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UNIVERSITÉ DE MONTRÉAL

STUDY OF RECOMBINANT PROTEIN PRODUCTION FROM TRANSGENIC *Nicotiana tabacum* PLANT CELLS IN A PERFUSION BIOREACTOR

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STUDY OF RECOMBINANT PROTEIN PRODUCTION FROM TRANSGENIC *Nicotiana tabacum* PLANT CELLS IN A PERFUSION BIOREACTOR

présenté par: MENESES RAMÍREZ Erick Alejandro

en vue de l'obtention du diplôme de : Maîtrise ès sciences appliquées

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

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DEDICATORY

To Marina, my Mother. Even at 4537 kilometres far away in the south, she is my counsellor, my inspiration and the best example of how to learn to fight against adversity.

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RÉSUMÉ

La culture de plantes transgéniques pour la production de protéines recombinantes est une activité qui est actuellement au centre de plusieurs études à cause de certains avantages économiques et techniques que cette culture offre par rapport aux cultures microbiennes et à celle des cellules de mammifères. La culture de plantes est peu dispendieuse, avec une mise à l'échelle facile et qui offre un risque minimal de contamination par des pathogènes qui affectent les cellules de mammifères.

La culture de lignées cellulaires de plantes transgéniques offre, en plus, une série d'avantages par rapport à la culture de plantes. Ces avantages incluent une commande fine des nutriments et de la croissance cellulaire, elle offre aussi une plus grande reproductibilité dans la qualité du produit par lot de production et un haut niveau de confinement dans la production de protéines recombinantes, qui entraine un respect dans les normes de biosécurité. Même si la culture de cellules des plantes en suspension offre les avantages précédents, le taux de production des protéines recombinantes demeure toujours faible dans cette plateforme cellulaire. Ceci est le principal problème qui empêche la production à des fins commerciales des protéines recombinantes dans des cellules de plantes en suspension cultivées en bioréacteur.

Dans ce projet de maitrise, une lignée cellulaire végétale transgénique stable (R514) qui produit l'anticorps de souris immunoglobuline gamma 1 (IgG1) a été obtenue à partir de la transformation génétique d'une lignée cellulaire en suspension de *Nicotiana tabacum*.

L'objectif de ce projet était de montrer que la lignée R514 produisait IgG1 dans le milieu extracellulaire, en tant que cultivée convenablement en bioréacteur, en utilisant un système de recirculation en mode cuvée, et aussi dans un système en perfusion. L'objectif précédent a été accompli et l'anticorps a été détecté et mesuré dans le milieu extracellulaire. La ligné R514 est caractérisé par un taux de croissance spécifique de 0.23 $\pm$ 0.07/jour pendant l'opération en mode cuvée, et 0.29 $\pm$ 0.11/jour pendant l'opération en perfusion (dans la période de croissance exponentielle). Le système a permis d'obtenir une masse sèche maximale de 14.9 g l^{-1}.

Une stratégie de culture a été également essayée en manipulant les nutriments d'importance dans le milieu de culture. La stratégie à consisté en deux périodes de culture qui impliquent des limitations dans les sources d'azote et du phosphate dans la culture.

La limitation en azote a été utilisée afin de favoriser l'accumulation de glucides dans les cellules. Les glucides servent de squelettes carbonés pour la biosynthèse des acides aminés qui feront partie des protéines incluant

l'immunoglobuline gamma 1. La limitation en phosphate visait à diminuer le taux de croissance cellulaire.

L'application de la stratégie précédente a permis de produire environ 3.49 mg d'IgG1 total dans un bioréacteur de 3.0 l (2.7 l de volume d'opération). En effet, dans une culture menée sans modifications des nutriments, il a été produit 1.5 mg d'anticorps dans le même volume de production. D'après les résultats obtenus, on peut penser que la modulation des pools de métabolites cellulaires est une avenue intéressante pour favoriser l'augmentation de la production des protéines recombinantes dans des cellules de plantes cultivées en bioréacteur.

ABSTRACT

The use of whole plant cultures for the production of recombinant antibodies has recently received a great deal of attention due to many technical and economical advantages in comparison with microbial and mammalian production systems. These advantages include: inexpensive crop culture, easily scalable process, and low risk of contamination with mammalian pathogens.

Plant cell culture of transgenic cell lines also offers a series of additional advantages over the whole plant approach, such as: precise control over feed nutrients and growth conditions, batch to batch consistency in product, and a high level of containment in the production of recombinant proteins. Despite of the previous advantages, production of recombinant antibodies in plant cell cultures exhibits a low yield compared to microbial and mammalian cell platforms. This is the main problem to overcome in order to produce recombinant proteins to be commercialized using a plant cell culture platform.

In this master's project, a stable *Nicotiana tabacum* derived cell line (R514) was obtained. The objective of this project consisted in proving that the cell line R514 is able to produce and to secrete the murine immunoglobulin gamma 1 (IgG1) into the extracellular medium when cultured in a perfusion bioreactor. This recombinant protein was also recovered continuously from the extracellular medium using affinity chromatography.

In a bioreactor culture, this cell line showed specific growth rates of 0.23 ± 0.07/day in batch operation, and 0.29 ± 0.11/day under perfusion operation (exponential growth period), reaching a maximum dry weight of 14.9 g l^{-1}.

A feeding strategy that included nitrogen and phosphate limitation was also tried in a bioreactor culture. Nitrogen limitation was done so as to increase sugar accumulation. These carbohydrates serve as carbon skeletons for the synthesis of amino acids, required for the biosynthesis of proteins such as the immunoglobulin gamma 1 (IgG1). Phosphate limitation was intended to limit cellular growth. This limitation permits the increase of cultivation time, but also the limitation of cellular density in the bioreactor.

The feeding strategy chosen permitted the production of about 3.49 mg of total IgG1 in a 3.0 litre bioreactor (2.7l of operation volume). Previous results are in contrast with 1.5 mg of total IgG1 obtained in a bioreactor conducted with full medium and without nutrient limitation. This leads us to believe that a posterior metabolic modulation of internal metabolite pools is an adequate alternative that deserves to be explored in order to increase the production of recombinant proteins in plant cell culture.

