whereas ajmalicine and total alkaloids levelled off after medium exchange.

5.2.2 NATURAL PRODUCT RELEASE IN 2L IMMOBILIZED CULTURES

The release observed with immobilized cells cultured as per the 1-STAGE PROCESS is depicted in TABLE 3. The biosynthesis, represented by the sum of cellular and medium alkaloid content, continually increased. Total alkaloid release in the 1-STAGE system did not depend on synthesis nor on extracelluar pH. Ajmalicine and serpentine were only observed in the medium at the last day of culture (120) where an acidification from 5.6 to 5.1 occurred. This may have been the result of cell lysis; however, the conductivity of the medium did not increase at this point indicating no excessive release of inorganic ions as would occur during lysis(120).

Significant release of total alkaloids was observed in the 2L immobilized cultures when cultivated according to the 2-STAGE PROCESS. Values ranging from 12% to 76% release of total synthesized alkaloids were observed (FIGURE16 and TABLE 3). The release could not be attributed to cell lysis since no increase in conductivity (indicative of high ion concentration in the medium) nor nitrate concentration were detected in the medium. Moreover, the spectra of

products detected in the medium was not equivalent to those found in the cells.

Percent release of total alkaloids, ajmalicine and serpentine peaked at staggered intervals between day 3 and day 12 (FIGURE 16). Total alkaloid release maximized during rapid cell growth at day 3. A slow linear decrease from 50% to 30% was observed between day 9 and day 21 except for a sudden decline during maximal biosynthesis at day 18 (TABLE 3). Relatively constant ABSOLUTE concentrations of total alkaloids were seen in the medium although increases in secondary metabolite production occurred. This caused a decrease in the relative release and implied that alkaloid secretion was not a function of the intracellular accumulation of the products.

Ajmalicine and serpentine were both released at day 6 prior to medium exchange (FIGURE 16). The maximal release for these alkaloids differed; ajmalicine peaked at day 9 with 50% release while serpentine reached its maximum 3 days later (day 9) with 57% excretion(FIGURE 16).

The ABSOLUTE concentrations of ajmalicine and serpentine were followed in different cultures in order to characterize the pattern of release during the culture. FIGURE 17A&B represent 2 separate cultures where ajmalicine, serpentine and pH were monitored. In FIGURE 17A , maximal ajmalicine level seemed to depend on acidity of the medium. The serpentine level remained constant from day 9 onward.

In FIGURE 17B the release of ajmalicine and serpentine were similar. The peaks of both alkaloids occurred at the lowest pH. The level of ajmalicine was lower in culture B than in culture A (800 $\mu g{\cdot}L^{-1}$ in FIGURE 17A vs. 180 $\mu g{\cdot}L^{-1}$ in FIGURE 17B). The pH attained 5.0 in FIGURE 17A while the lowest pH was only 5.7 in FIGURE 17B during the production

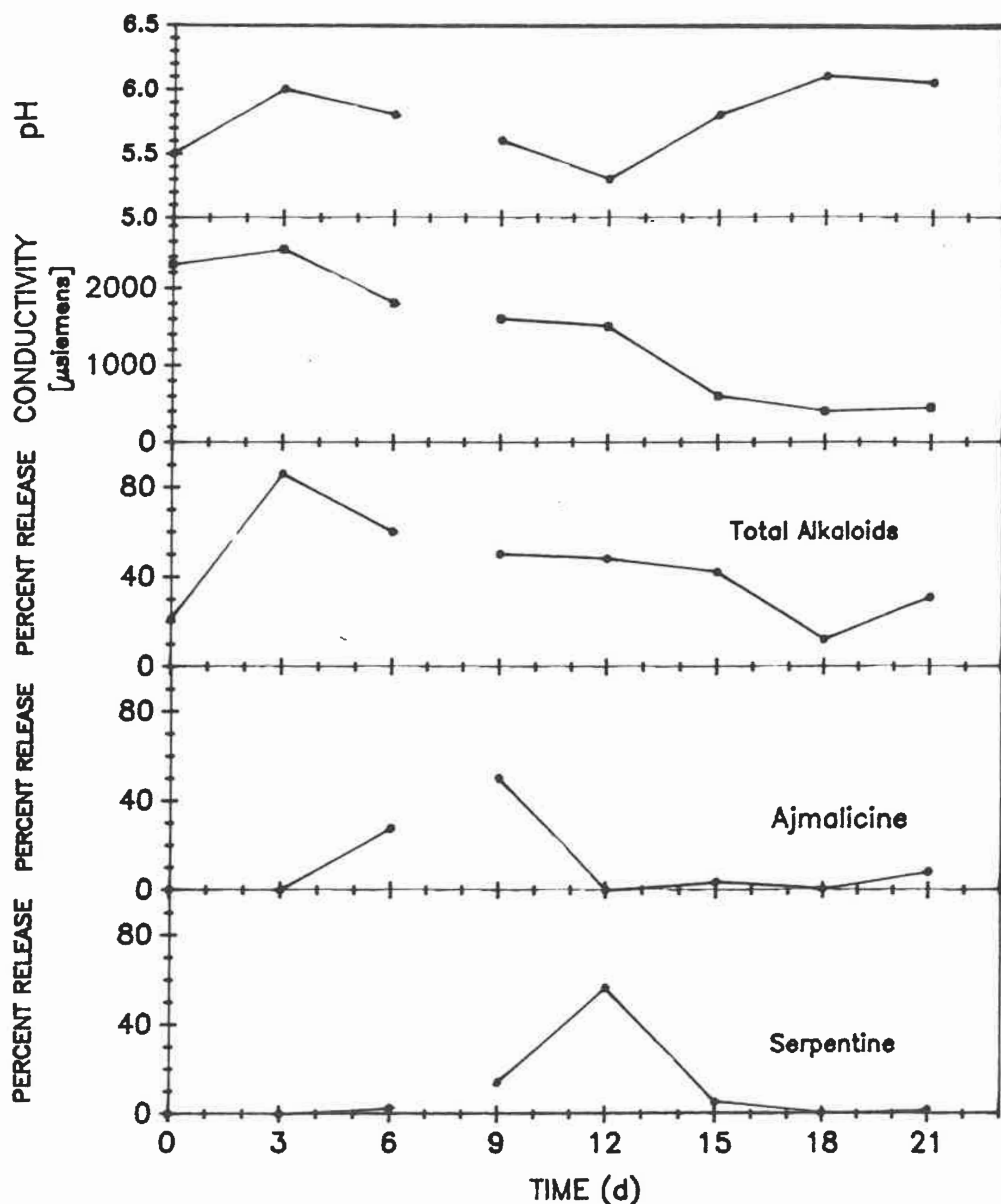


FIGURE 16: RELATIVE alkaloid release; total alkaloids, ajmalicine and serpentine in immobilized cultures (2 L). Cells were cultured according to the 2 STAGE process (6 days in AB5, 15 days in APM). Cultures were maintained at 28±1°C, aeration was kept at 0.07VVM (K_la~ 6h^{-1}) and stirring at 300 RPM.

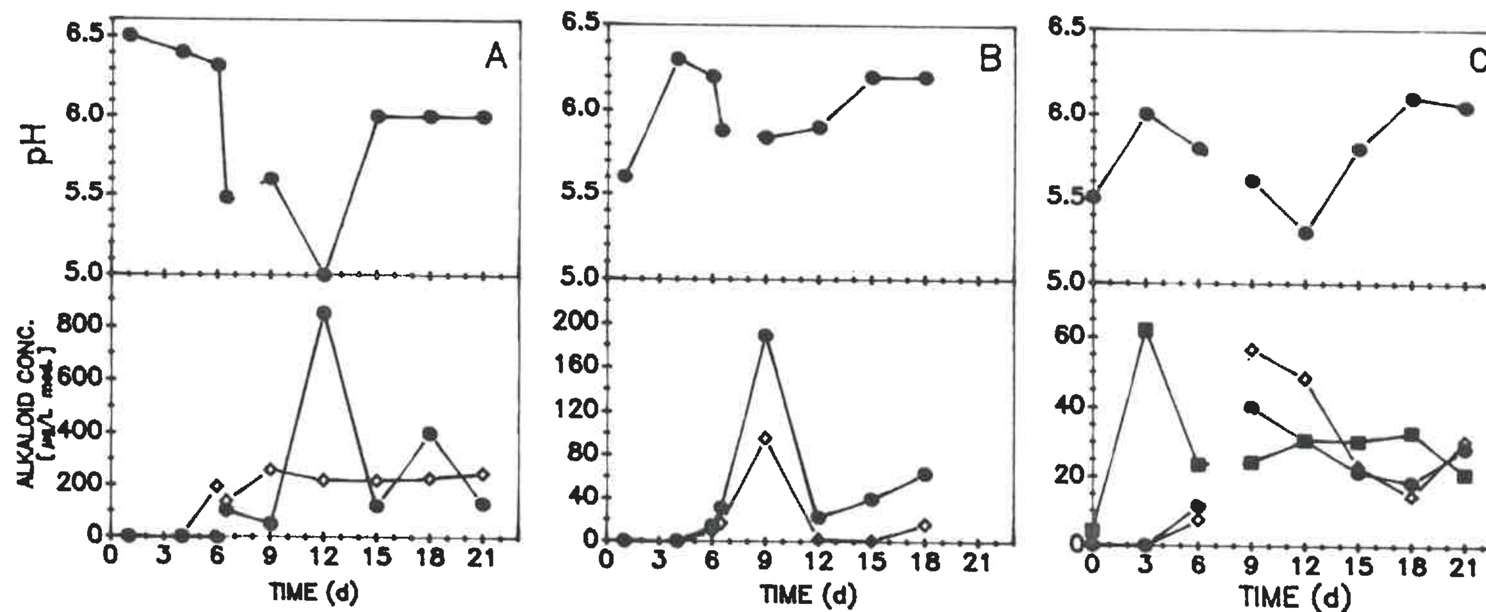


FIGURE 17 ; RELATIONSHIP BETWEEN PH AND ALKALOID RELEASE IN 2 L IMMOBILIZED CULTURES USING THE 2 STAGE PROCESS

A: ABSOLUTE ajmalicine & serpentine release of a 21–day culture.

B: ABSOLUTE ajmalicine & serpentine release of a 18–day culture.

C: ABSOLUTE ajmalicine & serpentine release; values obtained for each day corresponded to one bioreactor harvested.

● = ajmalicine, ◇ = serpentine, ■ = total alkaloids (mg/L).

period. This difference in pH may have been responsible for the 5 fold difference in alkaloid release. The inconsistent pH profile between the two cultures demonstrated high variability of incompletely monitored/controlled plant cell cultures used to produce secondary metabolites.

The culture in FIGURE 17C differed from those in FIGURES 17A and 17B because results obtained on a given day were extracted from separate cultures; seven 2 L immobilized cultures were run and harvested at different intervals. The release of ajmalicine and serpentine was similar to the culture monitored in FIGURE 17B. The lowest pH occurred at day 12 (pH 5.3) while serpentine release peaked at day 9. The ABSOLUTE alkaloid concentrations in these reactors were lower than that shown in FIGURE 17A and 17B. Decreasing culture pH seemed to parallel increasing release of ajmalicine and possibly serpentine in two of the three cases.

chapter 6

6.0 STIMULATED ALKALOID RELEASE

6.1 ALKALINIZATION EXPERIMENTS

6.1.1 GROWTH OF 6L IMMOBILIZED CULTURES USING THE 2-STAGE PROCESS

The 6L cultures were carried out as per the 2-Stage Process which consisted of a 6 day growth period in AB5 medium followed by a 15 day production period in APM medium. The culture parameters monitored included pH, conductivity, nitrate and carbohydrates. These were followed during the whole culture.

The behaviour of culture IA22 is presented in FIGURE 18. During the growth phase, the consumption rates for nitrate and carbohydrate were $2.22 mM \cdot d^{-1}$ and $1.7\ g \cdot L^{-1} \cdot d^{-1}$ respectively (TABLE 4). These were significantly lower than those found for the 2L immobilized cultures (TABLE 2). Carbohydrates, NO_3 and inorganic ions as detected by conductivity readings, were being consumed beyond the end of the growth period. Indicating sustained viability of the culture during the beginning of the production phase.

The final biomass yield on carbohydrate was 0.14 g dw/ g carbohydrate consumed. The culture reached a final dry weight of 27.9 g (TABLE 4). Inorganic ions, monitored off-line as conductivity, and carbohydrates were consumed up until day 12: thereafter, they remained constant. Thus, the cells reached stationary growth 6 days following medium exchange. The carbohydrate stabilized at 40 $g \cdot L^{-1}$ while conductivity remained at ~2000μsiemens.

The variations in NO_3 concentrations measured did not correlate with the conductivity measurements. It was demonstrated previously that these two parameters paralleled one and other (120). The abnormal phenomenon observed herein was caused by the interference of

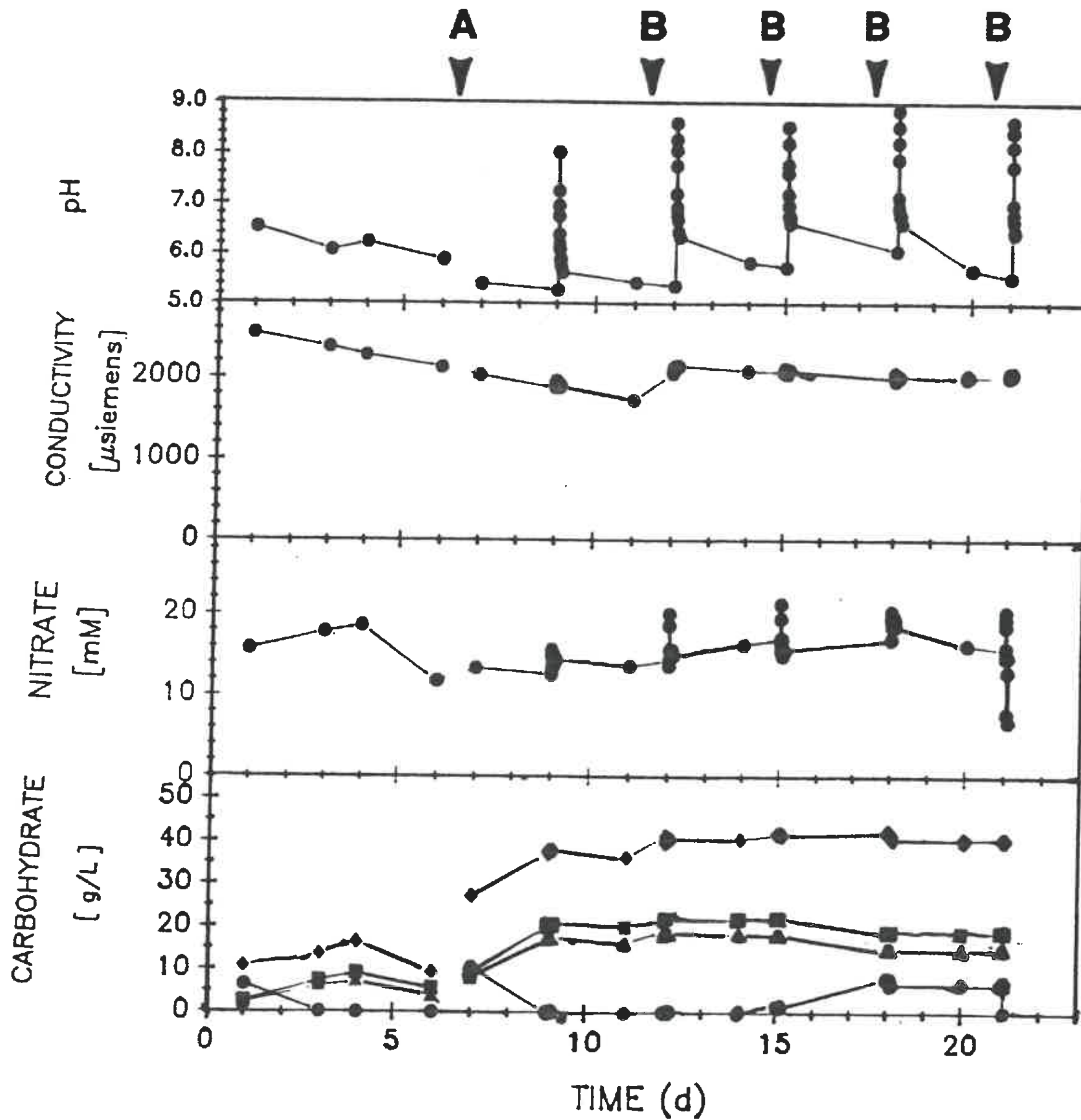


FIGURE 18: Culture IA22: Growth of MCR17 cultured according to 2 stage process in 6L SIPC. Alkalinizations were carried out at days 9,12,15,18 and 21. A=Medium exchange AB5 for APM. B= Addition of 20% APM. ● =Sucrose, ▲ =Glucose, ■ = Fructose, ◆ =Total carbohydrate.

TABLE 4

Comparison of culture characteristics of 2-Stage 6L immobilized systems

Culture	Culture Period (d)	Stimulation Experiment	NO3 Consumption (mM d^{-1})[1]	CHO Consumption (g L^{-1} d^{-1})[1]	Final Biomass (g)	$Y_{x/s}$
IA20	12	NONE	3.36	1.64	31.8	0.56
IA12	12	KOH	2.98	1.56	30.5	0.33
IA19	18	KOH	2.37	1.46	25.8	0.17
IA22	21	KOH	2.17	1.77	27.9	0.14
IA23	21	HCl	2.44	1.91	31.4	0.13

[1] Calculated during 6 day growth period in AB5 medium

KOH and/or high pH on NO_3 determination using the Orion probe as demonstrated in FIGURE 9 (section 4.3). Alkalinization did not induce cell lysis because conductivity remained constant following the modification of the pH (FIGURE 18).

Following three alkalinization pulses, there was an obvious decreases in sucrose hydrolysis at day 15. A similar behaviour was also noted in culture IA23. This may have been caused an interference of low [H^+] ions on invertase activity.

Cultures IA12 and IA19 showed similar nitrate and carbohydrate consumption rates as IA22 (TABLE 4). The high biomass yield on carbohydrate for IA12 was related to the shorter production period (12 days compared to 21 days for IA22). The conductivity remained constant following alkalinization. The erratic fluctuations of carbohydrate and NO_3 concentrations for IA12 (FIGURE 19) and IA19 (FIGURE 20) may have resulted from improper treatment of samples. For instance, NO_3 and carbohydrate concentrations were quantified after the samples for IA12 and IA19 were stored at -20°C which may have caused anomalities in the measurements.

As for IA22, the alkalinization of the medium in IA12 (FIGURE 19) and IA19 (FIGURE 20) did not cause cell lysis since pH was consistently reestablished, the conductivity remained stable and because the spectra of alkaloids detected in the medium was different from that observed intracellularly.