CONDENSÉ EN FRANÇAIS

Le marché biopharmaceutique actuel compte environ 125 protéines recombinantes approuvées pour l'Amérique du Nord et l'Union Européenne (Rader 2008). La plupart des protéines produites ont des applications cliniques. La culture de cellules de mammifères est présentement le système dominant pour la production de protéines recombinantes.

Les plateformes de production qui utilisent les cellules des mammifères ont plusieurs avantages, mais un système de production moins dispendieux pourrait rendre certaines protéines thérapeutiques plus accessibles à la population à faible revenu.

Un système idéal et économique pour la production de protéines recombinantes devrait accomplir cet objectif avec un faible investissement et un bas coût d'opération. Un tel système devrait aussi produire des protéines bioactives selon les bonnes pratiques de fabrication et en respectant des standards élevés de biosécurité. Finalement, ce système de production devrait produire la protéine recombinante d'intérêt avec un taux de production supérieur à ce que l'on trouve dans les plateformes qui utilisent la culture de cellules des mammifères.

La culture de cellules de plantes transgéniques en suspension possède toutes les qualités requises d'une plate-forme de production idéale à l'exception d'un taux de production élevée.

L'amélioration de la capacité de production d'une protéine recombinante par une lignée végétale est une tâche multidisciplinaire. Afin d'atteindre cet objectif, une vision d'ingénierie des procédés doit s'appliquer à des connaissances qui relèvent de la biologie ou de la biochimie. Ce document présente donc la description d'une approche d'ingénierie qui pourrait potentiellement mener à l'objectif visé.

Les objectifs spécifiques de ce projet sont :

- Évaluer la croissance d'une lignée végétale transgénique et la production d'une protéine recombinante en utilisant un bioréacteur en perfusion.
- Comparer les taux de production obtenus en utilisant deux stratégies de culture.

Dans le présent travail, un bioréacteur, précédemment développé pour la production de métabolites secondaires (De Dobbeleer *et al.* 2006), a été utilisé pour la production de protéines recombinantes à partir de suspension de cellules végétales. Le bioréacteur est opéré avec recirculation du milieu de culture, en mode cuvée ou continu. Ce système a été spécifiquement adapté

pour permettre l'extraction en continu de l'immunoglobuline gamma I de souris (IgG1).

Nicotiana tabacum a été modifié génétiquement (transformation avec *Agrobacterium tumefasciens*) pour obtenir la lignée cellulaire R514 qui produit de l'IgG1 extracellulaire biologiquement active. Il a été montré précédemment que l'anticorps IgG1 est produit dans les cultures de cellules de plantes (*Medicago sativa*) avec les mêmes caractéristiques et affinité que l'anticorps produit par des souris et par la culture des hybridomes (Khoudi *et al.* 1999). Les modifications post-traductionnelles de l'anticorps (N-glycosilations) faites en plantes sont similaires à ceux trouvées dans l'IgG1 humain (Bardor et al. 2003). L'IgG1 est un anticorps d'une haute valeur commerciale (5000 $US g^{-1}, Khoudi et al., 2003) fréquemment utilisé dans des test sanguins pour déterminer la présence de facteurs agglutinants.

Le bioréacteur utilisé dans ce projet est un bioréacteur de 2.7 L (3 L capacité maximale) développé au laboratoire (Figure 1). Les coefficients de transfert d'oxygène (K_L a) et les temps de mélange à différentes vitesses d'agitation ont été déterminés pour le bioréacteur.

Une culture en bioréacteur a été menée en mode cuvée pendant 4 jours pour déterminer l'adaptabilité de la lignée cellulaire végétale aux conditions de

culture en bioréacteur. Une variante du milieu de culture MS (Murashige-Skoog) a été utilisée dans cette expérience.

Dans une deuxième culture, une période en mode cuvée de 4 jours a été suivie d'une période de culture en perfusion (jours 4 à 9) à un taux de perfusion de 1 l $jour^{-1}$. Dans cette culture, l'anticorps présent dans le milieu extracellulaire a été extrait à l'aide d'une colonne de chromatographie d'affinité couplée à la sortie du bioréacteur. La même variante du milieu MS mentionnée précédemment a été utilisée dans cette culture. La figure 2 b. montre la croissance cellulaire pour ce cas.

Une troisième stratégie de culture comprenant trois périodes de culture a également été définie. La première phase était une période de culture en mode cuvée avec milieu MS (jours 0 à 4). Elle était suivie d'une période de perfusion (jours 4 à 8) à un taux de 0.5 l $jour^{-1}$. Pendant cette période, la concentration du phosphate dans le milieu de culture utilisé pour la perfusion était de 20,94 mg/L, ce qui correspond à 1/5 de la concentration retrouvée dans le milieu MS. De plus, ce milieu ne contenait pas de sources d'azote. Cette étape visait l'augmentation de la concentration des squelettes carbonés nécessaires à la synthèse d'acides aminés. Finalement, dans la dernière période de culture (jours 8 à 12) le taux de perfusion de 0.5 l $jour^{-1}$ a été maintenu, mais le milieu de culture utilisé avait 1/5 de la concentration des sources d'azote du milieu MS

et aucune source de phosphate. La limitation en phosphate cherchait à diminuer la croissance cellulaire.

Des données de vitesse de sédimentation et du volume de sédimentation cellulaire précédemment obtenues pour la lignée R514 avaient permis de déterminer les valeurs de perfusion employées.

Des échantillons de culture pour les trois cas précédents (60-80 ml) ont été prélevés à chaque 12 heures environ. Les mesures intra et extracellulaires ont été prises pour les cations (NH_4^+, K^+, Na^+, Mg^{2+}, Ca^{2+}), anions (Cl^-, NO_3^-, PO_4^{2-}, SO_4^{2-}), glucose, protéines totales solubles, IgG1 produit (ELISA) et acides aminés libres. Les biomasses humide et sèche, ainsi que le pH ont été mesurées.

Les résultats suivants ont été obtenus :

Croissance cellulaire

Les données de croissance cellulaire pour les trois cultures en bioréacteur sont présentées à la Figure 2. Ces données montrent que la première une période de culture (cuvée jours 0 à 4) permet l'adaptation des cellules et un début de croissance exponentielle (croissance spécifique de 0.23 ± 0.07 jour^{-1}). Dans cet intervalle, les cellules végétales forment des agrégats qui permettent l'opération en perfusion.

La stratégie de perfusion proposée dans l'expérience illustrée dans la Figure 2a montre une croissance exponentielle entre les jours 4 à 9 (taux de croissance spécifique 0.29 ± 0.11 jour^{-1}) avec une valeur maximale de masse sèche de 14.9 g l^{-1} au jour 11. D'après ces résultats précédents, on peut conclure que les cellules de *N. tabacum* peuvent être cultivées adéquatement dans un bioréacteur avec une boucle de recirculation.

Production en continu d'IgG1

La Figure 3 montre la production d'IgG1 pour chaque culture. Dans la Figure 3a on peut distinguer 3 périodes bien distinctes. La première période (jours 0 à 3) montre que la production d'IgG1 intra et extracellulaire varie autour de 0.34 et 0.22 mg respectivement (quantité totale d'IgG1 dans le bioréacteur). La deuxième période de culture (jours 3 - 8) montre une augmentation de la production d'IgG1 (0.81 jusqu'à 3.37 mg d'IgG1 total) en parallèle avec la croissance cellulaire (taux de croissance spécifique 0.29 ± 0.11 jour^{-1}). Finalement, pendant les jours 8 à 12, on peut noter que la production de l'IgG1 se stabilise 3.49 mg d'IgG1 total.

L'aspect le plus important de cette culture est la quantité totale accumulée d'anticorps (~3.6 mg).

La figure 3b montre la production intra et extracellulaire d'IgG1 dans la culture décrite par la figure 2b. Cette culture a été réalisée en deux étapes. La

première, du jour 0 au jour 5, montre une augmentation dans la production d'IgG1 (0.26 à 1.05 mg d'anticorps total). Dans la deuxième étape (jours 5 à 9), la concentration d'IgG1 est stable autour de 1.5 mg d'anticorps total dans le bioréacteur.

Concentration de nutriments

La stratégie de culture utilisée pour la culture illustrée dans les Figures 2a. et 3a. a permis de maintenir la concentration de glucose intracellulaire constante. Cette stratégie a également limité la concentration de phosphate inorganique intracellulaire. Cette dernière limitation n'a toutefois pu diminuer le taux de croissance cellulaire tel qu'attendu. Ces résultats suggèrent donc qu'une certaine modulation des « pools » de métabolites est possible dans la lignée cellulaire utilisée (Figures 4 et 5).

Conclusions

La lignée cellulaire végétale R514 a été cultivée adéquatement dans un bioréacteur opéré en mode perfusion. Le système permet la production d'anticorps biologiquement actifs et l'extraction en continu de protéines, grâce à un procédé de séparation chromatographique (colonne d'affinité). La plateforme est donc efficace tant pour la production de métabolites secondaires que pour la production de protéines recombinantes. De plus, elle permet l'utilisation de plusieurs lignées cellulaires différentes provenant de la luzerne (Jolicoeur,

résultats non publiés), du tabac (travail présent) et du pavot de Californie (De Dobbeleer et al. 2006).

La stratégie de récupération de l'anticorps dans le milieu de culture (chromatographie d'affinité) devra être améliorée dans l'optique d'une production à grande échelle de l'IgG1.

Les résultats montrent qu'une stratégie de perfusion, couplée à des changements dans la concentration des nutriments du milieu de culture peuvent moduler les « pools » métabolites internes et ainsi augmenter de façon significative la production de protéines.

Le bioréacteur utilisé permet une diminution des coûts de purification de la protéine (downstream processing, DSP) puisque la protéine est secrétée dans le milieu extracellulaire. Les coûts de purification des protéines recombinantes constituent, de manière générale, une fraction importante des coûts totaux d'un procédé de production et une plateforme permettant de réduire coût aura un avantage certain par rapport aux autres plateformes.

Les protéines secrétées sont toutefois susceptibles d'être dégrader par l'action des protéases. Il est donc important de récupérer les protéines recombinantes présentent dans le milieu de culture le plutôt possible.

La plateforme de production présentée est un système non destructif qui permet le contrôle de la composition du milieu de culture, la production et l'extraction de la protéine d'intérêt de façon efficace et en continu. Le bioréacteur est particulièrement utile lorsqu'il s'agit de récupérer des biomolécules à courtes durées de vie, étant donné que les molécules sont extraites en moins d'une heure suivant leur sécrétion dans le milieu de culture.

Recommandations

Le projet présente une série d'aspects qui méritent une étude plus approfondie et des travaux subséquents:

La récupération d'IgG1 dans l'étape de séparation par chromatographie d'affinité mérite plus de travail. Le procédé d'extraction chromatographique dans le système consiste en une colonne d'absorption par affinité qui contient une résine en lit fluidisé qui capte l'IgG1 présent dans le milieu de culture extracellulaire. Dans ce projet la sélectivité et l'affinité de la résine utilisée doit être améliorée; cette amélioration permettra d'augmenter le faible niveau de saturation de la résine (figure 3b.). Un autre aspect à étudier est le design hydrodynamique de la colonne d'absorption. Les paramètres d'opération de la colonne devront permettre une extraction efficace de la plupart des protéines produites dans le bioréacteur.

L'adaptation d'un modèle cinétique métabolique pour la lignée R514 permettra aussi déterminer de façon plus rationnelle les stratégies de culture et de perfusion. Étant donné que l'adaptation d'un modèle métabolique a besoin de mesures de nutriments, de métabolites et d'autres molécules, il est intéressant de considérer certains points dans ce type de mesures. Dans le projet présent, une méthode ELISA a été utilisée pour la détermination de l'IgG1. On devrait aussi explorer d'autres méthodes complémentaires, telles que résonance plasmonique de surface (SPR) pour comparer les données obtenues par ELISA.

Un modèle structural décrivant la production de l'immunoglobuline G1 à partir de la concentration des « pools » d'acides aminés est aussi nécessaire. La mesure de la concentration et l'identification des acides aminés doivent être faites de façon adéquate et précise. Pour mesurer de façon adéquate les concentrations d'acides aminés, des résultats préliminaires (Meneses, résultats non publiés) ont montré qu'une méthode permet la formation de composés stables à partir de ces acides aminés. Ces composés sont ensuite mesurés par chromatographie liquide couplée à de la spectrométrie de masses.