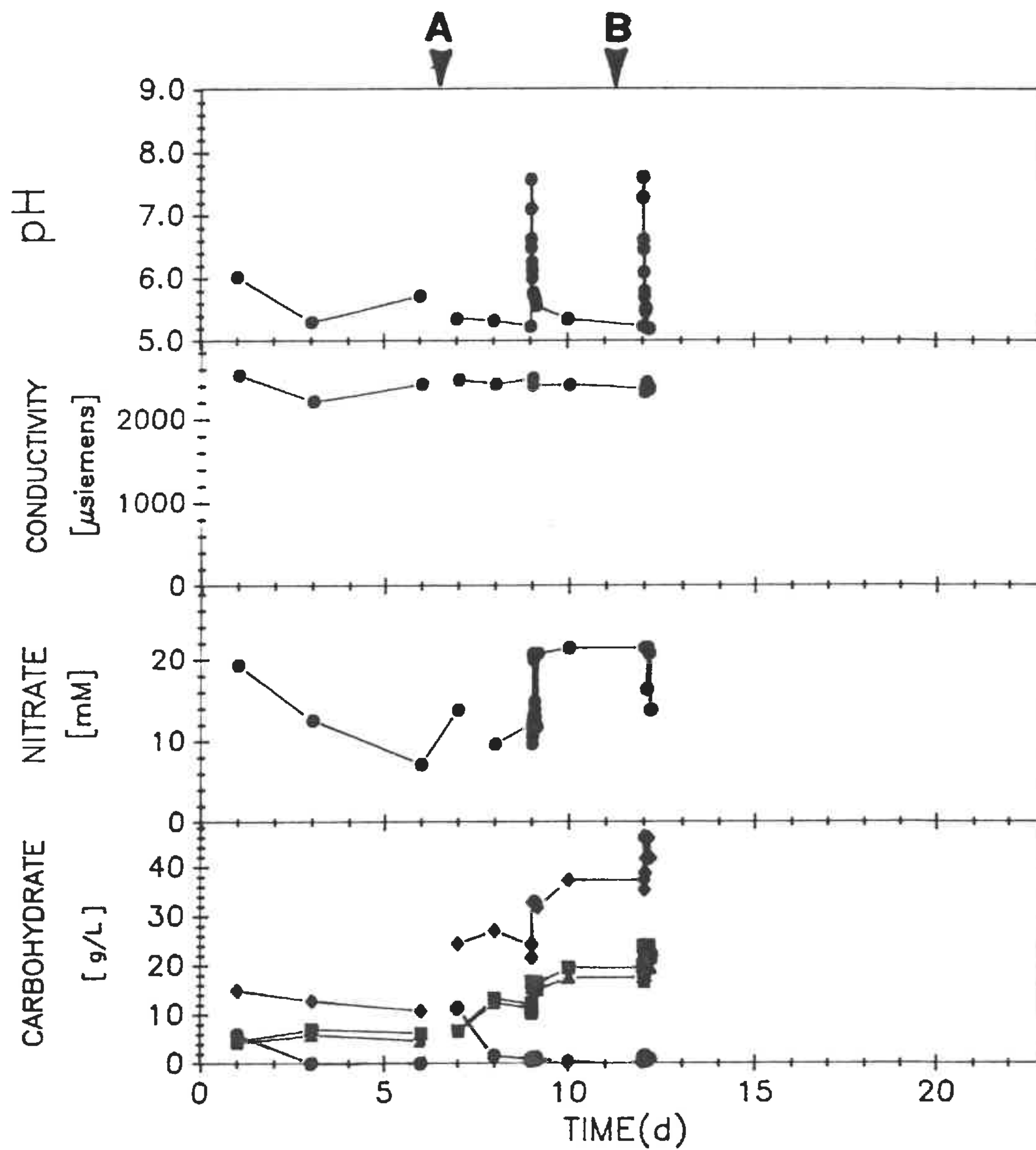


FIGURE 19: Culture IA12: Growth of MCR17 cultured according tp the 2 stage process in 6L SIPC. Alkalinizations were carried out at days 9 and 12. A= Medium exchange AB5 for APM. B= Addition of 20% APM. ● =Sucrose, ▲=Glucose, ■= Fructose, ◆=Total carbohydrate.

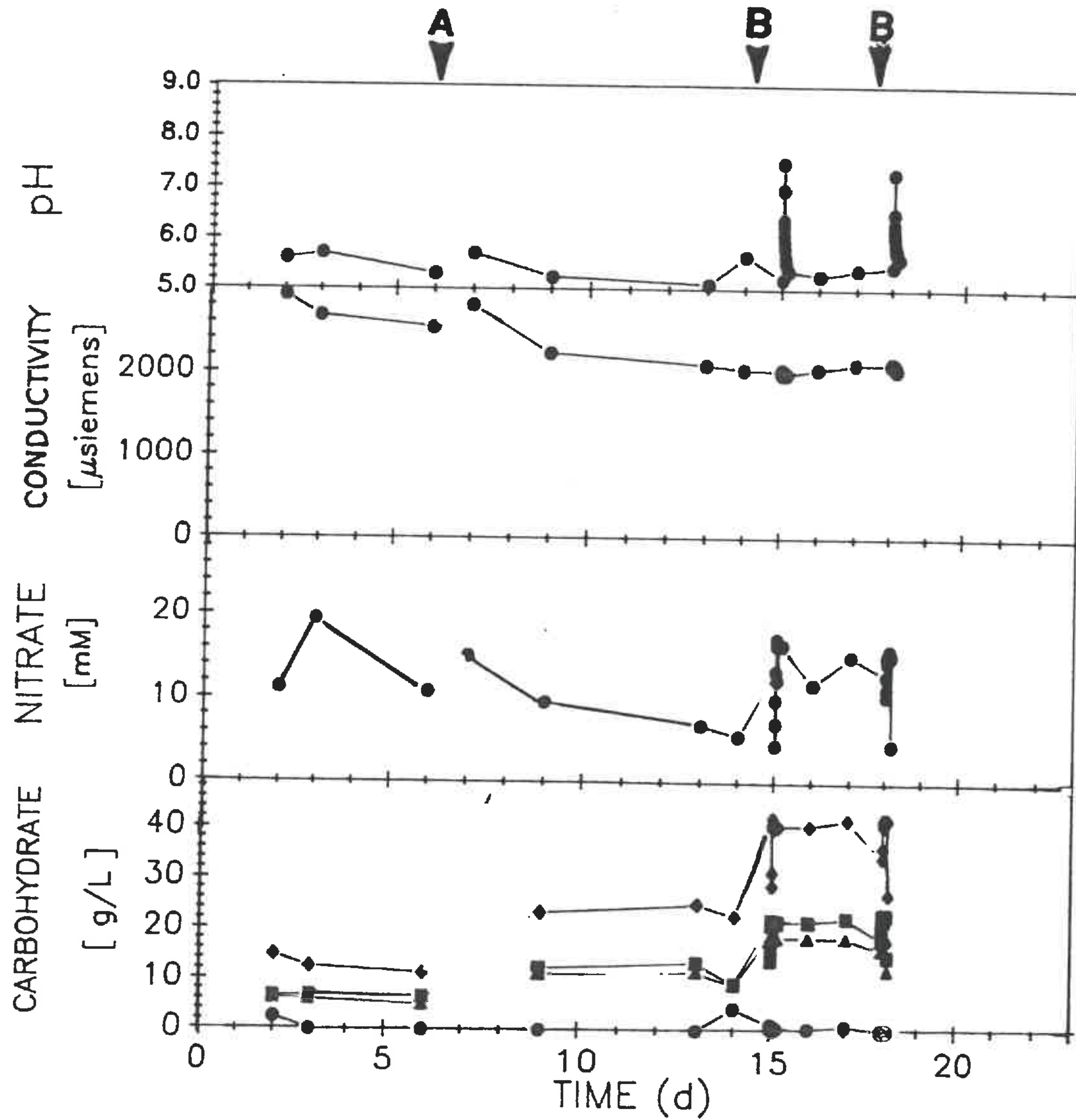


FIGURE 20: Culture IA19: Growth of MCR17 cultured according to 2 stage process in 6L SIPC. Alkalinizations were carried out at days 15 &18. A= Medium exchange AB5 for APM. B= Addition of 20% APM. ● =Sucrose, ▲ =Glucose, ■ = Fructose, ◆ =Total carbohydrate.

6.1.2 ALKALOID RELEASE

Results from the product release experiment IA22 are summarized in FIGURE 21. During the growth phase, there was a peak of released total alkaloids was observed at day 4 (11.4mg$\cdot L^{-1}$) which dropped to 6.2 mg$\cdot L^{-1}$ at day 6. A similar peak was observed in the 2L system at day 3 with 52 mg$\cdot L^{-1}$ (TABLE 3). The absolute concentration decreased when AB5 was removed and replaced with APM. This resulted from a dilution effect. In fact 8.6 mg$\cdot L^{-1}$ are released within 24 hours (from day 6 to day 7).

The addition of KOH caused significant efflux of alkaloids. During the first stimulation, increase of total alkaloids from 4.8 to 10.2 mg$\cdot L^{-1}$ was effected within 105 minutes of stimulation (FIGURE 22). The second pH change, at day 12, stimulated a five fold increase (from 2-10 mg$\cdot L^{-1}$) in total alkaloids in a much shorter period of time (10 minutes, FIGURE 23). Subsequent stimulations at days 15, 18 and 21 did not cause significant increases in the total alkaloid release (FIGURES 24-26).

Two quantifiable alkaloids, ajmalicine and serpentine, were also monitored (FIGURE 21). Prior to alkalinization both serpentine and ajmalicine were found in the medium at day 4 of culture which corresponded to the peak of total extracellular alkaloid concentration. The exchange of medium from AB5 to APM at day 6 incited an absolute release of 0.03 mg of ajmalicine and 0.05 mg of serpentine. The period between day 7 and day 9 showed a constant concentration of ajmalicine and serpentine in the medium, 0.01 an 0.02 mg$\cdot L^{-1}$ respectively (FIGURE 21).

The first change in pH at day 9 (pH 5.3-pH 8.0) caused a transient increase in serpentine concentration of 0.13 mg$\cdot L^{-1}$ (FIGURE 22) at 105 minutes while ajmalicine concentration

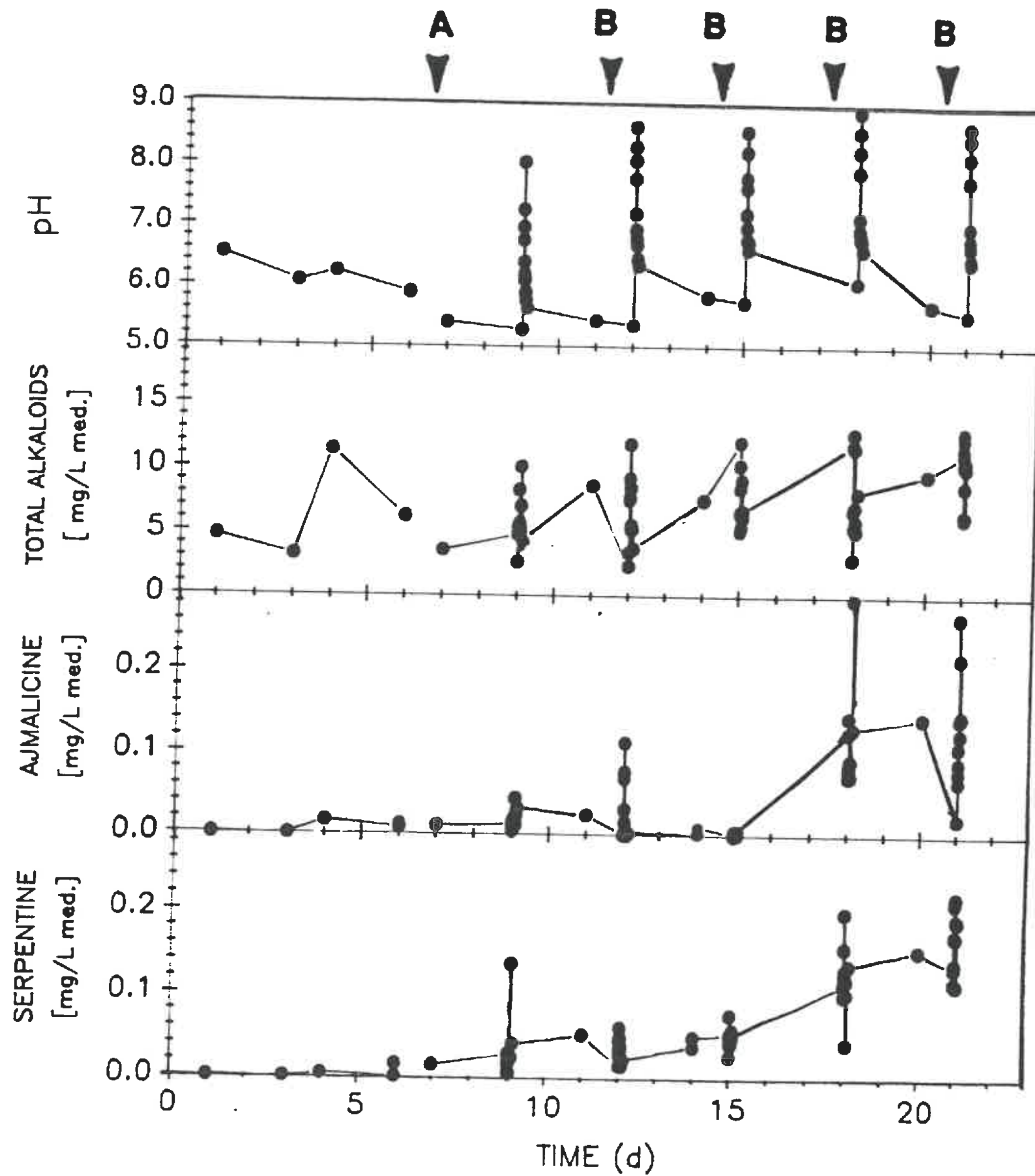


FIGURE 21: Culture IA22: Alkaloid release From MCR17 cultured with 2 stage process in 6L SIPC. Alkalinizations were carried out at days 9,12,15,18 and 21. A=Medium exchange AB5 for APM. B= Addition of 20% APM.

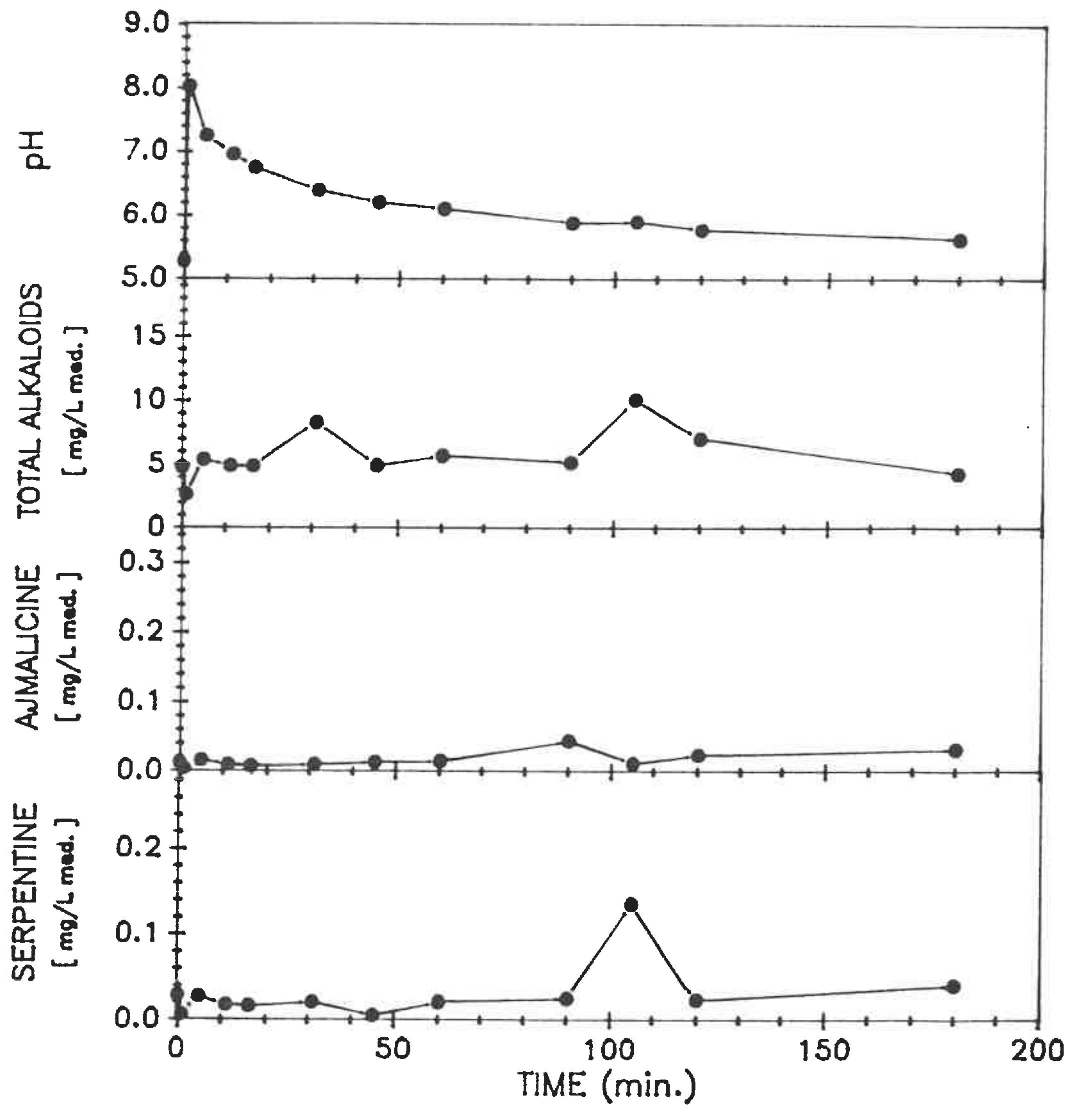


FIGURE 22: Culture IA22: Details of pH stimulated alkaloid release at day 9 during 3 hour period following alkalinization.

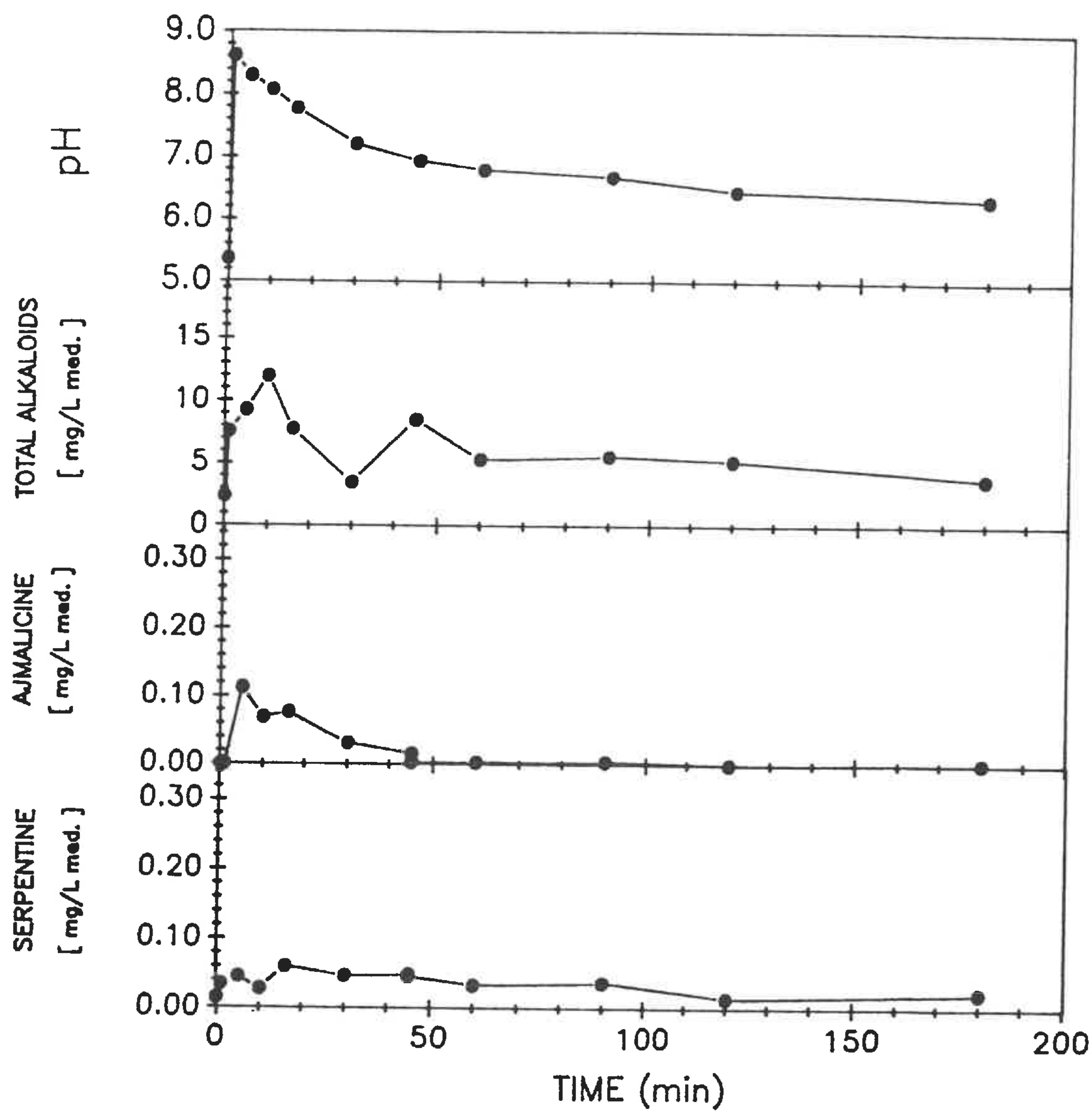


FIGURE 23: Culture IA22: Details of pH stimulated alkaloid release at day 12 during 3 hour period following alkalinization.

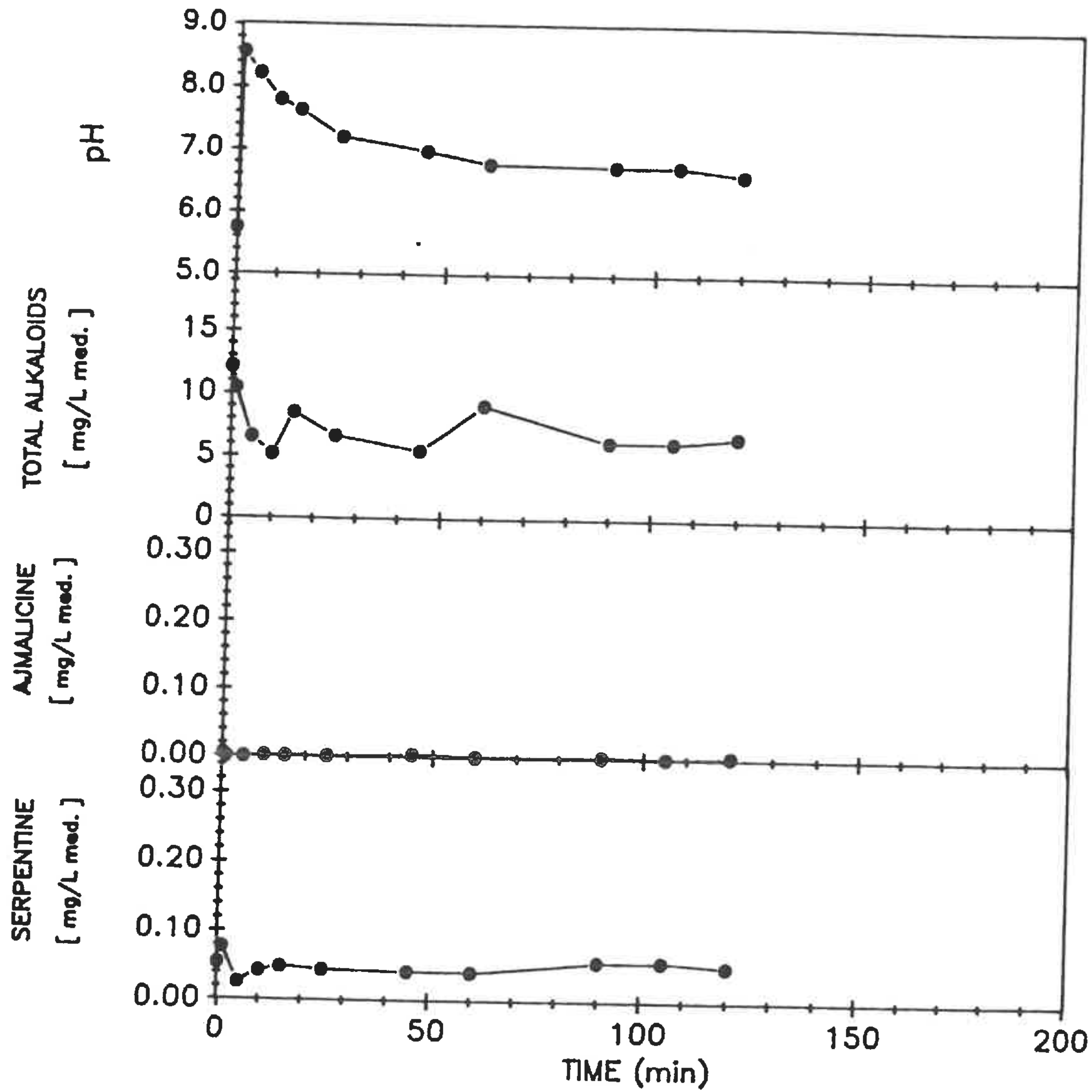


FIGURE 24: Culture 22: Details of pH stimulated alkaloid release at day 15 during 3 hour period following alkalinization.

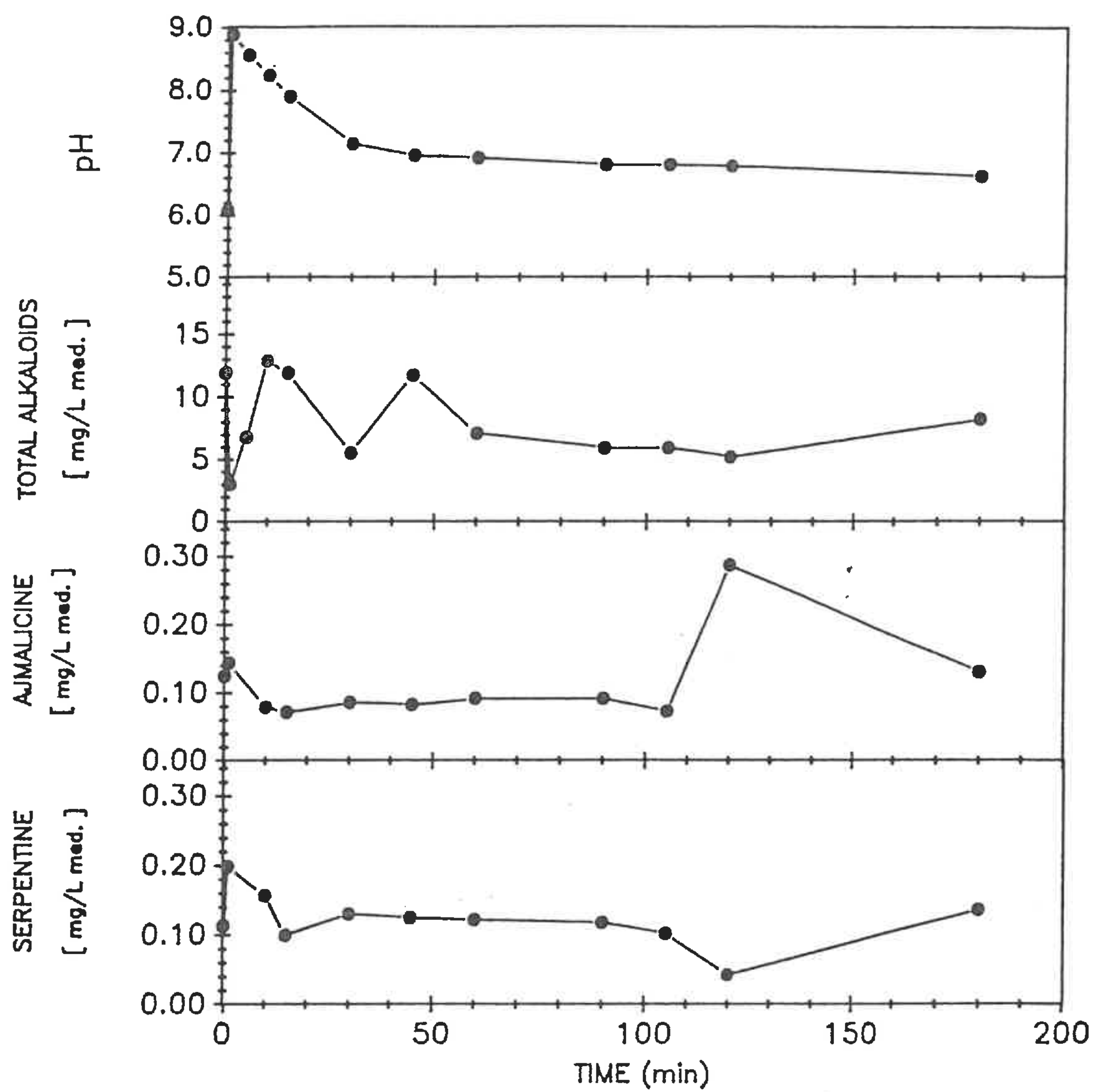


FIGURE 25: Culture IA22: Details of pH stimulated alkaloid release at day 18 during 3 hour period following alkalinization.

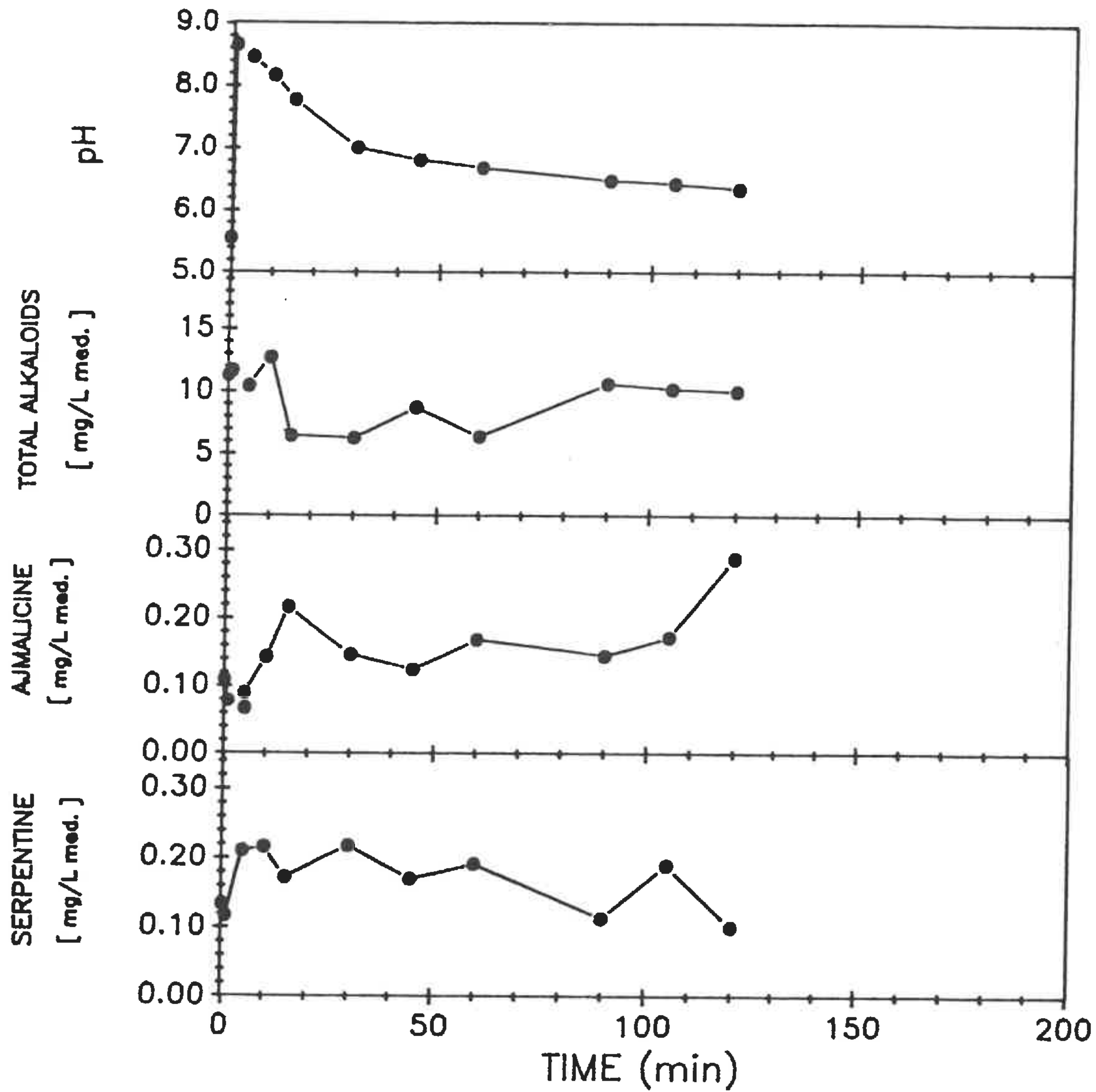


FIGURE 26: Culture IA22: Details of pH stimulated alkaloid release at day 21 during 3 hour period following alkalinization.

peaked at 90 minutes to 0.05 $mg \cdot L^{-1}$ (FIGURE 22). During the next 48 hours, the extracellular alkaloids remained constant. A noticeable decrease in ajmalicine and serpentine extracellular concentrations was effected following the addition of the 20% (v/v) of fresh APM (FIGURE 21). The second pH change at day 12 from pH 5.4 to pH 8.6, effected a 111 fold increased release of ajmalicine (0.001$mg \cdot L^{-1}$ to 0.111 $mg \cdot L^{-1}$) compared to a 4 fold increase (0.015 to 0.060 $mg \cdot L^{-1}$) of serpentine (FIGURE 23). The third stimulation at day 15 (ph 5.7 to 8.4) provoked insignificant alterations in the concentrations of ajmalicine and serpentine (FIGURE 24).

On day 18, alkalinization from pH 6.1 to 8.9 caused ajmalicine to increase from 0.13$mg \cdot L^{-1}$ to 0.29 $mg \cdot L^{-1}$ (t=120 min) while serpentine increased to 0.20$mg \cdot L^{-1}$ form 0.11 $mg \cdot L^{-1}$ in 1 minute (FIGURE 25). The final alkalinization (day 21) (pH 5.5 to 8.6), stimulated the ajmalicine levels to triple (0.10 $mg \cdot L^{-1}$ to 0.29 $mg \cdot L^{-1}$) while serpentine concentration increased from 0.13 $mg \cdot L^{-1}$ to 0.22 $mg \cdot L^{-1}$ (FIGURE 26). These last two stimulations resulted in similar magnitudes of release induction of the two alkaloids.

The level of serpentine consistently increased during the resting time between alkalinizations (FIGURE 21). Ajmalicine did not display this behaviour. Generally, short intervals were required to stimulate serpentine release (days 12, 15, 18 and 21) whereas ajmalicine release was more sluggish.

Other unknown alkaloids were also found to be released during alkalinization stimulation as shown in FIGURE 27. However, these could not be identified/quantified because standards were unavailable. FIGURE 27 presents a time course of HPLC chromatograms which can be used as a qualitative tool to observe relative quantities of unknown alkaloids at a given time during

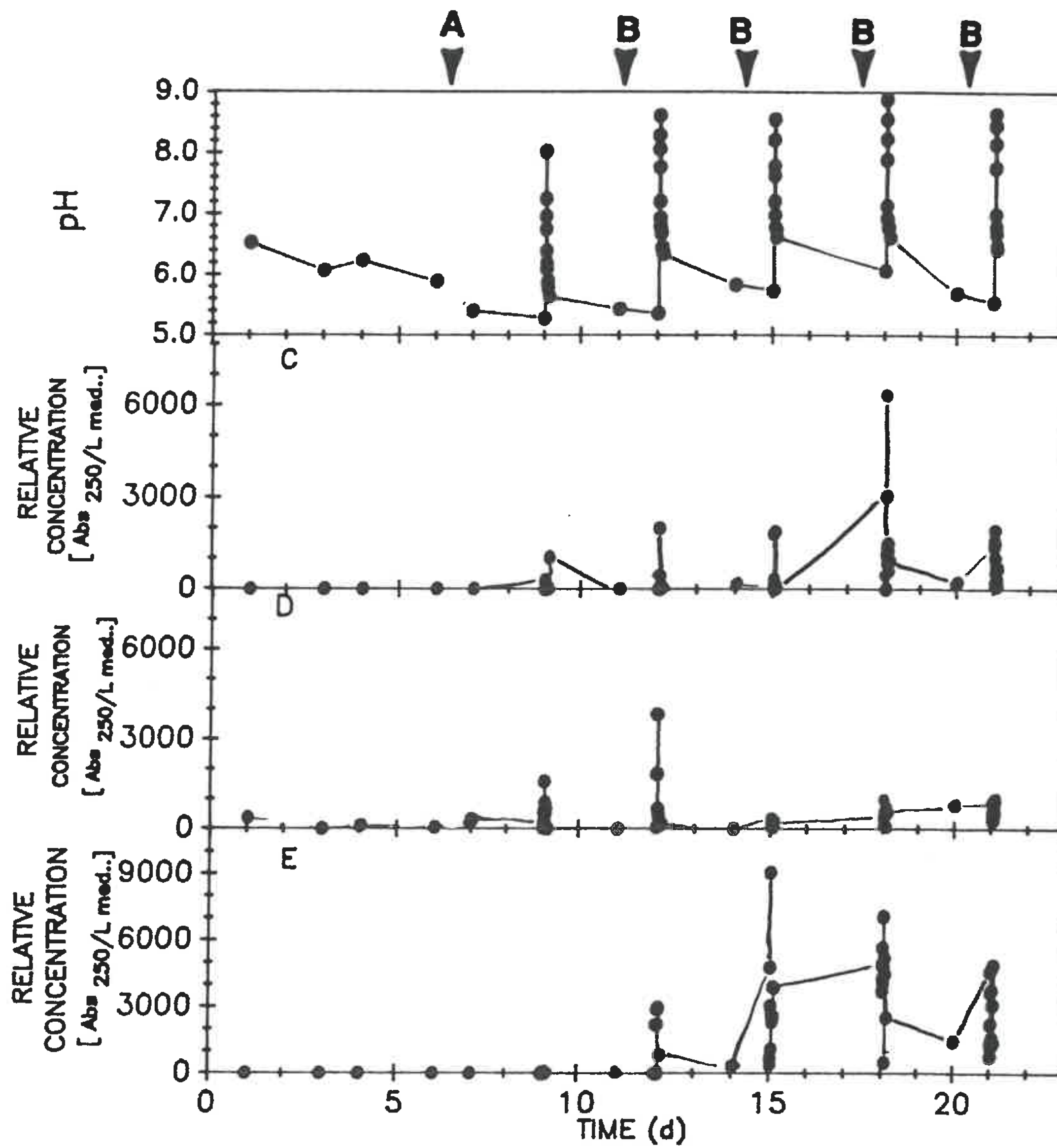


FIGURE 27 Time course release of unidentified alkaloids in the IA22 culture.

A= medium exchnage AB5 for APM.

B= addition of 20% APM.

C= Unidentified alkaloid eluted at 7.0 min.

D= unidentified alkaloid eluted at 21.0 min.

E= unidentified alkaloid eluted at 22.8 min.

the alkalinization at day 12. The relative concentrations of the 12 unknown compounds which displayed interesting behaviours during the alkalinization experiments were monitored during the whole culture. Some examples are included in FIGURE 28. A summary of the effectiveness of pH modification on these unknown alkaloid release is presented in TABLE 5 which illustrates whether unidentified compounds were present, increased or decreased following pH modification. Three hours after the first stimulation, 7 out of 12 compounds increased, 4 out of 12 decrease and 1 out of 12 remained undetected. On day 12, all of the compounds' extracellular concentrations increased during the addition of base of which 8 out of the 12 were not found prior to KOH addition. The fewest enhancements of alkaloid liberation occurred on day 21 where only 6 out of 12 demonstrated higher levels following alkalinization.