Il est importante mesurer d'autres glucides que le glucose. Les concentrations des métabolites intermédiaires des voies des hexoses et pentoses phosphates doivent être déterminées. Des résultats préliminaires (Meneses, données non publiées) ont montré qu'une méthode d'extraction simple avec des solvants

suivi d'une quantification par chromatographie liquide permettent une détermination de la concentration de ces sucres.

Une fois que le modèle métabolique cinétique sera adapté, on pourra alors déterminer une stratégie de culture qui permet de maximiser la production de l'anticorps. Cette stratégie permettra de définir le taux de perfusion ainsi que la composition du milieu à perfuser. Cette tâche relèvera du domaine de la recherche opérationnelle.

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CHAPTER 1: Introduction.

1.1 Overview.

The biopharmaceutical market includes over 125 recombinant proteins currently approved in the United States and the European Union (Rader 2008). Most of these proteins are produced for clinical applications. Cultivated mammalian cells have become the dominant system for the production of recombinant proteins.

Despite of all the advantages that the mammalian cell platform offers, it is clear that a more inexpensive production platform could make these recombinant therapeutic proteins more accessible to the general public.

This ideal, inexpensive system must be able to produce recombinant proteins with a low investment and operation cost. This production system should also provide bioactive proteins produced under good manufacturing practice procedures and bio-security standards. Finally, this ideal production platform must also be able to produce the recombinant protein of interest at the same or superior yield of those obtained in mammalian cell cultures.

Almost all of these requirements are already attained with transgenic plant cells cultured in suspension, with the exception of a high yield of recombinant protein production.

Improving the production capacity in a plant transgenic cell line is a multidisciplinary task, which involves both biological and engineering developments. In this paper, an engineering approach is described that could lead to this improvement.

1.2 Objectives

The project objectives are:

- To evaluate the growth of a transgenic plant cell line and the production of a recombinant antibody in a perfusion bioreactor.
- To compare the yields obtained using two different feeding strategies.

CHAPTER 2: Literature review.

This chapter will describe general aspects of the production of recombinant proteins in cell culture using diverse platforms. An emphasis will be placed on the production of recombinant proteins in plant cells.

2.1 Production of recombinant proteins in cell cultures.

Nowadays, the production of recombinant proteins in suspension cells is a common research and industrial activity. Expression of heterologous proteins, peptides, antibodies and secondary metabolites using bacteria, yeasts, mammalian and plant cells is an intensive topic of research and a source of new developments and discoveries in biological and engineering fields. Many bioreactor configurations are now available for the cultivation of suspension cells at an industrial scale.

The biosynthesis of recombinant proteins in plants cells cultured in suspension, however, is an alternative that is just beginning to be explored. In the future, plant cell culture will become an economically competitive alternative for the production of recombinant proteins in comparison with other well established production platforms such as mammalian, insect and bacterial cell cultures (Hellwig, 2004).

2.1.1 Bacterial cells.

Bacterial expression systems for the production of heterologous proteins present the ability to produce high levels of protein. Bacteria have the ability to grow rapidly at a high density and in inexpensive substrates. These cells are often well characterised genetically and offer a large number of cloning vectors and mutant host strains.

Nevertheless, many bacterial systems are not able to perform post-translational modifications. If these modifications are essential for bioactivity, bacterial expression is not the adequate system for the production of heterologous proteins.

The bacterium *Escherichia coli* is the preferred host for the production of recombinant proteins. However, other bacteria such as: *Bacillus brevis*, *Bacillus megaterium*, *Bacillus subtilis*, or *Caulobacter crescentus* can also be used as hosts.

This production platform can give yields as high as 3.0 mg l^{-1} of recombinant protein produced (Palva et al., 1983).

2.2.2 Mammalian cells.

Mammalian cells are the dominant system for the production of recombinant proteins for clinical applications. These cells provide the adequate protein

folding, assembly and post-translational modifications, which are necessary for the bioactivity of certain therapeutical proteins such as antibodies. In some cases, the production of recombinant proteins in mammalian cells has reached the gram per litre range. This yield value is equivalent to an increase of 100 fold when compared with productivities reported in the 1980's (Wurm, 2004).

There are two formats that have been employed for the production of recombinant proteins in mammalian cells: suspension cultures and adherent cells.

The production of erythropoietin in CHO cells is a good example of the production of recombinant proteins using adherent cells. This production platform enables product concentrations in the 50-250 mg l^{-1} range (Wurm, 2004).

CHO is a well studied cell line. It dominates the mass production of recombinant proteins in suspension cultures. Other cell lines are also used, such as the mouse myeloma-derived NS0 cells (Barnes *et al.,* 2004), BHK (Bödecker, 1994), HEK-293 (Bernard, 1999) and human retina-derived PER-C6 (Jones et al., 2003).

2.2.3 Plant cells.

The production of recombinant proteins in plant cell cultures shows economical, technical and bio-security advantages in comparison with other cell production platforms (Hellwig, 2004). Even when compared to the production in whole transgenic plants, cell culture is still advantageous due to its precise control of culture nutrients, growth conditions and product reproducibility.

Plant cell culture dates back to the 1950s (Sijmons *et al,.* 1990) where the aim of developing culture techniques using plants cells was the production of low molecular weight molecules. The description, characterisation and biological activity of these types of molecules, produced by secondary metabolism in plants, has been extensively studied by the chemistry of natural products (Gollin, 1999).

Many secondary metabolites have been reported with a wide range of biological activities. However, only a couple of molecules, Shikonin and Paclitaxel (Taxol), have been produced at a commercial scale using plant cell cultures (Ma *et al.*, 2003). The main problem with the isolation and purification of molecules derived from plant secondary metabolism is their low concentration in native cells.

The production of recombinant proteins in plant cells has been preferably conducted using several cell lines derived from *Nicotiana tabacum*, *Oryza sativa*, *Glycine max* and *Lycopersicum esculentum* species. However, the tobacco

cultivars Bright Yellow 2 and *Nicotiana tabacum* have been the most common for this purpose. Table 1 (adapted form Hellwig *et al.,* 2004) shows some proteins currently produced in plant cells. In the next chapter, Results, our production levels will be shown to be between those reported in the literature.