Culture IA12 displayed a different pattern of total alkaloid release during the growth period than IA22. This may have been affected by the high consumption rate of nitrate which occurred in this culture during the growth period. The high concentration of total alkaloid (17 $mg{\cdot}L^{-1}$) at day 3 coincided with increased ajmalicine release and natural acidification of the culture medium (FIGURE 29). The results for serpentine were not available for this culture. Prior to medium transfer of AB5 to APM, the pH increased while total alkaloid and ajmalicine both declined to 0.09 $mg{\cdot}L^{-1}$ and 0.01 $mg{\cdot}L^{-1}$ respectively. During the first three days in APM (from day 6 to day 9), the pH declined slowly while total alkaloid and ajmalicine concentration concomitantly increased.

The first alkalinization at day 9 induced an overall decrease in ajmalicine (0.023 to 0.008$mg{\cdot}L^{-1}$) and total alkaloid (11.7 to 5.6 $mg{\cdot}L^{-1}$) release. However, a transient peak occurred for both of these at 105 minutes. At day 10 of culture, 24 hours following the alkalinization the

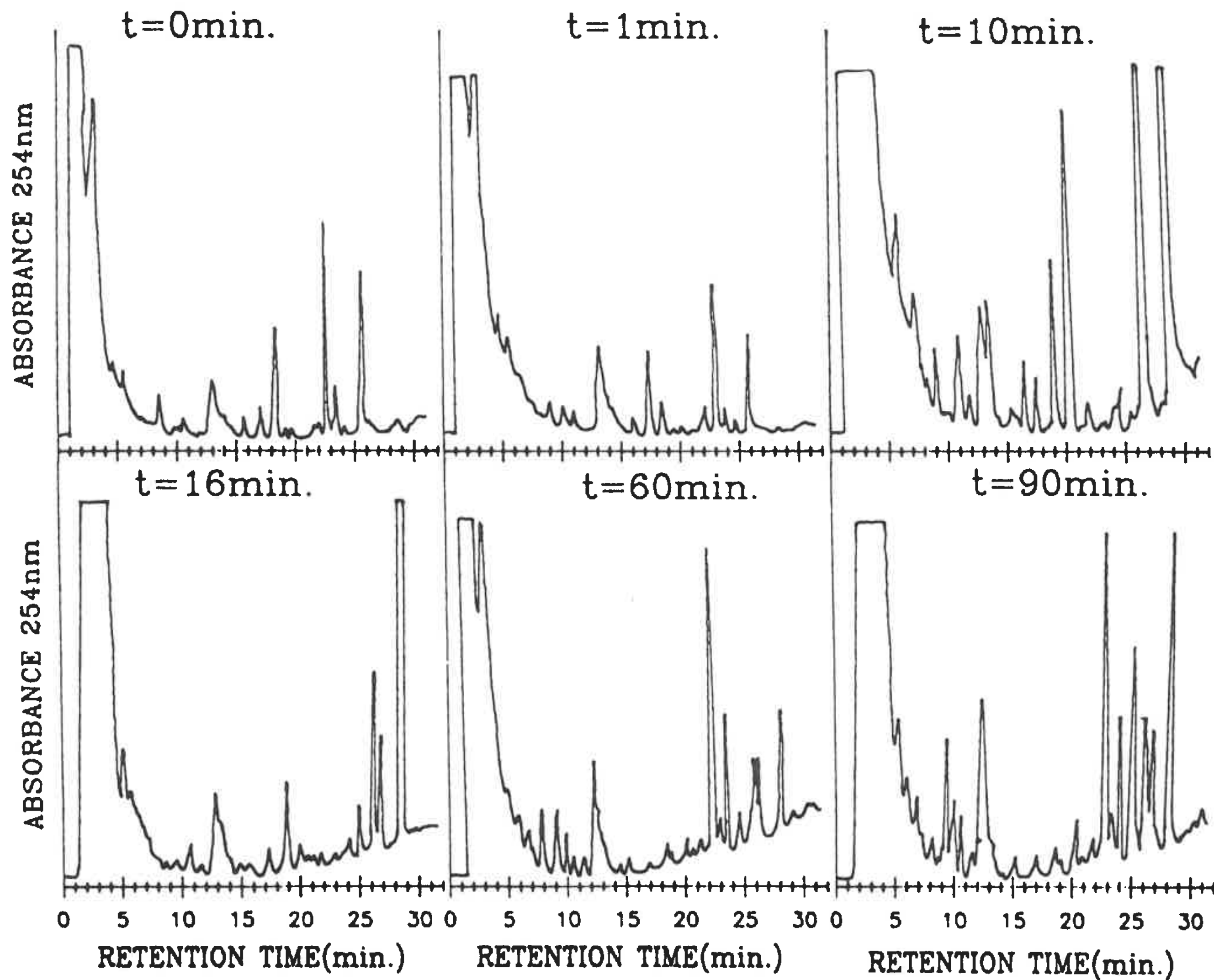


FIGURE 28: Comparison of alkaloid HPLC chromatograms during alkalinization pulse of culture IA22 on day 12.

TABLE 5

Effect of alkalinization on release of unidentified indole alkaloids

Retention time (min)	Day 9 pH 5.3-8.0			Day 12 pH 5.4-8.6			Day 15 pH 5.7-8.6			Day 18 pH 6.1-8.9			Day 21 pH 5.6-8.7		
	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]
7.0		X		X		X	X			X			X		
8.0	X		X	X		X		X		X			X		
9.5	X			X			X			X			X		
18.9	X		X	X		X	X	X	X			X		X	
20.1	X			X		X				X				X	
21.0	X		X	X					X	X			X		
22.8			X	X		X	X			X				X	
23.9		X		X			X			X				X	
23.5	X		X	X			X				X			X	
24.5		X		X		X		X		X		X			X
25.4	X			X		X		X		X			X		
26.9		X		X		X	X		X	X			X		
TOTALS	7	4	5	12	0	8	7	4	3	10	1	2	6	5	1

[1] I= Increase of extracellular concentration during three hour sampling period.
[2] D= Decrease of extracellular concentration during three hour sampling period.
[3] U= Undetected prior to alkalinization.

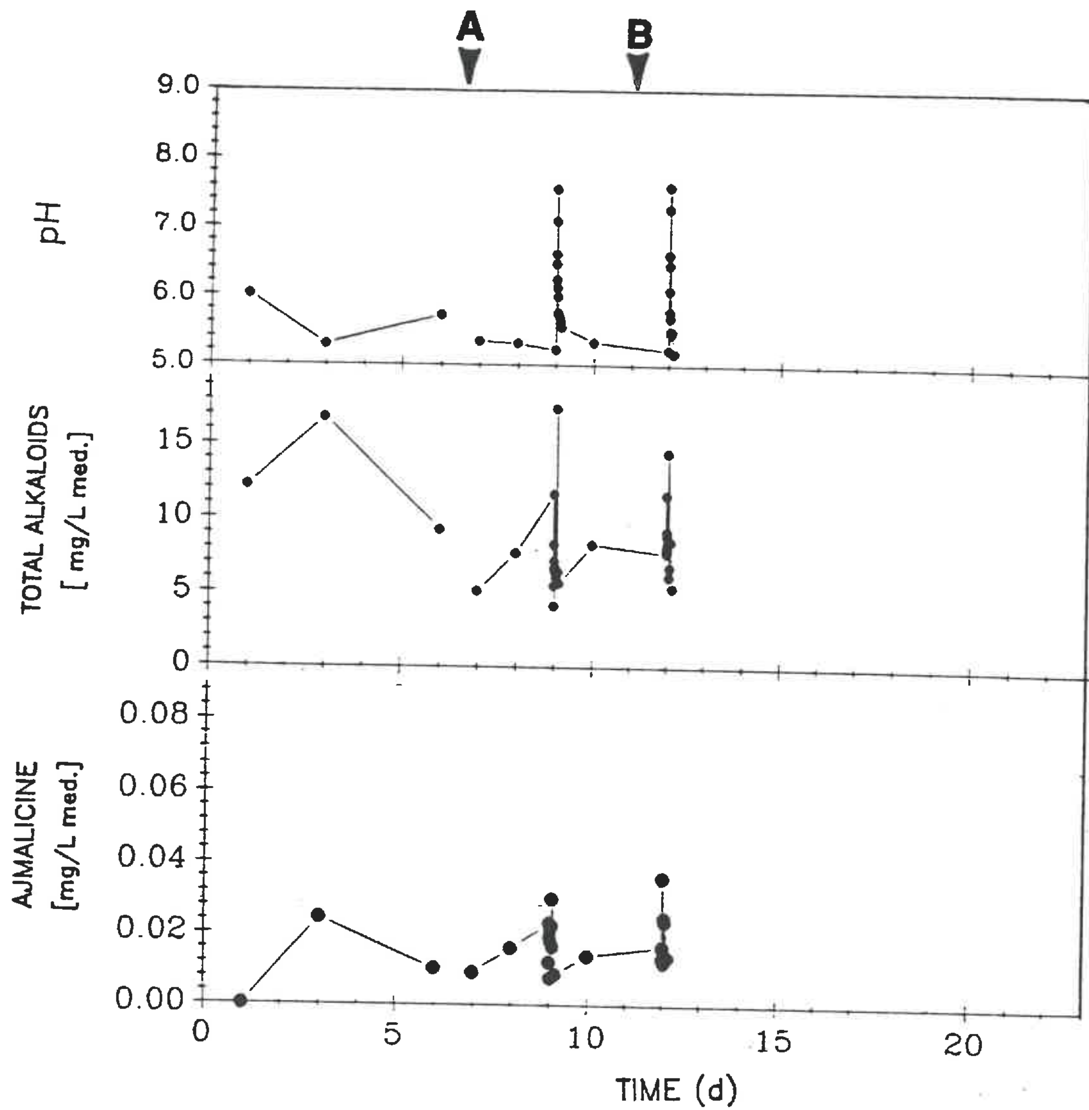


FIGURE 29: Culture IA12: Alkaloid release from MCR17 cultured with 2-Stage process in 6L SIPC bioreactor. Alkalinizations were carried out at days 9 and 12. A = Medium exchange AB5 for APM. B = Addition of 20% APM.

pH was reestablished and a slight release of total alkaloid and ajmalicine was observed. The 2nd stimulation at day 12 induced fluctuations in ajmalicine and total alkaloids with peak values greater than the starting levels (FIGURE 29). The positive effect was only transient and return to lower concentrations towards the end of the three hour sampling period was observed.

The maximal alkaloid concentration in IA19 (FIGURE 30) was seen at day 6 with 11.5 $mg \cdot L^{-1}$. This occurred during the growth period and coincided with an acidification of the culture medium. At day 9, three days following medium transfer, the level of alkaloids was maintained at 10 $mg \cdot L^{-1}$. Fresh medium was added at days 14 and 16. The first addition at day 14 did not incite any release of total alkaloids while at day 16 a total of 20 mg were released.

Day 15 was the time chosen to stimulate release by increasing pH from 5.2 to 7.9. This induced a slight increase of extracellular total alkaloids concentration(5.9 to 8.8 $mg \cdot L^{-1}$). The second alkalinization at day 18 stimulated excretion to a larger extent (10.6 to 18.4 $mg \cdot L^{-1}$) within 1 minute of pH change. The concentration returned to below the starting level by the end of the three hour sampling period.

The ajmalicine concentration showed a peak at day 6 which coincided with that of total alkaloids and a decrease in pH. No serpentine was detected in the medium during the growth period. The exchange of medium at day 6 did not incite increased release of either ajmalicine or serpentine. At day 13 the extracelluar concentrations of ajmalicine and serpentine were approximately equal (0.02 and 0.03 $mg \cdot L^{-1}$ respectively).

The addition of 20% fresh APM at day 14 caused release of ajmalicine and serpentine (0.095 mg and 0.062 mg). The first alkalinization stimulated sustained release of ajmalicine from 0.04 $mg \cdot L^{-1}$ to 0.06 mg L^{-1}. Serpentine release was transient and the initial level was reestablished.

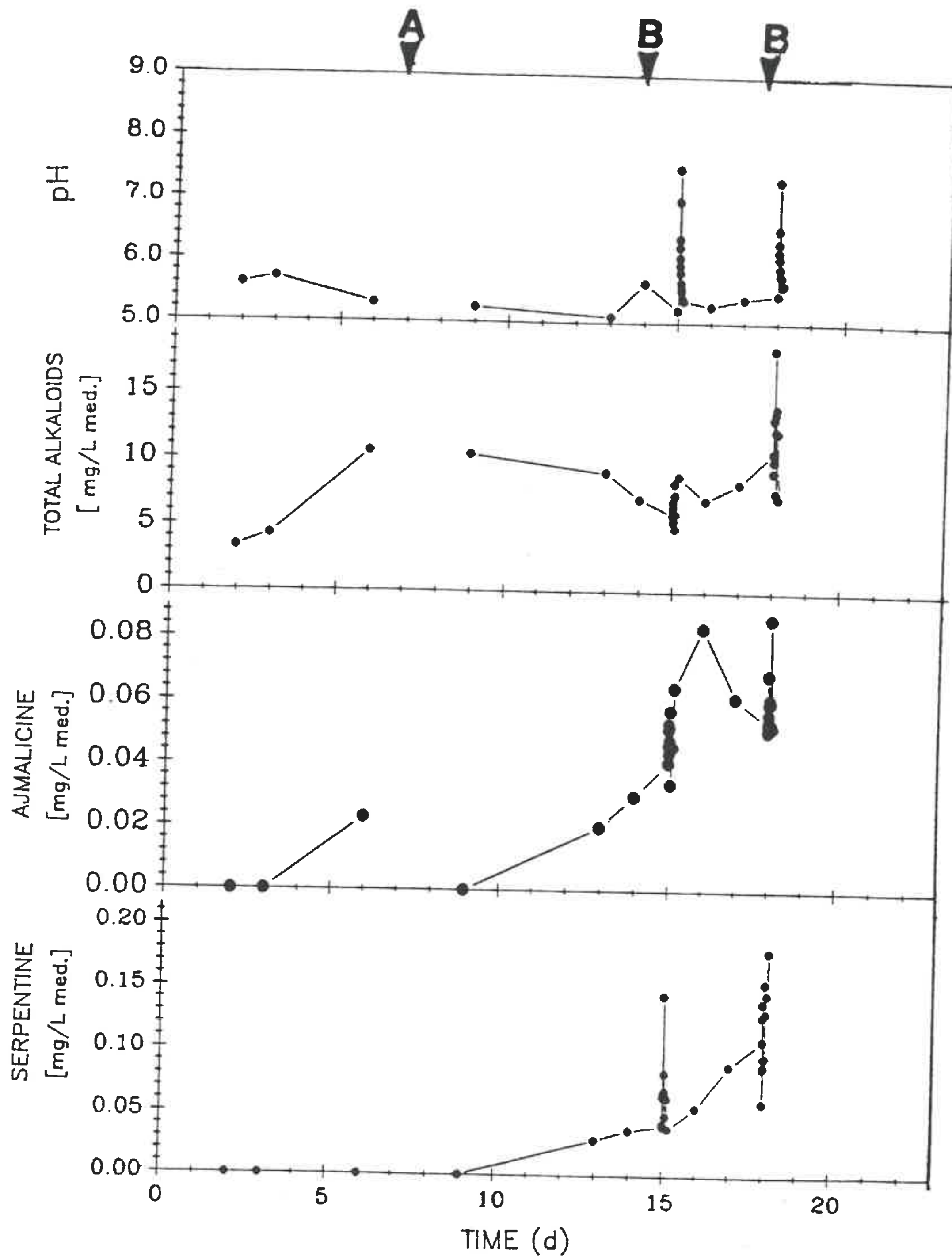


FIGURE 30: Culture IA19: Alkaloid release from MCR17 cultured with 2-Stage process in 6L SIPC bioreactor. Alkalinizations were carried out at days 15 and 18. A=Medium exchange AB5 for APM. B = Addition of 20% APM.

The concentration of these alkaloids increased 24 hours after stimulation. The final alkalinizationat day 18 stimulated release in both cases. The extracelluar concentration of ajmalicine increased from 0.053 to 0.095 $mg{\cdot}L^{-1}$ while serpentine increased from 0.11 to 0.18 $mg{\cdot}L^{-1}$.

6.2 ACIDIFICATION EXPERIMENT

6.2.1 GROWTH IN 6L IMMOBILIZED CULTURE USING THE 2-STAGE PROCESS

The growth parameters of the 6L, 2-stage culture IA23 were monitored as shown in FIGURE 31. During the first 6 days (growth phase), high consumption of carbohydrate and NO_3 was observed (TABLE 4). Similar consumption was seen in the 2L reactors ($2.72 g{\cdot}L^{-1}{\cdot}d^{-1}$ for carbohydrate and 2.5 $mM{\cdot}d^{-1}$ for NO_3) (120). The medium exchange at day 6 effected an increase in NO_3 from 13.3 mM to 15 mM and an increase in carbohydrate concentration from 8 $g{\cdot}L^{-1}$ to ~50 $g{\cdot}L^{-1}$.

Nutrients were consumed during the first three days of the production period at rates of 2.4 $mM{\cdot}d^{-1}$ and 6.5 $g{\cdot}L^{-1}{\cdot}d^{-1}$ for NO_3 and sugars respectively. During the period from day 9 to day 21, feed cycles of APM caused increases in the NO_3 concentration (14-20%), carbohydrate concentration (4%-10%) and conductivity level(10%).

At day 18, reduced hydrolysis of sucrose was observed. This may have been the effect of declined invertase activity as observed for IA22. The biomass yield ($Y_{x/s}$) for the whole culture was 0.13 g dw/ g carbohydrates consumed and the total final biomass in the reactor was 31.4 g dw. This corresponded to a final biomass concentration of 6 g $dw{\cdot}L^{-1}$. The specific growth rate could not be calculated because of the inaccessibility of the immobilized biomass during the

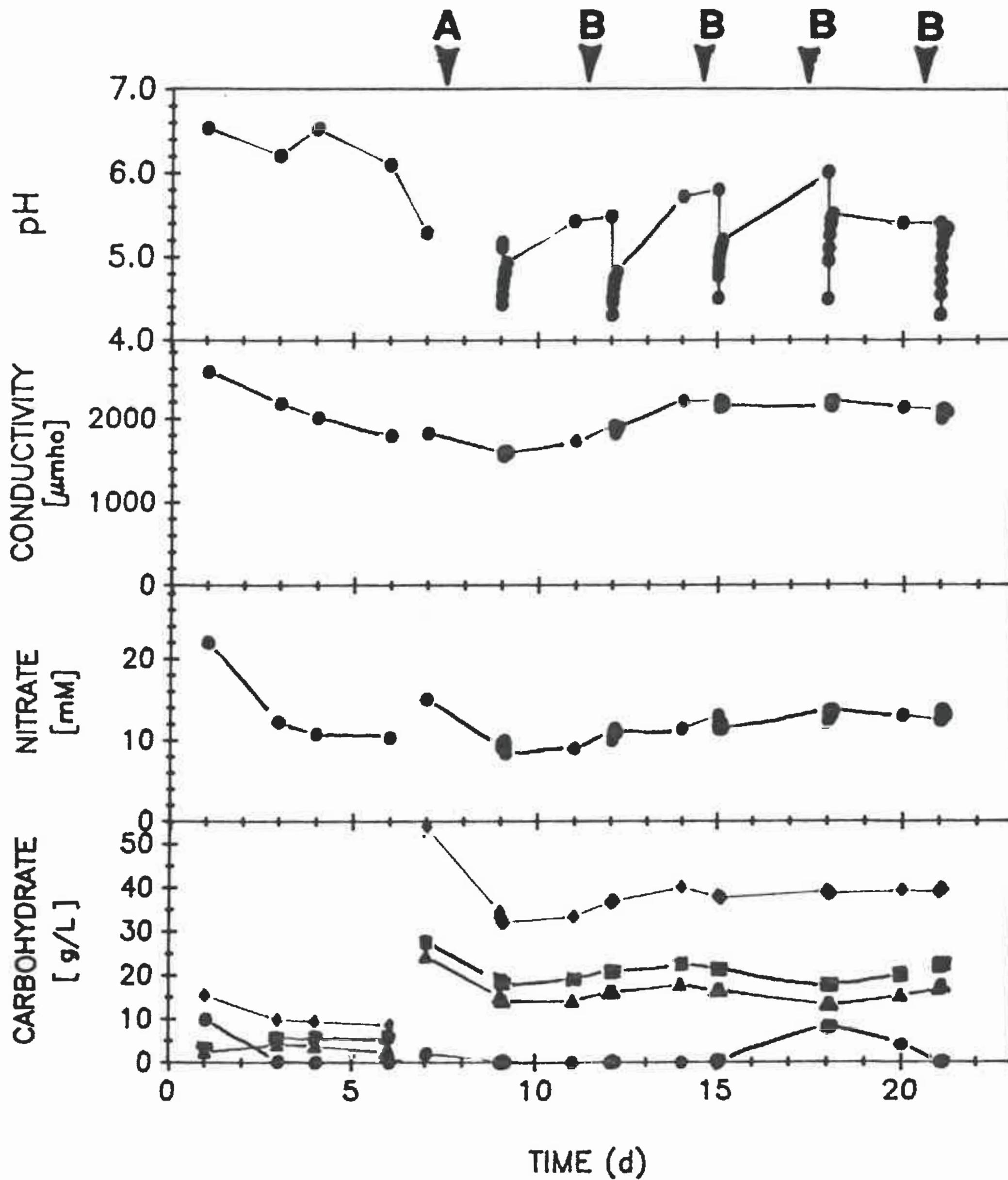


FIGURE 31: Culture IA23: Growth of MCR17 cultured according to the 2 stage process in 6L SIPC. Acidifications were carried out at days 9,12,15,18 and 21. A=Medium exchange AB5 for APM. B= Addition of 20% APM. ● =Sucrose, ▲=Glucose, ■= Fructose, ◆=Total carbohydrate.

experiment.

Decreases of 1 to 1.5 pH units induced by acid addition were all compensated for by the cells. Within one hour after the change in pH, the culture pH returned to within 0.2 to 0.8 units of the original value (FIGURE 31). This demonstrated the repeated buffering capacity of the cells except for the lagging pH reestablishment at day 12 which may have indicated slower metabolic activity of the cells. In this case, however, the initial pH was attained 48 hours following pH modification.

6.2.2 ALKALOID RELEASE

Results of indole alkaloid release stimulated by medium acidification are presented in FIGURES 32 to 37. The pH profile and alkaloid release during the whole culture are presented in FIGURE 32. During the growth phase, maximal total alkaloids (8.2 $mg{\cdot}L^{-1}$) detected in the medium was achieved at day 6 (FIGURE 32).

Medium transfer (AB5 to APM) at day 6 effected a dilution of extracellular alkaloid concentration from 8.1 $mg{\cdot}L^{-1}$ to 4.2 $mg{\cdot}L^{-1}$. Nonetheless ,during this transition, a total of 8.1 mg of alkaloids was in fact released by the cells. The total alkaloid concentration remained constant from day 6 to day 9 (FIGURE 32), The first acidification at day 9 stimulated a 2.3 fold increase (4.0 to 9.4 $mg{\cdot}L^{-1}$) of total alkaloids within three hours (FIGURE 33) . The extracellular alkaloid level remained stable until day 11 at which time APM was added to refill the culture for the next acidification; this medium addition effected the release of 22.4 mg of total alkaloids.

The second experiment at day 12 induced a 47% increase in medium total alkaloid concentration from 10 to 15 $mg{\cdot}L^{-1}$ (FIGURE 34) within approximately 60 seconds. The

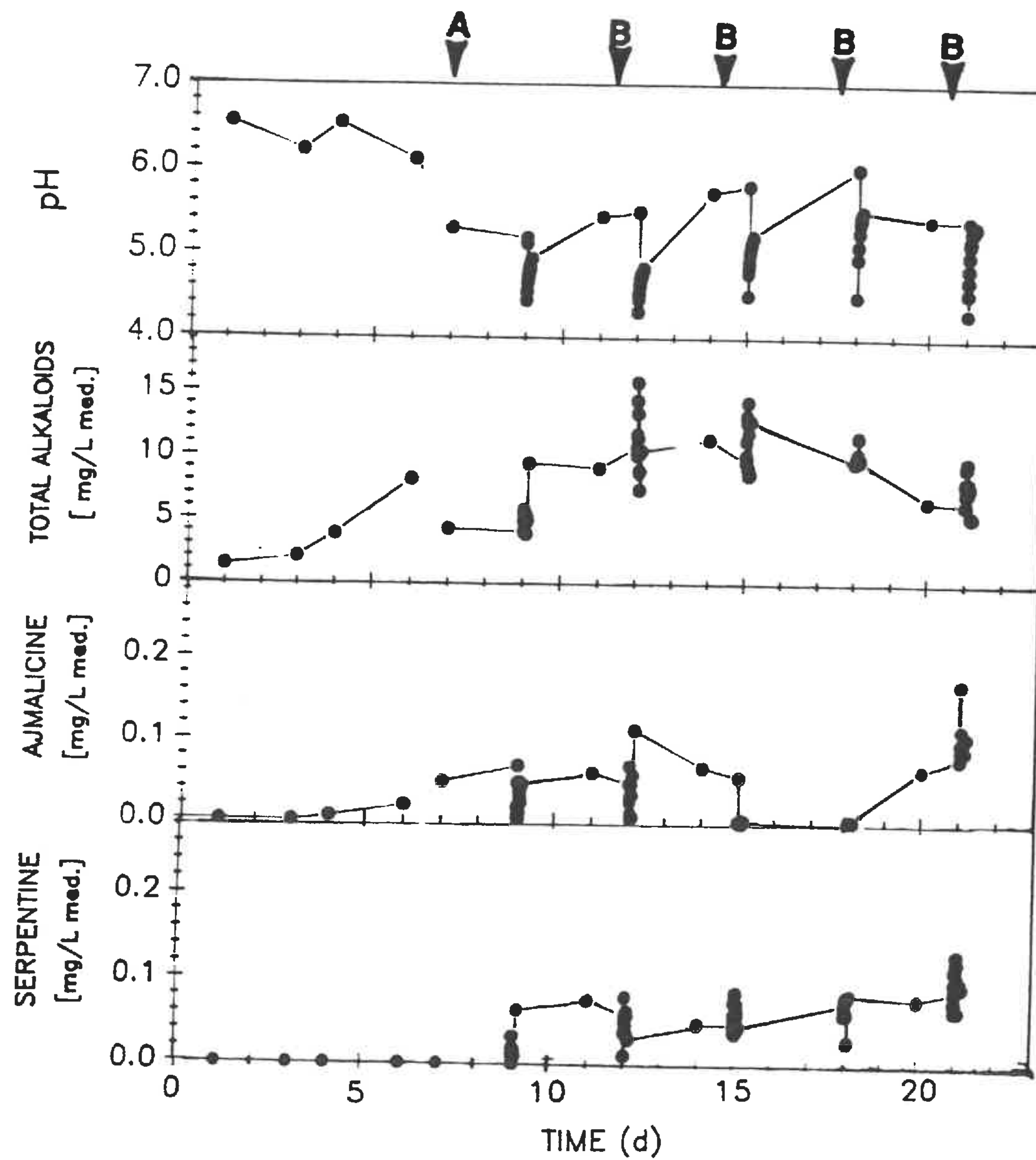


FIGURE 32: Culture IA23: Alkaloid release fromMCR17 cultured with 2 stage process in 6L SIPC. Acidifications were carried out at days 9,12,15,18 and 21. A=Medium exchange AB5 for APM. B= Addition of 20% APM.

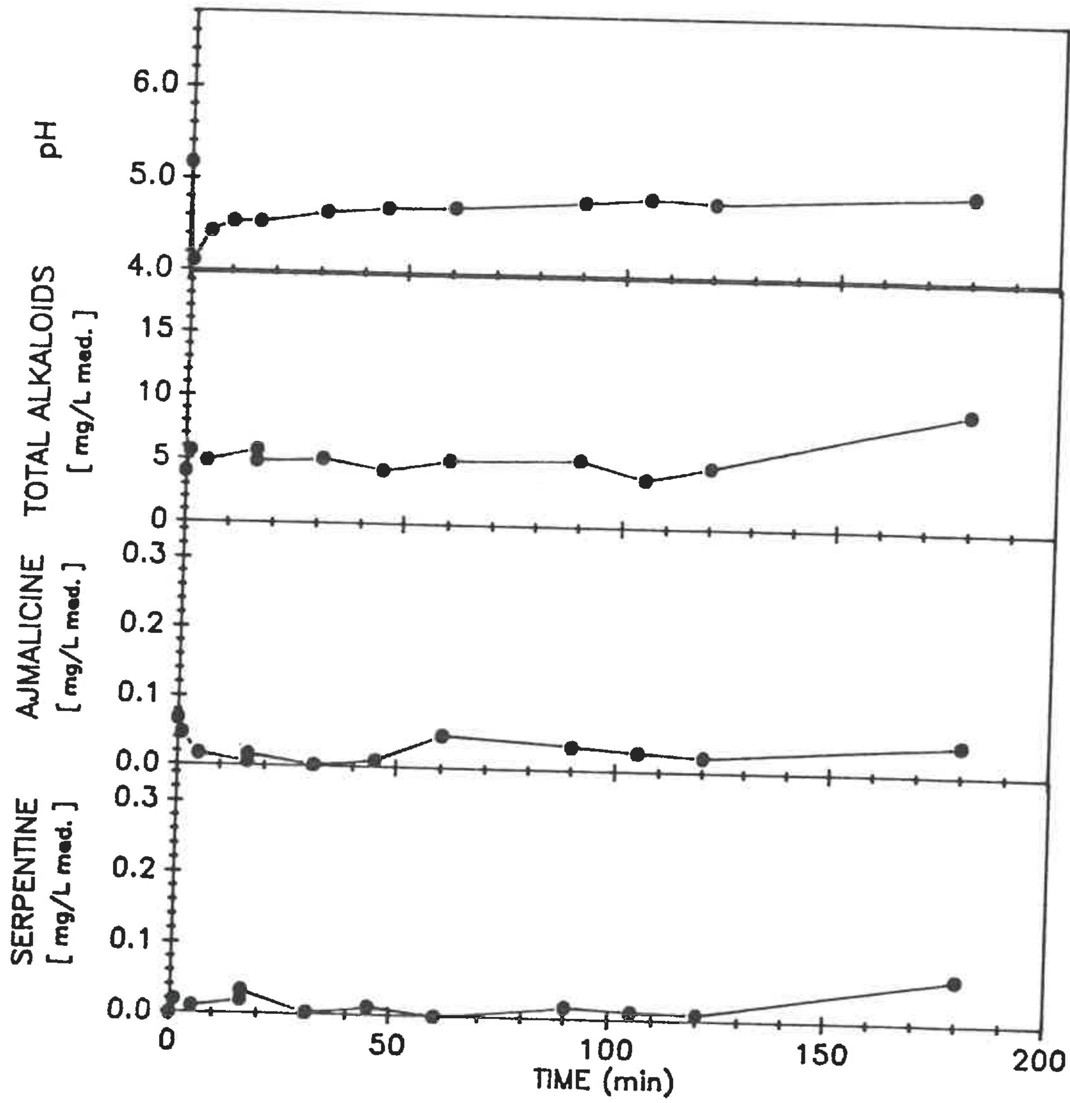


FIGURE 33: Culture IA23: Details of pH stimulated alkaloid release at day 9 during 3 hour period following acidification.

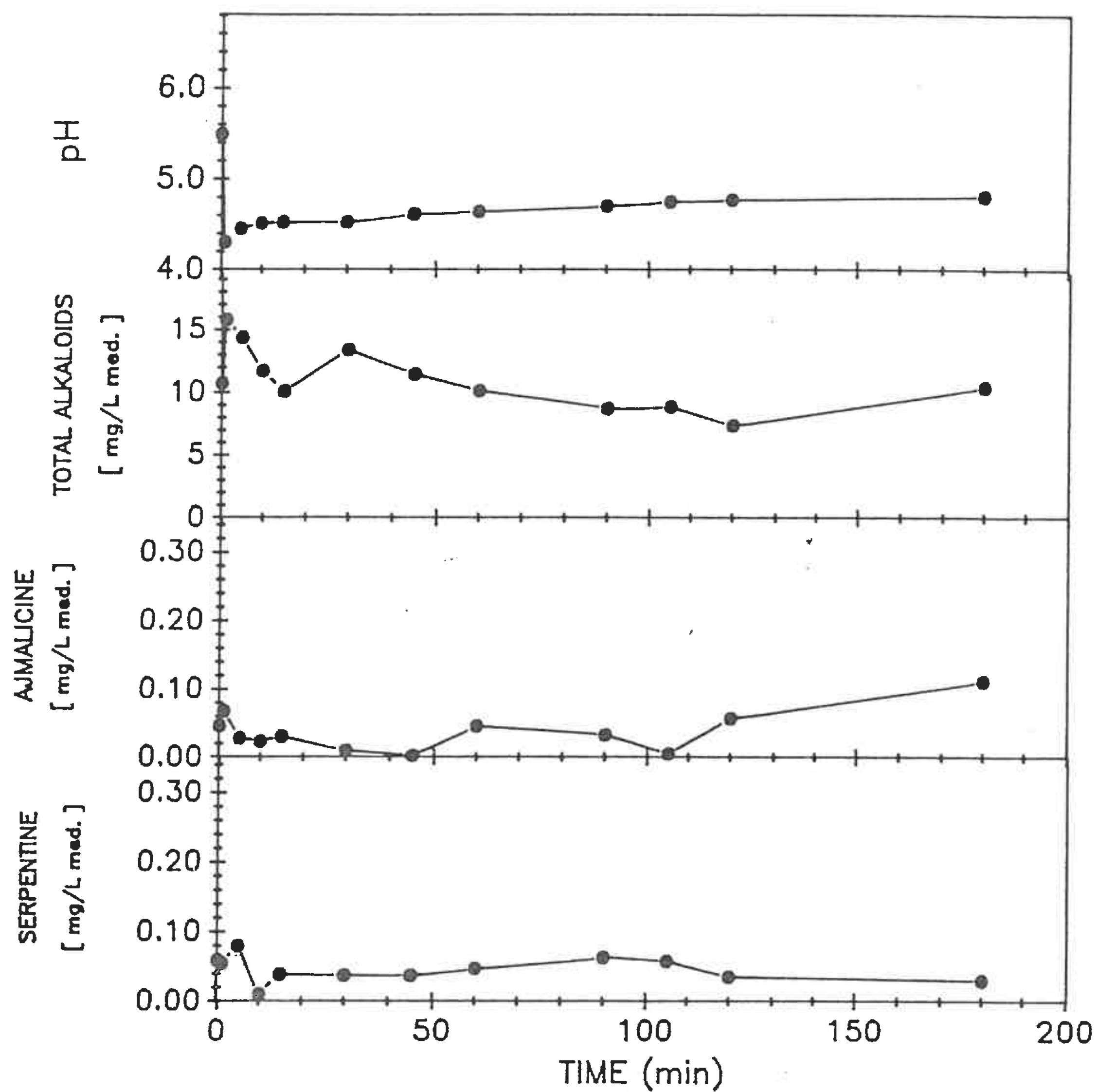


FIGURE 34: Culture IA23: Details of pH stimulated alkaloid release at day 12 during 3 hour period following acidification.

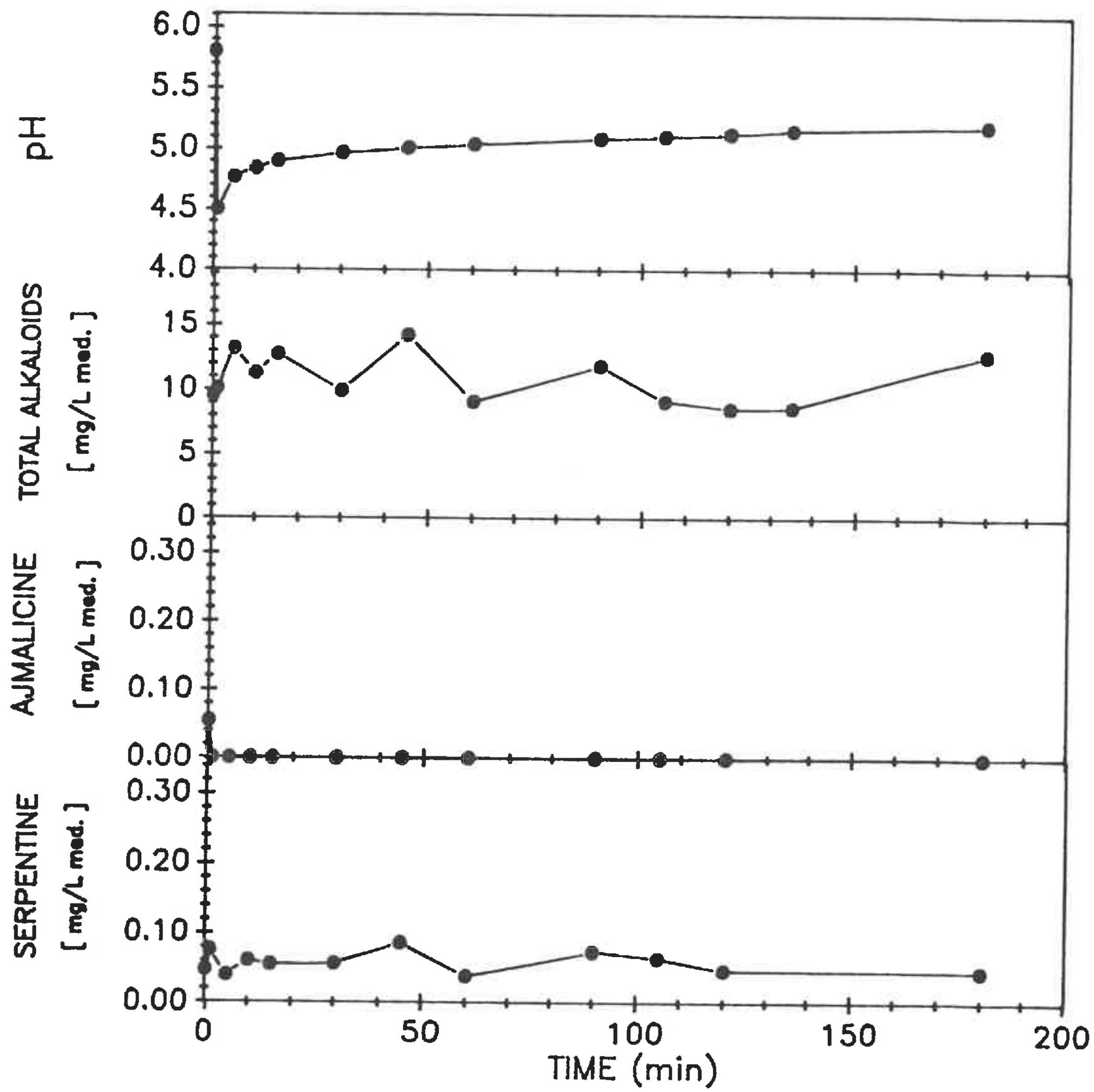


FIGURE 35: Culture IA23: Details of pH simulated alkaloid release at day 15 during 3 hour period following acidification.