2.2.3.1 Advantages of plant cell cultures.

Plant cell culture presents a series of economical and technical advantages. Plant cell culture is, as bacterial culture, inexpensive. Given the fact that plants are autotrophic, they require a minimal consumption of complex growth factors. This leads to lower prices associated with plant cell culture media compared to highly complemented media used in mammalian cell cultures. Contrary to bacterial cultures, plant cells can undergo post-translational modifications in protein products. These modifications are required in most cases to ensure the bioactivity of the protein produced and to prevent possible deleterious responses of the immune system in eventual pharmaceutical products.

Indeed, plant cells can synthesize complex proteins such as glycoproteins (*ie.* immunoglobulins) and interleukins. Many of the transgenic glycoproteins produced in plants present structural similarities to those found in natural host cells.

Table 1. Recombinant proteins produced in plant cells.

Protein produced	*Cell host*	*Promoter*	*Localization, yield*
Human serum albumin (Sijmons, 1990)	*N. tabacum* suspension culture initiated from transgenic plants	Modified CaMV 35S	Secretion/apoplast targeting, 0.25 μg mg^{-1} protein in supernatant
scFv antibody fragment (Firek, 1993)	*N. tabacum* suspension culture initiated from transgenic plants	CaMV 35S	Secretion, up to 0.5μg l^{-1}up to 0.5% of TSP
Human erythropoietin (Matsumoto, 1995; Matsumoto, 1993)	*N. tabacum* cv BY-2 suspension culture	CaMV 35S	Secreted, 1 pg g^{-1} FW
Mouse monoclonal heavy chain γ (Magnuson, 1996)	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Native heavy-chain secretion signal, ca. 10 μg l^{-1}, with stabilization up to 350 μg $l^{-1}$with PVP
Mouse $IgG_{2b/κ}$ (Fischer, 1999)	*N. tabacum* cv Petite Havana SR-1	Enhanced CaMV 35S	15 μg g^{-1} FW, ~0.3% TSP
Heavy chain mAb (La Count, 1997)	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted up to 10μg l^{-1} with stabilization up to 350 μg l^{-1}
Bryodin 1 (Francisco, 1997)	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted up to 30mg l^{-1}
Human interleukin-2 and interleukin-4 (Magnuson, 1998)	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted (native signal peptides), 8-180 μg l-1of culture broth
Recombinant ricin (Sehnke, 1999)	*N. tabacum* suspension culture	CaMV 35S	25-37.5 mg l^{-1}
scFv antibody fragment (Torres, 1999)	*Oryza sativa* cv Bengal (rice) callus culture	Maize ubiquitin	Apoplast targeting (optimized Ig leader peptides)
Full size IgG2b/κ (Fiscjer, 1999)	*N. tabacum* cv Petite Havana SR-1	Enhanced CaMV 35S	0.3% of TSP or 15 μg/ g wet weigth
Human α-1 antitrypsin (Terashima, 1999)	*O. sativa* cv Taipei 309 suspension culture	RAmy 3D	Secreted 85 mg l^{-1} in shake flask, 25 mg l^{-1} in bioreactor
biscFv antibody fragment (Fischer, 1999)	*N. tabacum* cv BY-2 suspension culture	Enhanced CaMV 35S	Cytosolic (at detection limit), apoplast-targeted (up to 0.0064% of TSP), ER retained
Human granulocyte-Macrophage colony-stimulating factor (hGM-CSF) (James 2000)	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted/targeted to apoplast ~250 μg l^{-1} extracellular, ~250 μg l^{-1} intracellular (based on culture volume)
scFv antibody fragment (Ramirez, 2000)	*N. tabacum* suspension culture initiated from transgenic plants	CaMV 35S	Apoplast targeting (sporamin secretion signal). 1 mg l^{-1} extracelullar, 5 mg l^{-1} intracellular
Human α-1 antitrypsin (Huang, 2001)	*O. sativa suspension culture*		*Up to 200 mg l^{-1} (calli* suspended to 40% (vol/vol)) cell density in induction medium.
Hepatitis B surface antigen (HBsAg) (Smith, 2002)	*Glycine max* cv Williams 82 (soybean) and *N. tabacum* NT-1 suspension culture	RAmy3D	Intracellular up to 22 mg l^{-1} in soybean ~2 mg l^{-1} in tobacco
hGM-CSF (Lee, 2002)	*N. tabacum*	$(ocs)_3$ mas	1.6 to 6.6 μg l^{-1} upon homogenizing the entire culture broth
Human Isozyme (Huang, 2002)	*O. sativa* cv Taipei 309 suspension culture	RAmy3D	Intracellular (although RAmy3D signal peptide was used) up to 3%-4% of TSP
IL-12 (Kwon, 2003)	*N. tabacum* Havana suspension culture	Enhanced CaMV 35S	Secreted, up to 800 mg l^{-1} of supernatant
hGM-CSF (Kwon, 2003)	*Lycopersicum esculentum* cv Seokwang (tomato) suspension culture	Enhanced CaMV 35 S	Secreted, up to 45 mg l^{-1} of supernatant
HBsAg (Sunil, 2003)	*N. tabacum* cv NT-1 suspension culture	*A. thaliana* ubq3	Secreted, up to 800 mg l^{-1} of particulate HBsAg
mAb against HBsAg (Yano, 2004)	*N. tabacum* cv BY-2 suspension culture	CaMV 35S	Secreted, ~50/50 between supernatant and cells, total max 15 mg l^{-1}

TSP, total soluble protein; ER endoplasmic reticulum; PVP polyvinylpyrrolidone; FW, fresh weight.

Protein yields in plant cell cultures are more or less constant when the cultures are performed under similar conditions (ie. nutrient feed and bioreactor operation conditions). Unlike whole plant harvesting, protein yield does not depend on climate or soil quality. Contamination with pesticides, mycotoxins, and possible by-products derived form secondary metabolites is also diminished.

Another advantage is the ease of purification of the product, especially in cells where the protein is secreted (Doran 2000, Fisher *et al.,* 1999).