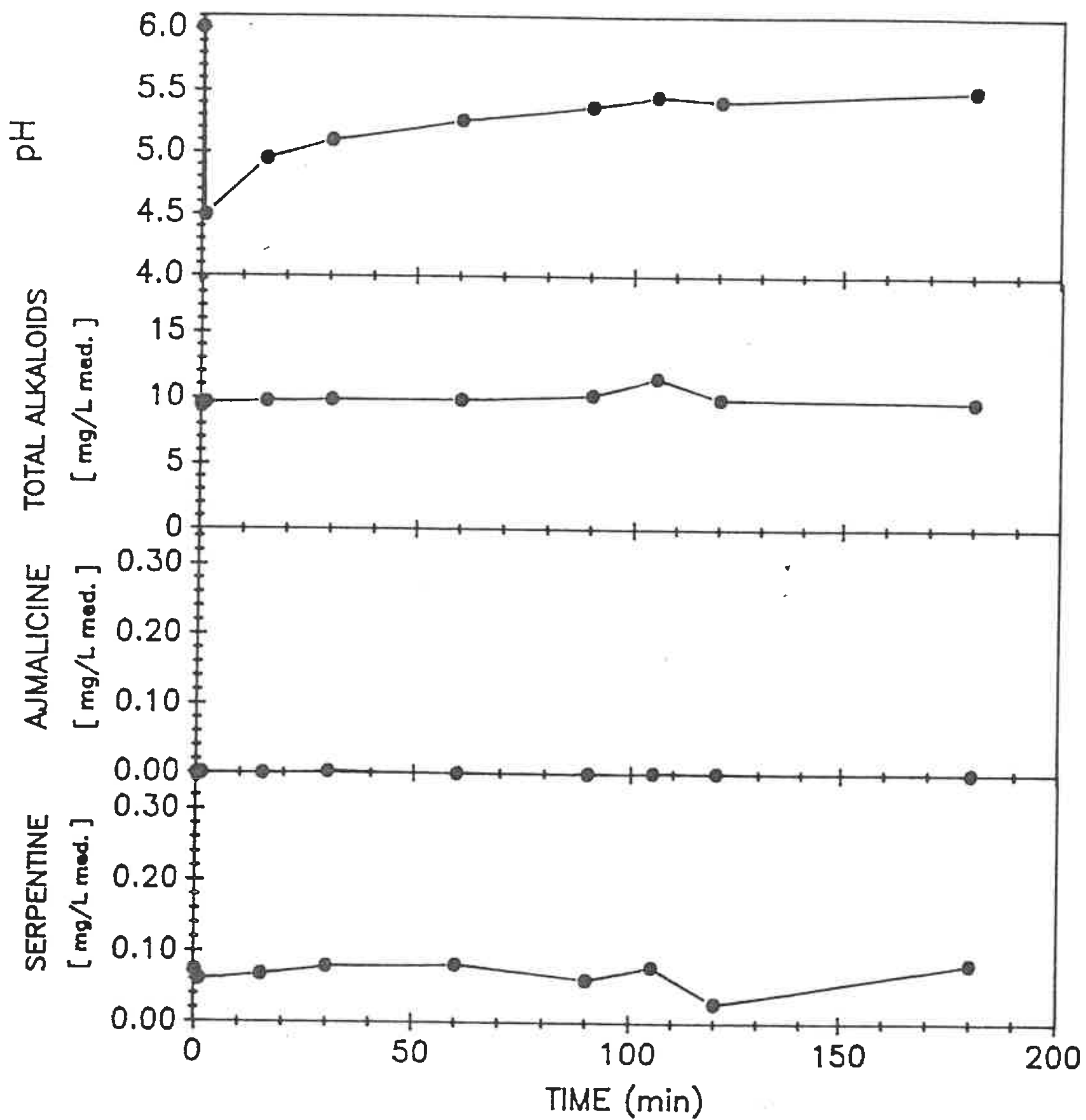


FIGURE 36: Culture IA23: Details of pH stimulated alkaloid release at day 18 during 3 hour period following acidification.

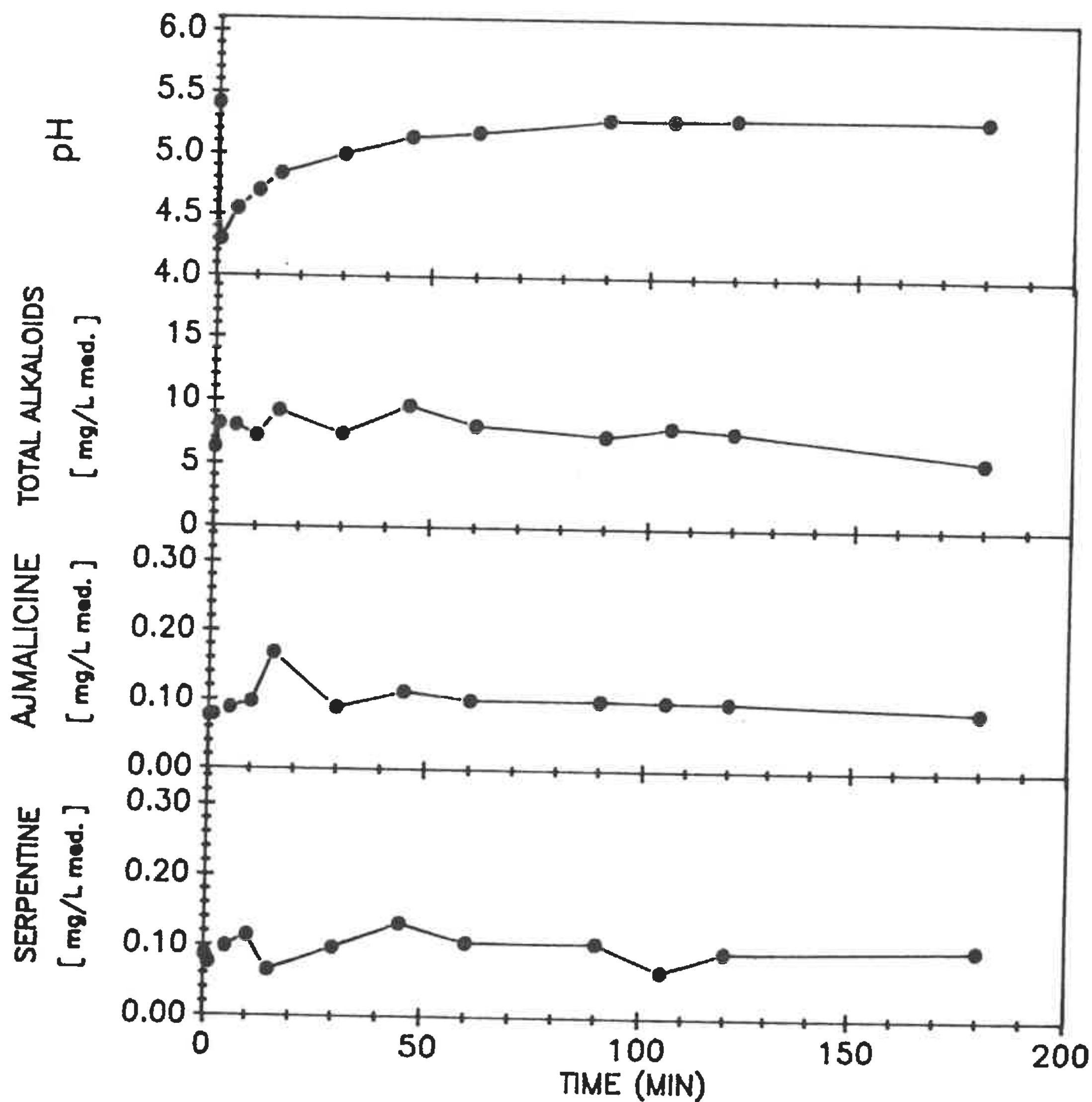


FIGURE 37: Culture IA23: Details of pH stimulated alkaloid release at day 21 during 3 hour period following acidification.

alkaloid concentration returned to the initial level within 60 minutes. A similar but weaker response (from 9.5 to 13.2 $mg{\cdot}L^{-1}$ in 5 minutes) was observed at day 15 (FIGURE 35). A pH modification from 6.0 to 4.5 did not significantly alter the extracellular alkaloid content at day 18 (FIGURE 36) while release of total alkaloids was seen at day 21 (from 6.4 to 9.2 $mg{\cdot}L^{-1}$) (FIGURE 37).

The identifiable alkaloids detected in the medium were ajmalicine and serpentine. During the first three days of culture, neither ajmalicine nor serpentine were found extracelluarly. Ajmalicine was first detected at day 4 where pH dropped from 6.5 to 6.1. Medium exchange caused release of 0.22 mg of ajmalicine between day 6 and 7 (FIGURE 32). Acidification at day 9 (FIGURE 33) significantly decreased the medium ajmalicine concentration while at day 12 a pH drop of 1.2 units(from pH 5.5 to 4.3) induced an overall increase from 0.05 to 0.12$mg{\cdot}L^{-1}$. At day 15 (FIGURE 35), the extracellular ajmalicine in fact decreased to an undetectable limit. The acidification at day 18 was ineffective at causing ajmalicine release (FIGURE 36). The fifth acidification at day 21 induced the most pronounced alteration of ajmalicine levels; an increase from 0.077 to 0.17 $mg{\cdot}L^{-1}$ occurred (FIGURE 37). Many products found intracellularly were detected in the medium at harvest (day 21) (FIGURE 38). However, no observable changes were observed in NO_3 concentration nor in conductivity levels thus cell lysis was excluded as the reason for significant ajmalicine release.

The acidification at day 9 provoked a first appearance of serpentine (0.02 mg·L-1) (FIGURE 33) which seemed to be degraded or be taken up by the cells .Thereafter, a slow increasing trend was observed and a maximum of 0.06 mg·L-1 of serpentine was attained at the end of the three hour sampling period. This level was maintained for the next 2 days then decreased following

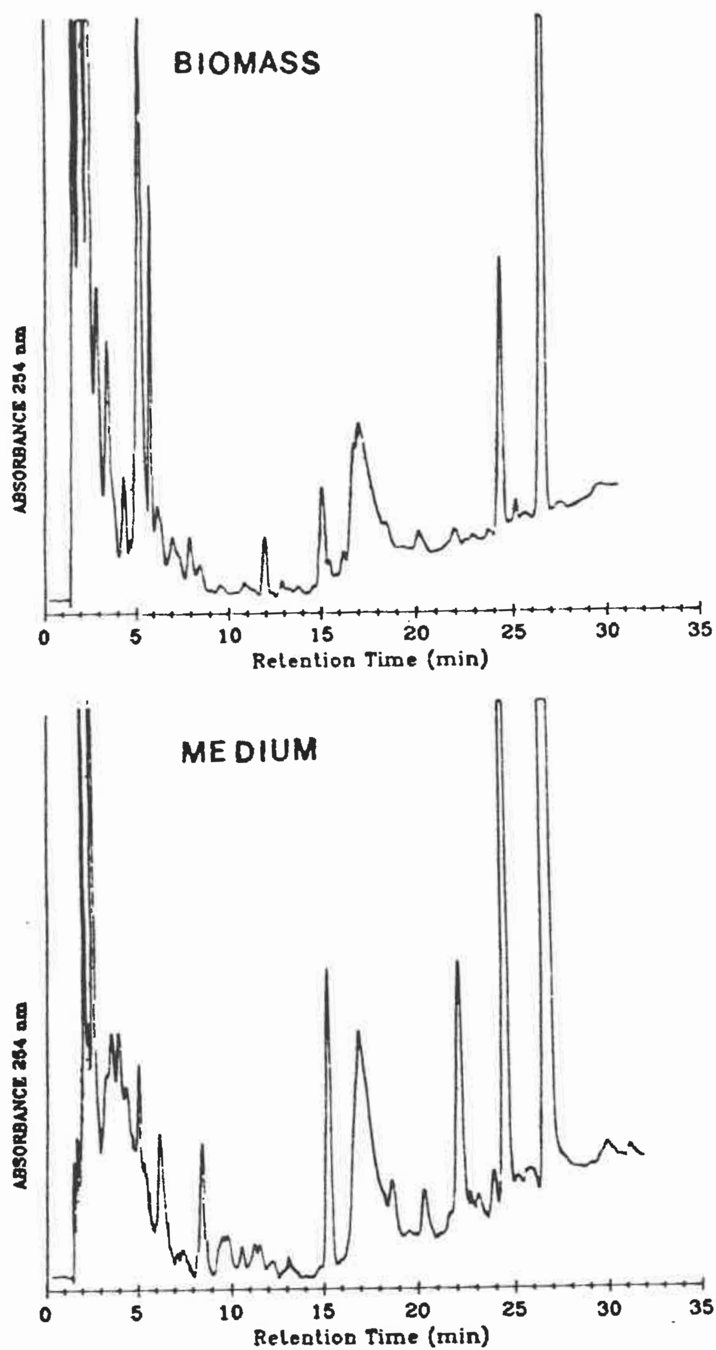


FIGURE 38: Comparison of alkaloids in the medium and in the biomass of the acidified culture IA23 at harvest.

APM replenishment at day 11. The second acidification (day 12) induced a decrease in the extracellular levels of serpentine (FIGURE 34) while at day 15 the concentration increased from 0.04 to 0.08 $mg{\cdot}L^{-1}$, one minute after HCl addition (FIGURE 35). The serpentine seemed to be degraded or taken up by the cells by the end of the sampling period. The acidification at day 18 did not effect release of serpentine. On day 21 the pH was decreased from 5.4 to 4.3 which resulted in two peaks in extracellular serpentine levels. The first at 10 minutes reached 0.115 mg·L-1 and the second occurred at 45 minutes attained 0.133mg·L-1 serpentine (FIGURE 37).

Some as yet unidentified alkaloids were quantified on a relative basis in order to determine whether acidification had a positive effect on their release. The effects of decreasing pH are summarized in FIGURE 39 and TABLE 6.

The first stimulation at day 9 caused an increase in 2 out of 14 alkaloids while 8 out of 14 were undetected. A large percentage (62.5%) of the undetected alkaloids appeared at day 12 prior to external pH adjustment which was 48 hours following acidification. The time course evolution of unidentified extracellular alkaloids as detected by HPLC on day 12 are depicted in FIGURE 40. These can only be used qualitatively to compare the presence or absence of a given product. Significant release of alkaloids were observed as early as 5 minutes. The alkaloids detected at ~25 minutes started to increase at t=90 minutes and it remained a significant alkaloid component of the medium until and possibly beyond 150 minutes.

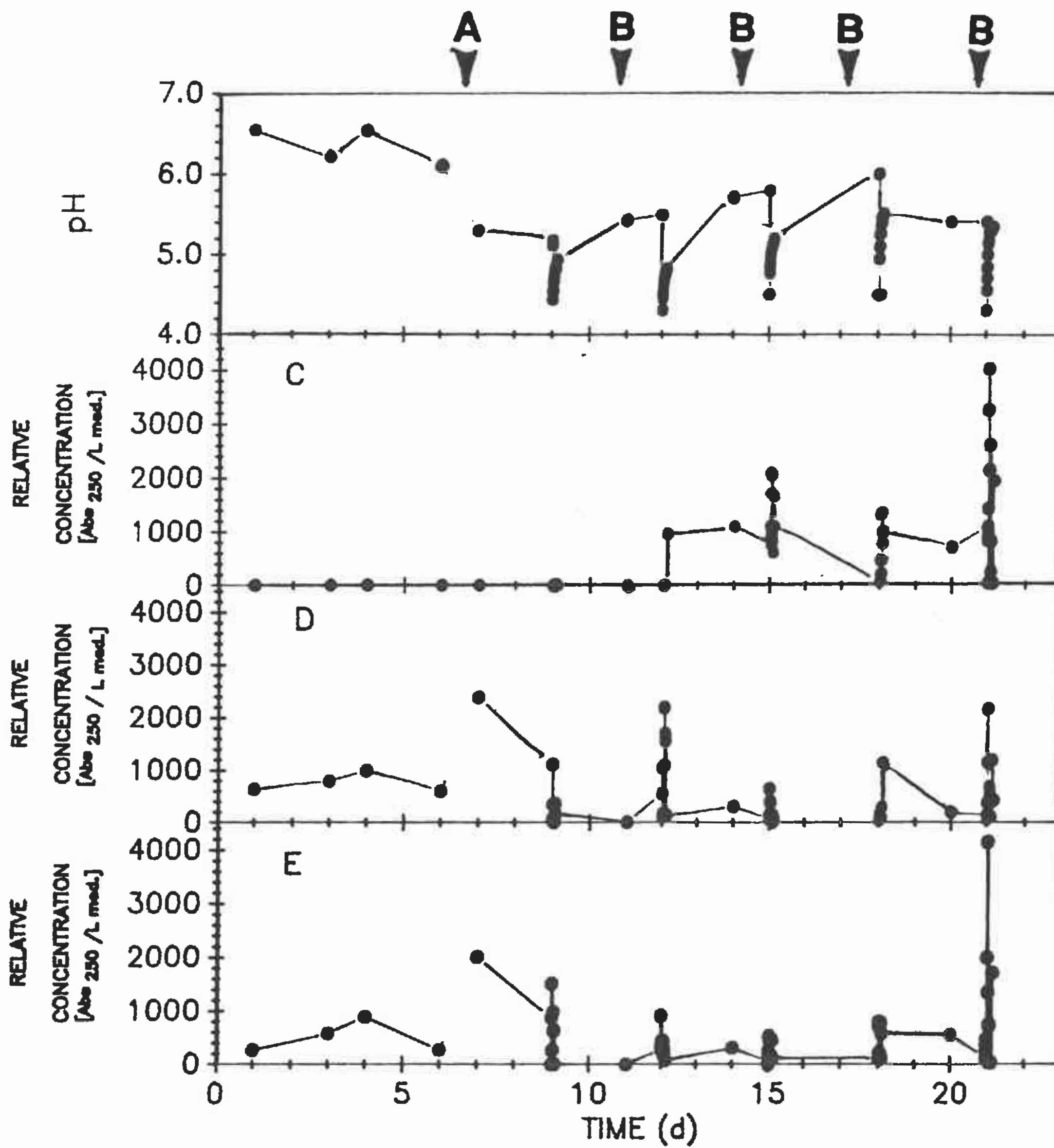


FIGURE 39: Time course releasae of unidentified alkaloids in the IA23 culture.
A= medium exchnage AB5 for APM.
B= addition of 20% APM.
C= Unidentified alkaloid eluted at 7.8 min.
D= unidentified alkaloid eluted at 8.6min.
E= unidentified alkaloid eluted at 9.2 min.

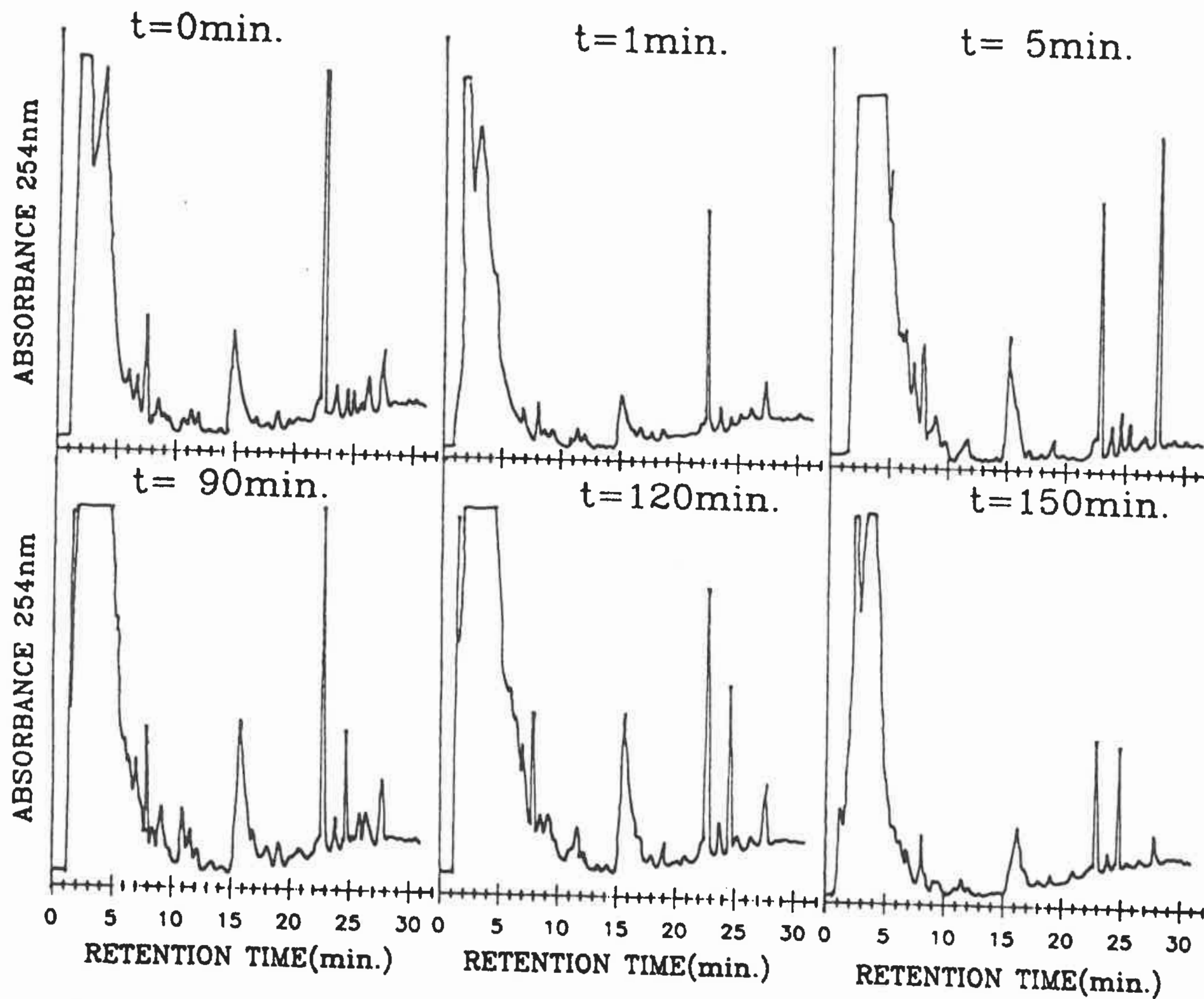


FIGURE 40: Comparison of alkaloid HPLC chromatograms during acidification of IA23 on day 15.

TABLE 6

Effect of acidification on release of unidentified indole alkaloids

Retention time (min)	Day 9 pH 5.2-4.11			Day 12 pH 5.5-4.3			Day 15 pH 5.8-4.5			Day 18 pH 6.0-4.5			Day 21 pH 5.4-4.3		
	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]	I[1]	D[2]	U[3]
5.8			X	X					X		X			X	
7.8			X	X			X			X			X		
8.6		X				X	X			X			X		
9.2	X			X			X			X			X		
11.2			X	X			X				X		X		
12.0	X		X	X		X	X					X	X		
21.4			X		X		X				X			X	
23.2			X	X			X				X			X	
24.9			X	X			X			X			X		
25.5		X			X		X				X		X		
26.7			X	X				X			X		X		
27.5		X			X		X				X			X	
19.8			X	X			X			X				X	
28.6		X			X		X		X	X			X		
TOTALS	2	4	9	9	4	2	12	1	2	6	7	1	9	5	0

[1] I= Increase of extracellular concentration during three hour sampling period.
[2] D= Decrease of extracellular concentration during three hour sampling period.
[3] U= Undetected prior to alkalinization.

chapter 7

7.0 DISCUSSION

7.1 GROWTH CHARACTERISTICS OF *CATHARANTHUS ROSEUS* CELLS

The larger culture system had lower maximal biomass for similar culture conditions. A comparison of the cultures using the 2-stage process and cultured at 27°±1°C showed marked differences. For instance, the maximal biomass concentrations were 6g $d.w.\cdot L^{-1}$, 12 g $d.w.\cdot L^{-1}$ and 20 g $d.w.\cdot L^{-1}$ for the 6L immobilized culture, the 2L immobilized culture and the 200 mL suspension culture, respectively.

The differences may have stemmed from the inconsistent process conditions between the systems. The oxygen-transfer coefficient (k_La) value recommended for plant cell cultivation has been quoted to be 15 h^{-1} (20). Previous mass transfer studies(36) have measured the K_la for the 2L immobilized culture and the 6L immobilized culture (APPENDICES 1 and 2). The 200 mL suspension cultures were measured to have a k_La ~10 h^{-1}. The 2 L was operated at 0.07±0.02VVM and mixed with a magnetic stirrer at 300 RPM which resulted in a K_La of 6h^{-1}. The 6 L reactor was aerated at 0.3-0.5VVM which gave a K_La of 6-8h^{-1}. All measurements were done in water @ 27±1°C.

The lower K_La values did not directly imply oxygen limitation. The cultures are not stunted by lack of O_2 as long as the O_2 transfer rate (**OTR**) is greater than the O_2 uptake rate (**OUR**):

$$OTR= \frac{dc}{dt} = K_La*(C^*- C) \qquad (6)$$

$$OUR= Q_{O2}*X \qquad (7)$$

Where k_L= mass transfer coefficient ($cm\cdot h^{-1}$);

a = gas liquid interfacial area per unit volume (cm^2/cm^3);

C^*= is the saturated liquid concentration of O_2 ($0.25 mmole\cdot L^{-1}$);

C = is the actual O_2 concentration (mmole·L^{-1});

Q_{O2}= specific O_2 uptake rate (mmole O_2·g dw^{-1}·h^{-1});

X = cell concentration (g d.w.·$L^{-1)}$

Direct specific uptake rates were not determined for MCR17 in the 1 and 2-Stage processes. Values for k_La as per appendices 1 and 3 were measured using water as the liquid phase. Changing the composition of the medium greatly influences the solubility of O_2 (C^*). For instance increasing the sucrose from 0.4mM to 1.2mM (0.43 g·L^{-1}) causes a drop in C^* by 27%. In the case of APM the sucrose concentration was 50 g·L^{-1}. The fact that solubility of O_2 during the production phase in APM was greatly reduced implied that there was likely O_2 limitation in all cases.

A phenomenon which possibly hindered the growth in the 6L SIPC culture was excessive CO_2 stripping. Low partial pressures of CO_2 have been implied to interfere with normal growth of *C. roseus* cells (37,51,61,64,66). The higher aeration in the large immobilized cultures (0.4 vs. 0.07VVM) has a tendency to decrease the partial pressure of CO_2 (66) which could have been responsible for a 2 fold lower biomass maximum (6 vs. 12 g dw L^{-1}) and lower yields of biomass on carbohydrate ($Y_{x/s}$ = 0.13 vs. ~0.23) in the 6Lcompared to the 2L.

The inferior yield factors ($Y_{x/s}$) obtained for the immobilized cultures may have been the result of restricted unidirectional growth allotted to these cultures. The diffusion of growth limiting substances was probably the more important factor influencing primary metabolism. The transport of nutrients through the biofilm is a function of the biofilm thickness, uptake rate and diffusivity. It was reported that the time required to pass through this resistant solid interface is ~4h (36). Since the fastest doubling time of MCR17 is ~2d, one can assume that diffusion may

not have limited cell metabolism.

7.2 NATURAL INDOLE ALKALOID RELEASE IN SUSPENSION CULTURES AND 2L IMMOBILIZED CULTURES

The 1-Stage suspension cultures grown in APM released a greater percentage of their alkaloids compared to the surface immobilized cultures in APM which conflicted with previous claims that immobilization increases alkaloid release (36). However, cell lysis was likely responsible for this increase in the suspension cultures. The level of alkaloids released into the medium of the suspension cultures increased starting at day 6 whereas the immobilized cultures displayed a peak of extracelluar alkaloids between days 6 and 9 (16.0-12.0 mg·L^{-1}). The absolute alkaloid concentrations achieved in the suspension and the immobilized cultures never exceeded 20 mg·L^{-1} (TABLE 3). The release of ajmalicine and serpentine were low for these cultures which may have been the result of low productivity of the 1-STAGE process.

The 2-STAGE process displayed high fractional release during the growth period in **AB5**. At day three, maxima of 31% (17.0 mg•L^{-1}) and 76% (52 mg•L^{-1}) release were observed for the suspension and the 2L immobilized cultures respectively. The high release was transient and at day 6, prior to medium exchange, the level of relative release decreased in both systems. At this point the release was greater in the 2L SIPC (60%) than in the suspension cultures (5%). This may have been due to adhesion of the cells onto the immobilization matrix since significant secretion of unidentified substances has been shown to occur as cells bind to the man-made geotextile (124). This may alter the formation of normally impermeable membranes and increase their permeability. If this is the case, consistently higher release of intracellularly stored products such as alkaloids will occur. Higher relative releases occurred in the immobilized cultures; an

average of 44% release was calculated during the production phase in 2L SIPC whereas the suspensions had an average release of 16%.

Increased release has been observed previously with immobilized cultures (35,98,115). For instance, ionotropic gelation with polycationic chitosan caused increased release of intravacuolar oxalate (52). It was reported that 80% of ajmalicine was released due to sodium alginate encapsulation (98). Provoked excretion may have resulted from transient alterations of the cells caused by interactions of the immobilizing matrix with the cells (52).

The patterns of indole alkaloid release in the immobilized system and in the suspension cultures were dissimilar. The SIPC showed declining relative release (FIGURE 16) whereas the relative release was constant for the suspension culture (FIGURE 15). In the 2L culture, the absolute concentrations of total alkaloids found in the medium were constant during the production period; the concentration was maintained below 25 $mg \cdot L^{-1}$. The transport mechanisms were possibly saturated and or feedback inhibited. Slowed extracellular sequestration of the alkaloids was further substantiated when a sudden rise in synthesis at day 18 occurred without concomitant increase in the extracellular content; the extracellular content in the medium remained unchanged at $22 mg \cdot L^{-1}$. This may demonstrate that the absolute extracellular concentration was limited by the toxicity of the secondary metabolites and/or there was turnover and reuptake of the alkaloids so as to maintain this level. Another hypothesis was that a constant level of extracellular alkaloids were detected because only those cells closest on the outer surface of the matrix could release these products. The surface area did not significantly increase during the production period, thus, the indole alkaloids remained unaltered.

The suspension cultures demonstrated different behaviour. Between days 9 and 15 the

intracellular content increased almost 4 fold while the extracelluar concentration rose by 7 times (9.0-65.0mg·L-1). This showed that release in the suspension cultures was likely a function of intracellular content.

According to the above mentioned discrepancies between the two cultures, it seemed that the mechanisms governing extracellular release were dependent on the system in which the cells were cultured. The cell physiology of the two culture systems differed as seen by varying nutrient consumption rates. Immobilization increases the level of aggregation which may have allowed increased communication between the cells which incited increased transport.

A fundamental physical difference of the two cultures was the development of a biofilm layer in the surface immobilized system. In freely suspended cells, alkaloids can be released by moving from the vacuole to the cytoplasm then across the cell wall into the extracellular space. Alkaloids in the immobilized system must follow the same path, however, once outside the wall, the alkaloid must diffuse through/around a multitude of cells. Although, the biofilm is a highly hydrated and permeable film (~$6.6x10^6 cm^2/s$) the alkaloids may have been taken up by the cells and sequestered into the vacuole or possibly degraded. It requires ~4 hours for a substance to move through the biofilm and it has been reported that ajmalicine was partially degraded within the same amount of time when added to cell suspension cultures (102).

The ABSOLUTE release of ajmalicine was probably dependent on the acidity of the medium (FIGURE 17A &17C); as the pH decreased the ajmalicine concentration rose in two out of three cases. This effect has been observed previously (7,83,87,88,99,102) and could be attributed to reversal of vacuolar trapping. As the H^+ concentration increases, equation 1 predicts a decrease in the C_i/C_e ratio:

$$C_i/C_e = \frac{1 + 10^{[pKa - pHi]}}{1 + 10^{[pKa - pHe]}} \quad (1)$$

This should effect a higher liberation of intracellularly stored alkaloids. The most significant factor which could be manipulated is (pK_a - pH_e). As the external pH decreases the denominator increases and the external concentration concomitantly rises.

The release of serpentine was similarly influenced. However, when the extracellular content is exceedingly high (FIGURE 17A; 300ug·L) the level of release was unaffected by pH modification. In this case, the release of serpentine may have been subject to feedback inhibition and/or liberation may have been equilibrated between synthesis, release and degradation.

7.3 STIMULATED RELEASE OF INDOLE ALKALOIDS

7.3.1 EFFECTS OF ALKALINIZATION ON CULTURE

The addition of KOH to the cultures IA12, IA19, and IA22 did not cause noticeable cell deterioration because of the following characteristics of these cultures: firstly conductivity readings did not increase, the fluctuations in NO_3 concentrations resulted from interference of unusually high pH with the measurements with the Orion probe. This explanation was corroborated by the lack of concomitant increase in conductivity. The second characteristic which supported retained cell viability following KOH addition was the fact that nutrients were continuously consumed upon APM addition. The third was that pH increases were neutralized to initial values within 24-48 hours after treatment.

7.3.2 EFFECT OF ALKALINIZATION ON PRODUCT RELEASE

The addition of KOH induced significant changes in extracellular alkaloid

concentrations. When alkaloid concentration was decreasing and base was added, increase in excretion was observed. On the other hand, when constitutive concentration was increasing and alkalinization was effected, the extracellular concentration generally declined. An exception to this was when ajmalicine levels were increasing at day 15 in IA19; alkalinization further increased release of ajmalicine. This indicated that the best suited time to incite ajmalicine release by alkalinization was towards the end of production period when cellular content was high.

Sudden alkalinization caused transient increases of serpentine levels in all cases of KOH addition studied; however, the serpentine concentrations were generally reestablished to the initial concentration three hours following the pH modification. The long term effect of alkalinization (24 to 48 after stimulation), caused a more sustained release of serpentine, increasing extracellular concentrations by an average of almost 2 fold.

The swift restoration of extracellular levels may be the result of many phenomena. For instance, alkaloid turnover has been reported to occur (92) and decrease of alkaloid content may be effected by exoenzymes known to be released into the culture medium (92) when levels are exceedingly high. Another possibility was that alkaloids could be translocated intracellularly into storage cells (92) once extracellular pH is reestablished. A consistent increasing trend of extracellular alkaloid concentration was observed 48 hours after alkalinization. These two phenomena implied that there was a dual system involved in transport. The first being a rapid transient release while the second required

more time and incited a permanent level of extracellular alkaloids.

The rapid biomechanism of alkaloid release was difficult to assess since transport in the biofilm was believed to be dampened by slow diffusion. A possible explanation for this quick release was that translocation through the cells was not involved and that rapid release was the result of desorption of alkaloid from the outer surface of the biofilm. Cell wall adsorption of berberine alkaloid has been reported (100). Reabsorption may be the cause of decreased level of alkaloids in the medium within a short time following alkalinization.

Comparison of cultures IA12 and IA19 showed some interesting points. Culture IA12 was alkalinized early in the production phase (day 9) and excretion was not substantially stimulated. The decrease in alkaloid concentration as induced by alkalinization may have been the result of insufficient biosynthetic capacity of the cells and/or the depletion of intracellular stores. Culture IA19 was alkalinized for the first time at day 15, the 9th day of production, which induced sustained alkaloid release. Therefore, alkalinization should be effected in the later stages of production to cause a more positive and maintained release of alkaloids.

The pattern of total alkaloid release was not reproduced upon successive alkalinizations of the same culture. The variability could be attributed to the modification in the physiological state of the cells, which may infer alterations in secondary metabolism and/or product accumulation of the culture.

7.3.3 EFFECTS OF ACIDIFICATION ON CULTURE

Conflicting results render the determination of the effect of acid addition on cell viability difficult. Estimated average nitrate and carbohydrate uptake rates in the acid stimulated cultures were 130% and 70% higher than calculated for the alkalinized culture. Slight increases in the conductivity and nitrate levels were noticed on the 9th day and at the 12th day. The conductivity remained stable from day 15 onward. Surprisingly, at harvest (day 21), similar intracellular and extracelluar alkaloid spectra indicated cell lysis. The lowest pH effected was 4.1 at day 9, a more moderate acidification would likely have been more appropriate

7.3.4 EFFECT OF ACIDIFICATION ON PRODUCT RELEASE

Acidification caused variable alkaloid release responses. The first three acid additions positively effected release of total alkaloids and serpentine, however, ajmalicine concentrations were significantly decreased. The second acidification incited a increase in the extracellular concentration of total alkaloids, ajmalicine and serpentine.

The ion trap model as explained by equation 1 predicted a higher level of release of ajmalicine and serpentine upon acidification. Research has been carried out in order to confirm or negate the role of ion trapping on vacuolar compartmentation of alkaloids. Acidification of the culture medium in suspended cell cultures from pH 7.9 to pH 4.9 diminished the ajmalicine C_i/C_e ratio from 66 to 47.5 (102). This was the result of a combination of decreased intracellular levels and increased extracellular levels.

Another group studied the effect of decreasing pH on the release of ajmalicine and serpentine (87). A continuous 2L cell suspension system was used, fresh medium of pH 4.3 was fed into the reactor. It required 6 days of continuous operation to attain the lowest pH level of 5.5. At

this time maximum release of ajmalicine (85%, 0.14 mg·L^{-1}) was effected while serpentine release remained constant at 30% (0.03 mg·L^{-1}). The pH was autoregulated back to pH 6.3 and ajmalicine release levelled off at 64%. It was concluded that decrease in pH stimulated excretion of ajmalicine as was predicted by the "ion trap model". This conclusion was misleading because the cell viability was very low (10 to 50%) and the biosynthesis of ajmalicine and serpentine decreased by ~15% and 65% respectively (87).

On the contrary, results from this work indicated that a sudden decrease of pH from~6.0 to ~4.3 in immobilized cultures did not evoke efflux of ajmalicine. This was opposite to what the ion trap model predicted. The inconsistencies in the release of ajmalicine upon acidification in the suspension and immobilized systems was not surprising because the immobilized state of the cells could have introduced morphological changes in the biomass which conceivably altered the mechanisms governing alkaloid compartmentation. This in turn, may have affected the ion trapping of alkaloids in the vacuole. The buffering capacity of the immobilized cells and dissimilar mass transfer across the biofilm were other parameters causing comparison of the suspension and immobilized cultures difficult. The harshness of acidification may have also interfered with normal cell metabolism causing different secondary metabolite production and/or accumulation.

7.4 COMPARISON OF NATURAL ALKALOID RELEASE IN THE 2L IMMOBILIZED CULTURES AND STIMULATED RELEASE IN THE 6L IMMOBILIZED CULTURES

The fundamental differences between the protocol used for the 6L cultures and the 2L cultures were the following :

1. Inoculum size
2. Aeration rate (VVM)
3. Nutrient consumption

The 6L reactors were inoculated with 20% (v/v) while the 2L reactors were inoculated with 10%. Inocula were grown in medium containing 2,4-D as auxin; thus, a higher percentage of 2,4-D carry over was introduced into the 6 L immobilized cultures and 2,4-D has been reputed to be deleterious to secondary metabolite synthesis in *Catharanthus roseus* cell cultures which substantiated the lower production in the 6L cultures.

The aeration rate in the 6L reactor varied from 0.3 to 0.5 VVM while the 2L reactor was aerated at 0.07VVM. This discrepancy caused 2 parameters of the culture to differ. The first was the oxygen transfer coefficient (6L: k_La=6-8 h^{-1} and 2L: K_La=6h^{-1}). The lower K_La may have possibly interfered with cell proliferation and may have hindered secondary metabolite synthesis (111). The other characteristic which was different was the partial pressure of CO_2 dissolved in the medium. High VVM incited more CO_2 stripping which interfered with normal growth (61).