2.2.3.2 Plant cell culture characteristics.

Many strategies can be used to culture plant cells *in vitro*. The cultivation of plant cells includes the suspension cell culture, hairy roots, shooty teratomas and immobilized cells. Suspension cell culture is the principal strategy used because this system can be easily adapted in large-scale bioreactor cultivation.

Plant suspension cells are obtained by culturing the friable callus tissue in culture media in shaker flasks or bioreactors. The idea is to form single cell cultures or cultivations with small cell aggregates. A callus is an undifferentiated tissue obtained after cultivation of explants in solid medium that contains a mixture of plant hormones to help maintain an undifferentiated state. Plant cells are subsequently cultured in liquid media containing the same hormonal balance and are supplemented with factors to promote fast growth.

When the plants in the callus are transgenic, there is no need for additional genetic manipulation, selective stress may be applied. Otherwise, wild type cells would have to undergo genetic transformation with recombinant plasmids by co-cultivation with *Agrobacterium tumefasceins* or by particle bombardment.

Compared to bacterial cultures, plant cell cultures have lower cell densities and growth rates (Taticek *et al.,* 1994). Indeed, microbial growth rates range from 0.1 h^{-1} to 1 h^{-1}, whereas suspension plant cell rates in for example, *Catarantus roseus,* range from 0.019 h^{-1} to 0.028 h^{-1}. Both cases show higher rates in native cells lines. In the case of the tobacco cultivar BY-2, values of a growth rate of up to 0.044 h^{-1} have been observed (Nagata, 1992). This cell line also exhibits growth synchronicity and low nicotine level when compared to other cultivars (Murashigue, 1962).

In the case of Oxygen Uptake Rate (OUR), plants cells are also characterised by values that are lower than those found in bacteria. This can be beneficial in settings where the growth rate must be controlled. Typical OUR's for plant cells range from 1 to 3.5 mmol l^{-1} h^{-1} (Taticek *et al.*, 1994) while values from 5 to 90 mmol l^{-1} h^{-1} are reported for bacterial cultures (Hellwig, 2004).

2.2.3.3 Recombinant protein production and promoters.

Tobacco cultivars such as NT-1 and BY-2 are commonly used as host cells to produce recombinant proteins. These cell lines have been chosen because they

present a high growth rate and are easily transformed and propagated. However, certain aspects such as proteolytic activity, deserve greater study in these cell lines (Hellwig, 2004).

Other cell lines in suspension have been established for recombinant protein production using plants such as: rice (Terashima *et al.*, 1999; Torres *et al.*, 1999; Huang *et al.,* 2002; Huang *et al.,* 2001), soybean (Smith *et al.*, 2002) and tomato (Kwon et al., 2003). These cell lines have been tried in an effort to see if they may favour by-product molecule content, faster growth, higher expression levels, and greater efficacy in secretion compared to tobacco derived cell lines.

The promoter is a key component in the construction design for recombinant protein expression. The promoter can affect the yield by controlling the rate of transcription. The most widely used promoter is the Cauliflower Mosaic Virus (CaMV) 35s or its enhanced version (Hellwig, 2004). Other promoters can be seen in Table 1. Most of the promoters listed in Table 1 are constitutive except for the rice α-amylase *RAmy3D* promoter, which is induced by sugar starvation. An important aspect to consider in promoter selection is the presence or absence of a leader peptide. Whether the leader peptide sequence comes from a plant protein or from another type of cell is irrelevant since both accomplish the same objective. In the case of secreted proteins, human proteins can express their endogenous leaders in plant cells (Hellwig, 2004).

2.2.3.4 Improving recombinant protein production.

The production of recombinant proteins in plant cells varies from 0.0064% to 4% of total soluble protein or about 0.5 μg l^{-1} to 200 mg l^{-1} (Table 1). However, these values are difficult to compare, because extraction and quantification methods are not standardized and sometimes results obtained using different techniques are not the same (i.e. Enzyme Linked Immunoassay *vs.* Surface Plasmon Resonance measurements of IgG1).

The main problem in plant cell culture is the production level of recombinant proteins. This value is normally one or two orders of magnitude below values required to make the process viable. However, the supernatant of plant cell culture offers two important advantages: ease in the recovery step and facilitated separation of the medium from cells (i.e. separation columns in separation systems, De Dobbeleer *et al.,* 2006).

Tables 2 and 3 (adapted from Hellwig, 2004) show different methods used to improve the protein yield in plant cell culture. These methods consist primarily in changing the properties of the culture medium and modifying the process development.

2.2.3.5 Protein recovery and downstream processing.

Downstream processing involves all the procedures used to separate and purify the protein from the culture media or biomass. Depending on the purity degree desired in the final product, protein recovery can represent up to 80% of the total production cost.

Most of the time, isolation and purification techniques must be designed to an individual case. However, the product can sometimes be isolated using a standardized approach, for example, using affinity chromatography to separate recombinant proteins which contain a general engineered epitope tag (Stoger, 2005).

Secreted targeted proteins offer an advantage compared to intracellular ones, because they provide a starting material (supernatant) with a lower contaminating metabolite and intracellular protein content when compared to cytosolic or internal compartment targeted proteins. Purification of extracellular targeted proteins has the disadvantage that considerable quantities of medium must be processed in the purification step. This is the case when the recombinant protein titre in the medium is very low. High dilution rates also usually lead to a higher degree of degradation of the recombinant protein.

Table 2. Medium changes to improve the production of recombinant antibodies in plant cell cultures

Medium additive	*Expression host*	*Expressed Protein*	*Effect*
PVP (Lacount, 1997; Magnuson, 1996)	*N. tabacum* NT-1 suspension culture	Heavy chain monoclonal antibody	Addition of 0.75% PVP 360.000 increased secreted product accumulation 35-fold
BSA, NaCl (James, 2000)	*N. tabacum*cv BY-2 suspension culture	Human-granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Enhanced secreted product accumulation 100% (BSA), or 50% NaCl
BrefeldinA (Sharp, 2001)	*N. tabacum* suspension culture initiated from transgenic plants	Mouse IgG1	Inhibited the secretory pathway and thereby prevented degradation of secreted protein, increased mAb accumulation 2.7 fold
Reducing Manganese (Tsoi, 2002)	*N. tabacum* suspension culture initiated from transgenic plants	Mouse IgG1	In manganese-reduced medium the stability and accumulation of secreted IgG1 was increased – 1.7 fold
Pluronic antifoam, PEG (Lee, 2002)	*N. tabacum* cv Havana SR1 suspension culture	hGM-CSF	Pluronic antifoam addition increased the growth rate almost two fold, PEG 8000 increased hGM CSF accumulation fourfold
Gelatin, PVP, PEG (Lee, 2002)	*N. tabacum* cv Havana SR1 suspension culture	hGM-CSF	2% gelatin increased accumulation 4.6-fold, PVP and PEG showed no effect.
Gelatin, PVP, PEG (Kwon, 2003)	*N. tabacum* cv Havana	IL-12 heterodimer	2% gelatin increased IL-12 accumulation sevenfold

Media additives can act in different ways, for example, as enhancer of protein synthesis, enhancers or inhibitors of secretion, inhibitors of intracellular protein degradation, or as extracellular stabilizing agents. The effect of such additives must be determined empirically for each culture system and recombinant protein, and it should be noted that the addition of these compounds may, in some cases, interfere with downstream process processing. PEG, polyethylene glycol; BSA, bovine albumin serum.

Table 3. Process Engineering approaches to improve the yield of recombinant antibodies

Bioreactor type, scale and fermentation mode	***Expression host***	***Expressed protein***	***Effect***
Semicontinous production of Chloramphenicol- acetyltranferase CAT in repeated batch and continuous fermentation at 0.25 d^{-1}in a bubble column. (Hogue, 1990)	*N. tabacum* suspension culture	Chloramphenicol-acetyltranferase (CAT)	Constant product concentration in harvest
Shake-flask, semicontinuous perfusion/ continuous mode, replacing 33% of the medium every 12h, equivalent to 0.66 d^{-1} (La Count, 1997)	*N. tabacum,* NT-1 suspension culture	Heavy-chain monoclonal antibody	+/- constants levels of secreted heavy chain mAb in harvest
Stirred tank bioreactor, 10 litters continuous, 0.2 d^{-1} (Des Molles, 1999)	*N. tabacum,* cv BY-2 suspension culture	Carrot acidic invertase	Increased overall productivity fourfold
Encapsulation of suspension cultured cells in alginate, shake flask (Bodeutsch, 2001)	*N. tabacum,* NT-1 suspension culture	Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Increased levels of hGM-CSF 107 to 178 µg l^{-1}
Shake flask, periodic harvesting using hydroxyapatite-resin (Sharp, 2001)	*N. tabacum* suspension culture initiated from transgenic plants	Mouse IgG1 monoclonal	Periodic harvesting increased overall yield

Alternatives to the classical purification schemes such as expanded bed absorption (EBA) may also help, in some cases, to capture the protein produced in the extracellular medium. This technique has been used industrially to purify recombinant proteins from several microbial and animal cell cultures and as a result an adaptation to plant cell cultures deserves further study. A final approach that can be tried is that of a two phase cultivation system, which would enable an *in situ* protein extraction approach.

Intracellular protein recovery is a more difficult task. This procedure includes an initial step of cell and membrane disruption. Therefore, wet milling, sonication, pressure homogenisation and enzymatic disruption are methods that should be explored for this primary step. After disruption, a clarification extract must be done using the techniques previously discussed in the extracellular purification step.

CHAPTER 3: Results. Continuous production of murine IgG1 by *Nicotiana tabacum* suspension cells in a recirculation loop bioreactor.

3.1. Introduction

Sixteen recombinant immunoglobulins have already reached the biopharmaceutical market and over 200 are currently under clinical trials (Raju, 2003; Reichert et al., 2005). In order to face the demand in monoclonal antibodies, various production platforms have been developed and evaluated for their ability to produce bioactive antibodies at lower costs. Among the various cell systems that have been proposed, the plant cell platform is gaining interest. Plant cell culture has been widely studied for the production of recombinant proteins that are bioactive, including human proteins and antibodies (Sijmons et al., 1990; Firek et al., 1993; Matsumoto et al., 1995; Suehara et al.1996; Lee et al., 1997; Magnuson et al., 1998; James et al., 2000; Ryland et al., 2000; Sharp and Doran 2001; Hellwig et al., 2004). Suspension cultures of plant cells offer a series of advantages for the production of recombinant proteins, for example: completely defined and inexpensive culture media, which does not promote the development of human viruses, prions and pathogens, in comparison with the animal cell production platform. It is also possible to work with cryopreserved master cell banks (Miele, 1997). However, the mammalian cell platform is now reaching volumetric production of over 4 g L^{-1} (Wurm, 2004) and this is highly competitive for the other platforms. Development of efficient transformation

systems for plant cells has allowed a significant increase of production yields in recombinant proteins, which make the plant platform competitive with other expression systems (Ma et al., 2003).

In the present work, a bioreactor previously developed for secondary metabolite production (De Dobbeleer et al., 2006) was used for the production of recombinant proteins from plant cell suspensions. The bioreactor is operated with medium recirculation, in batch or continuous feed mode, and is specifically designed to allow the continuous extraction of the antibody of interest. *Nicotiana tabacum* was modified to produce biologically active Immunoglobulin gamma 1 (IgG1) antibody. It has been demonstrated previously that a fully functional IgG1 protein can be effectively produced in plant cell culture (*Medicago sativa*) with the same specificity and affinity of those obtained in mice and hybridoma cultures (Khoudi et al., 1999). Post-translational modifications of the antibody, such as N-glycosilation, produced in plant cells are similar to those found in human IgG1 (Bardor et al., 2003). IgG1 is an antibody with a high commercial value (5000 \$US g^{-1}, Khoudi et al., 1999) and is frequently used in blood tests to determine the presence of agglutinant factors.

3.2. Material and methods

3.2.1 Suspension cell culture

A stable *Nicotiana tabacum* R514 cell line was established from NT1 cell lines transfected with *Agrobacterium tumefaciens*, strain AGL1, provided by MEDICAGO Inc. This strain contains a plasmidic DNA sequence coding for murine IgG1.