The lower consumption rate of NO_3 in the 6L cultures as compared to the 2L cultures

(2.67mM d^{-1}, 3.33mM d^{-1} respectively) caused a higher level of NO_3 to remain in the medium during the production phase in APM. Essentially all NO_3 in 2L cultures was depleted by day 15 and maximal alkaloid production was attained three days later (120). The NO_3 levels remained between 5-10 mM during the course of the production period in the 6L reactors. Depletion of NO_3 has been correlated with increased secondary metabolite enzymatic activity (85). Therefore, high level of this nutrient could have decreased indole alkaloid production. The 6L SIPC cultures were generally less productive than the 2L cultures. This was the result of high 2,4-D carry over, high basal levels of NO_3 and possibly high CO_2 stripping.

Taking into the consideration the lower production in the 6L system, the percent release of alkaloids seemed to compare. The 6L SIPC culture attained 2 fold lower biomass per liter and the product yield was 4 fold lower in the 6L culture. This should resulted in a 8 fold lower concentration of alkaloids in the medium if the percent release were comparable. In fact, the total extracellular alkaloid concentration was 10 fold lower.

The average release the 2L system was 44%. If alkalinization in the 6L reactor stimulated a further 60 to 70% release in extracellular alkaloid concentration it would suggest that 100% of the intracellular stores were released.

The NATURAL release of ajmalicine in the 2L reactor was related to the acidity of the culture medium similar to the prediction of the ion trap mechanism. However, when acidification was carried out the release of ajmalicine was not reproducibly stimulated.

This may have been due to the transient nature of the acidification process caused by the natural buffering capacity of the cells.

7.5 COMPARISON OF ALKALINIZATION AND ACIDIFICATION ON RELEASE OF INDOLE ALKALOIDS IN THE 6L IMMOBILIZED CULTURES

A summary of the efficiency of product release in the 6L, 2-STAGE cultures is presented in TABLE 7. The total quantities of indole alkaloids accumulated in the biomass at the end of a culture are compared to products present in the medium. The percent releases were then calculated as per equation 6.

The highest relative release of total alkaloids at harvest occurred in culture IA19 at day 18 (59.4%) following alkalinizations at days 15 and 18. The maximal product yield of 4.2 mg·g dw^{-1} occurred at day 18 similarly to the peak (18 mg·g $dw^{-1)}$ that was observed in the 2L SIPC reactors cultivated by the 2-STAGE process (122).

The percent releases were comparable at harvest with 44% release for IA22 and 43% release for IA23 (TABLE 7). The most noticeable difference was the low product yield for the acidified culture. It seemed that acid interfered with production [Y_P= 1.8 mg·g dw^{-1}] to a greater extent than base [Y_P=3.1 mg·g dw^{-1}].

Reproducibility is difficult in plant cell culture. Two cultures grown under similar culture conditions can display significant differences in their growth and production. For instance, in IA19 (FIGURE 30) the extracellular level reached 10mg·L^{-1} prior to stimulation while IA22 released only half this concentration. The estimated average nitrate levels were ~18mM, 15mM ,15mM, 12mM for cultures IA12, IA19, IA22 and IA23 respectively. This variability of NO_3 concentrations demonstrated the difficulty in reproducing exact culture conditions from one

TABLE 7

DISTRIBUTION OF TOTAL INDOLE ALKALOIDS, AJMALICINE AND SERPENTINE BETWEEN CELLS AND MEDIUM AT THE END OF 6L IMMOBILIZED CULTURES.

Culture	Culture Duration (d)	Stimulation Experiment	Total Indole Alkaloids				Total Ajmalicine				Total Serpentine			
			m[1] (mg)	X[2] (mg)	%R[3]	$Y_{A/X}$[4]	m (mg)	X (mg)	%R	$Y_{A/X}$	m (mg)	X (mg)	%R	$Y_{A/X}$
IA12	12	Alkalinization at days 9 and 12	15.3	37.0	29.3	1.7	0.06	0.05	54.5	0.004	0	0.49	0	0.016
IA19	18	Alkalinization at days 15 and 18	64.8	44.4	59.4	4.2	0.47	0.10	82.4	0.022	0.89	0.21	80.9	0.042
IA20	12	-	29.3	34.0	46.3	2.0	0.09	0.03	75.0	0.004	0	0.12	0	0.004
IA22	21	Alkalinization at days 9,12,15,18 and 21	38.2	48.8	43.9	3.1	1.44	0.18	89.0	0.058	0.95	0.37	71.9	0.047
IA23	21	Acidification at days 9,12,15,18 and 21	25.4	33.4	43.2	1.8	0.52	0.05	91.2	0.017	0.47	0.18	72.3	0.020

(1) m : Alkaloids detected in the medium.

(2) X : Alkaloids detected in the biomass.

(3) %R : Percentage of alkaloids released into the medium.

(4) $Y_{A/X}$: Product yield ($mg \cdot gdw^{-1}$)

culture to another. The result of having different NO_3 levels were significant to the product expression in *C. roseus* cell culture(120).

The results presented in this study showed that both acid and base stimulations yielded transient and/or permanent increases in product release, estimated to attain ~100 % of production without significant apparent damage to the cultures. These results indicate that alkaloid sequestration within the vacuole of *C. roseus* cells was partly reversible by pH alterations.

7.6. LITERATURE COMPARISON OF AJMALICINE AND SERPENTINE LEVELS

Variability in cell lines and discrepancies in culture parameters, particularly composition of culture medium rendered the comparison of results quoted in the literature difficult. The maximal ajmalicine in the 6L reactor was 0.29 $mg \cdot L^{-1}$ while peak concentrations of serpentine was 0.21 $mg \cdot L^{-1}$. Both of these peaks occurred in IA22(base). Similar levels of ajmalicine occurred 180 minutes following KOH addition at days 18 and 21. Serpentine peak occurred at day 21, 10 minutes following alkalinization.

The influence of variability in cell line and of unstandardized production media composition are demonstrated in the following examples. In one case, maximal levels of 0.09$mg \cdot L^{-1}$ and 0.05$mg \cdot L^{-1}$ were reported for ajmalicine and serpentine respectively (28,29). A different cell line released 100 times more alkaloid: ajmalicine = 9.1 $mg \cdot L^{-1}$ and serpentine = 6.5 $mg \cdot L^{-1}$ (19). In all cases, cell suspension cultures were employed but different media were used. Previous results were available for MCR17 in the 6L bioreactor (36) reported serpentine peaks of 0.03 $mg \cdot L^{-1}$ while extracellular ajmalicine concentration reached 0.05 mg·L-1 when the single stage process in APM was used (36). The results obtained in this study compare to those

quoted from (36) however a different 2-STAGE culture process was utilized.

CHAPTER 8

8.0 CONCLUSIONS AND RECOMMENDATIONS

Primary metabolism is dependent on culture system. The growth parameters such as nutrient consumption, specific growth rate and yield were different for the 200 mL suspension culture, the 2L immobilized culture and the 6L immobilized culture. Inconsistent culture parameters such as working culture volume, pCO_2, dissolved O_2, aeration rate and degree of aggregation rendered direct comparison of the three culture systems difficult. The larger systems require optimization with stringent control of DO_2 and pCO_2 to evaluate the usefulness of the different systems for plant cell tissue culture. Controlled dissolved O_2 and CO_2 supplementation are being studied presently.

The production of indole alkaloids was maximized by the 2-STAGE process. The low product yields in the 6L reactor were attributed to high 2,4-D carry over, incomplete nitrate consumption and possibly low dissolved O_2 and CO_2. Decreasing initial nitrate levels during production could increase the product yields. Development of a new production medium or selection of a higher producing cell line would bring about higher levels of secondary metabolite production.

Elicitation of *Catharanthus roseus* cell cultureshas been reported to rapidly increase the production and release of indole alkaloids (12). This technique would be highly facilitated in the immobilized culture since interference from the biomass upon removal of potentially deleterious substances would not occur. This may potentially be an alternative method to stimulate production.

Once production has been optimized, intensive study is required to understand alkaloid release mechanisms. On a absolute level, release is highest in the smaller system (200 mL

suspensions). This can be attributed to the higher product yields found in these cultures (120). The flux of alkaloids seems to be a function of biosynthesis in the 200 mL cultures. Limitations of release are observed for the SIPC cultures. A maximum of 33 mg·L-1 is recorded for the 2L system and 18-19 mg·L-1 for the 6L reactor. This plateau may be overcome decreasing potentially toxic effects of secondary metabolites using on-line harvesting of products.

The NATURAL ajmalicine release increased when the medium was acidic. The maximal extracellular level was 45 ug·L^{-1} for 200 mL (pH=6.0) suspensions, 40ug·L^{-1} for the 2L (pH 5.6) and 67.5ug·L^{-1} for the 6L immobilized culture (pH 5.17).

High relative release of total alkaloids, ajmalicine and serpentine occurred with the 2 STAGE process in the 2L system. This indicated the positive effect of cell aggregation on release of intracellular products. The generally elevated release observed in the immobilized systems may eliminate the need for further stimulation.

The 6L system was offered as a first model in the study of the effects of pH alteration on the release of alkaloids. The stimulated release of alkaloids by pH alterations was unpredictable. The ion trap could not explain the observed extracellular fluxes. The reasons for the unpredictability are unclear.

The first stimulations in cultures IA12, IA22 and IA23 were carried out at an inopportune times when secondary metabolism was beginning (day 9 which was day 3 of the production period). The first alkaloid release should be undertaken when alkaloid production was maximum. High alkaloid production has been recently shown to occur 3 days following NO_3 depletion (120). This may be used to determine the exact moment to initiate alkaloid release.

Base may be the preferred stimulus because it did not affect cells while acidification as carried

out in culture IA23 gave conflicting results as to whether lysis occurred. If acidification was done moderately the results may have been less controversial.

A more predictable effect of pH alteration on alkaloid flux is essential to determine the effectiveness of this method on product excretion. Transient fluctuations in alkaloid levels may be the result of buffered pH of the extracellular medium. This suggested the necessity of controlled pH changes to determine a more reliable alkaloid release response. Selection of a cell line which expresses high release of alkaloids could also increase the value of this immobilized process.

Alkaloid production, as carried out by an immobilized process, creates product recovery problems due to dilute concentration of the desired product in the effluent medium. A method which could aid in concentrating the product prior to extraction is alkaloid adsorption onto polymeric substances which is able to concentrate the alkaloids by 2 orders of magnitude (77).

Analytical improvements such as ameliorated extraction procedures would facilitate alkaloid analysis; lengthy liquid-liquid extractions were undertaken. A suitable solid-liquid extraction protocol could increase efficiency of alkaloid determination.

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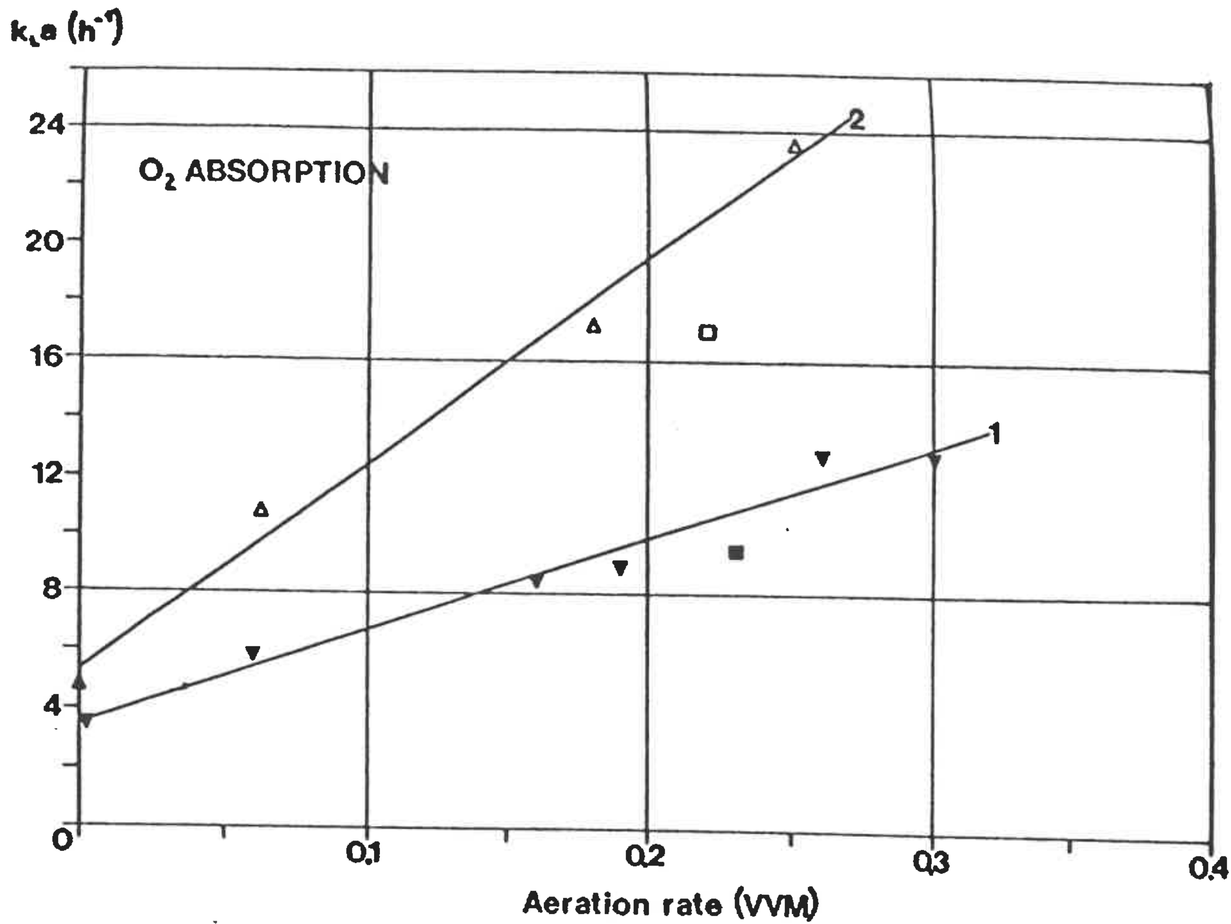
APPENDIX 1



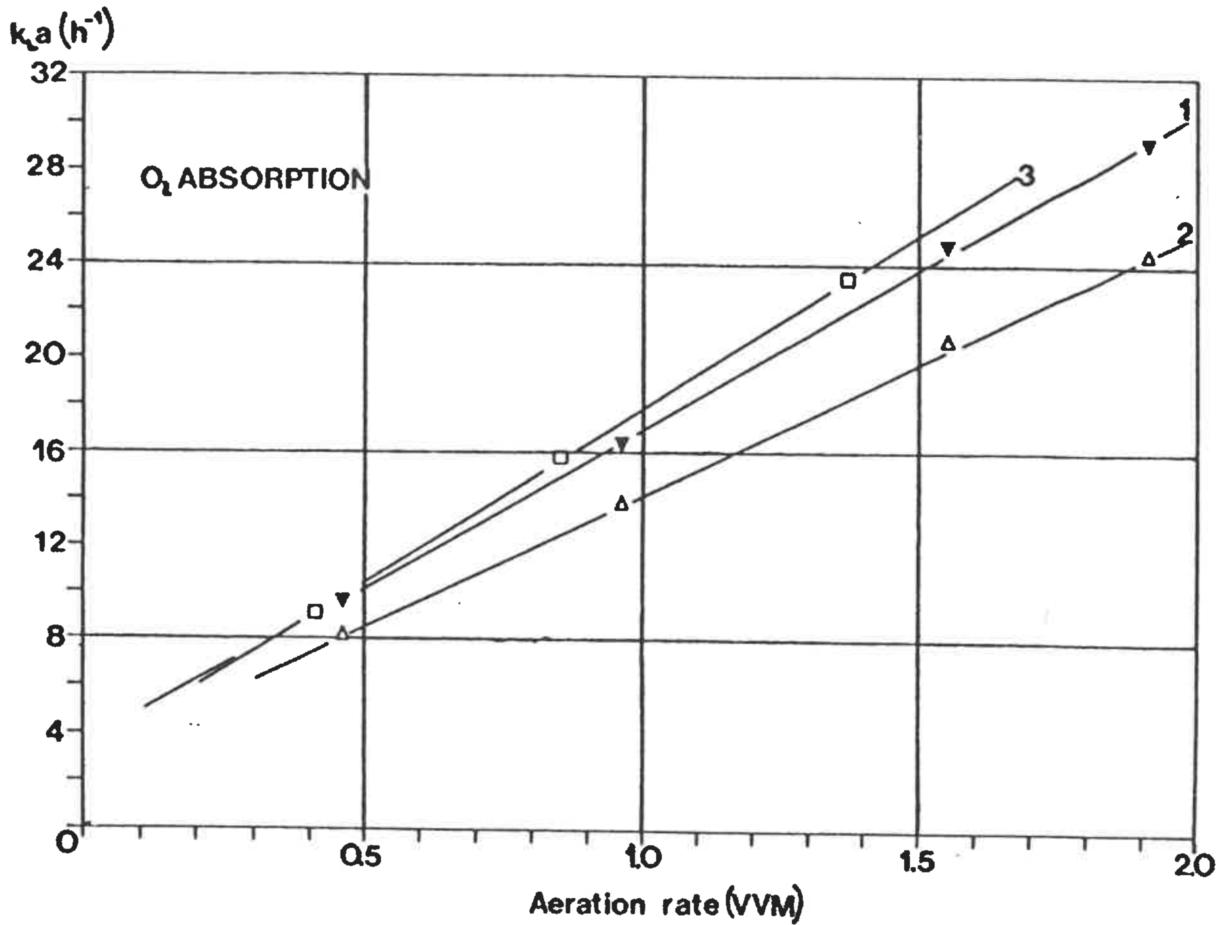
Oxygen transfer capacity (K_La) of 2L surface immobilized bioreactor as a function of the aeration rate at the mixing rate of 300 RPM (Re_i~ 29645).
▼= 2L level (16 cm) with the immobilizing structure. △ = 2L level (16 cm) without the immobilizing structure. ■ = 1.6L level (13.5 cm) with the imobilizing structure. □ = 1.6 L level (13.5 cm) without the immobilizing structure (36).

APPENDIX 2

Nutrient composition of media B5 and APM.

Nutrients	B5 (mg/L)	APM (mg/L)
Macronutrients		
NH_4NO_3	---	720
KNO_3	2500	950
$CaCl_2$	150	220
$MgSO_4$ $7H_2O$	250	185
KH_2PO_4	---	68
$(NH_4)_2SO_4$	134	---
NaH_2PO_4 H_2O	150	---
SUCROSE	20000	50000
Micronutrients		
KI	0.75	0.375
H_3BO_3	3.0	2.4
$MnSO_4$ H_2O	10	7.0
$ZnSO_4$ $7H_2O$	2.0	4.05
Na_2MoO_4 $2H_2O$	0.25	---
$(NH_4)_6Mo_7O_{24}$ $4H_2O$	---	0.0925
$CuSO_4$ $5H_2O$	0.025	0.01
$CoCl_2$ $6H_2O$	0.025	---
EDTA-ferric salt	43.0	55.9
pH	5.5	5.5
Glycine	---	2.0
Vitamins and hormones		
Inositol	100	100
Nicotinic acid	1.0	5.0
Pyridoxine HCl	1.0	0.5
Thiamine HCl	10.0	0.5
IAA	---	0.1752
Kinetin	0.1	---
2,4-D	0.1-1.0	---
Folic acid	---	0.5
Biotin	---	0.05
6-Benzylaminopurine		

APPENDIX 3



Oxygen transfer capacity of 6L modified airlift bioreactor (IA) as a function of the aeration rate. (Volume of liquid = 5.2 L, height of liquid = 30.5 cm).
▼ = DO probe in the external layer. △ = DO probe in the immobilizing matrix.
□ = without the immobilizing structure (36).

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