The transformed cell line was cultured in Murashige-Skoog (MS) medium containing 30 g/l Glucose (sigma G-7021), 0.5 g/l 2,3 dichlorophenoacetic acid (Sigma-Aldrich D7299), 0.25 g/l Kinetin (Sigma-Aldrich, K0753) and 1.3 mg/l thiamine HCL (sigma T-1270). Pluronic (0.1 g/l) and Simeticone (0.4 g/l) were also added to the medium as antifoam agents.

The pH of the medium was adjusted to 5.7 with 1M KOH before sterilisation. The suspension cell flask culture obtained after 7 days (15 g at 70% CSV) was transferred into a 500 ml Erlenmeyer flask containing 135 ml of fresh medium. After inoculation, cells were kept at 25 $\pm$ 3 °C in the dark, using an orbital shaker (130 RPM).

3.2.2 Bioreactor configuration

The bioreactor used in these experiments is shown in Figure 1. This is a 2.7 L in-house bioreactor (3.0 L maximum capacity) with a stainless steel lid (316-L).

The vessel (12.5 x 27 cm^2) and two separation columns (90 x 26 mm^2) were made from Pyrex glass. Geometric ratios for this bioreactor are similar to those in De Dobbeleer et al. (2006). A double helicoidal ribbon (Jolicoeur et al., 1992) was used for agitation (120 mm height x115 mm O.D., 22 mm width). Dissolved oxygen was measured using a polarographic probe (Mettler Toledo, InPro 6800) connected to an acquisition data system (Virgo, Longueuil, Québec, Canada). The membrane of the probe was placed 10 cm below the liquid culture surface and the columns were separated by 1 cm from the top of the helicoidal ribbon.

Oxygen transfer coefficients (K_L a) and mixing times were established for this bioreactor at different rotation speeds (30, 45, 60 and 90 rpm counter-clockwise rotation). Porous spargers were placed at 1 cm above each column end to diminish the cell entrainment in perfusion operation. The bioreactor was autoclaved, sterilised (121°C, 1 atm, 90 min) and inoculated in a horizontal laminar flow hood under sterile conditions (20% Fresh Weight/ Medium mass), using suspension cells from a 7 day old culture.

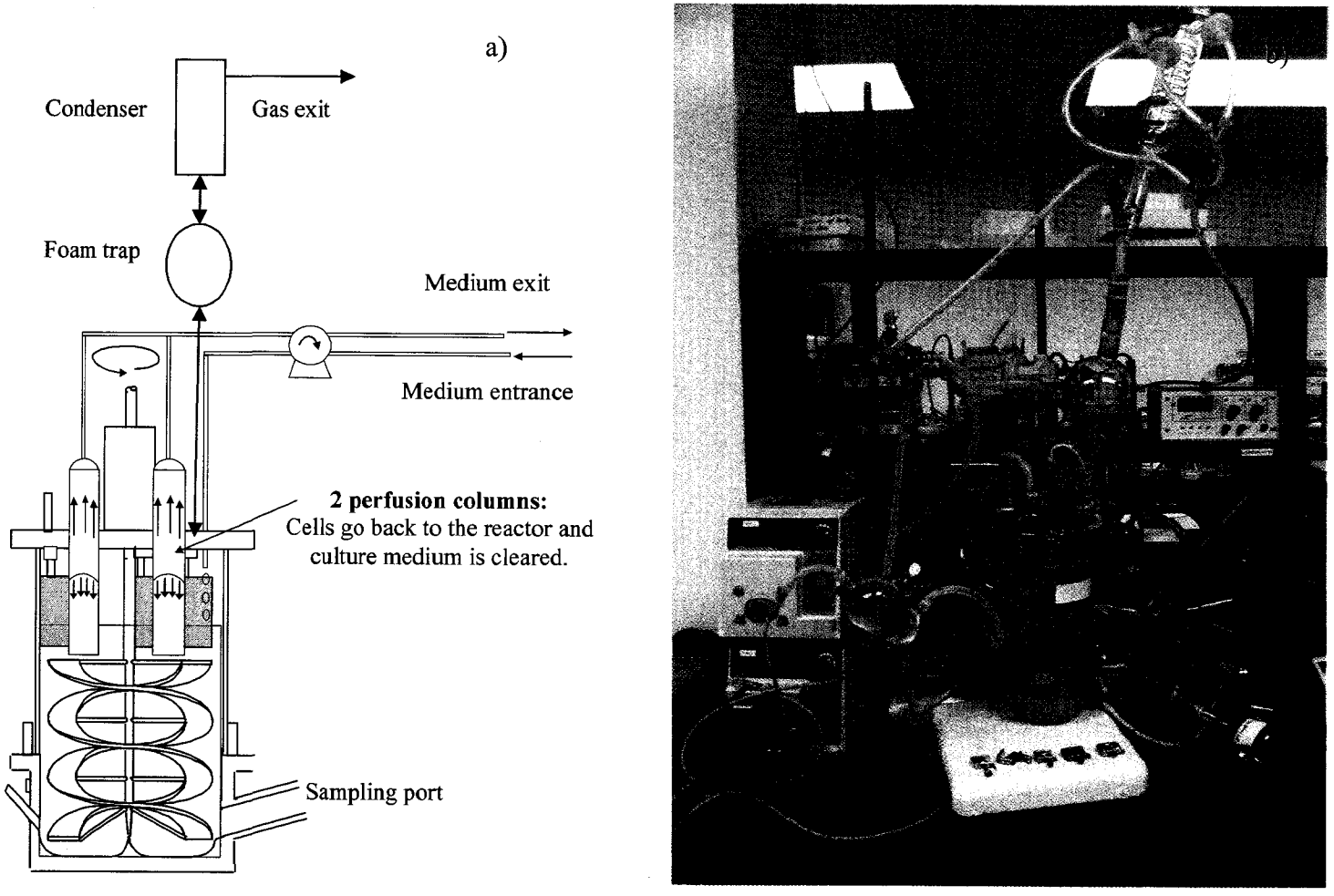

Figure 1. Bioreactor configuration. a) Scheme for the continuous extraction bioreactor used for the production of C5-1 with tobacco cells in suspension.
b) Continuous extraction bioreactor in operation.

Following K_L-a and mixing time measurements, an initial rotation speed of 30 rpm was fixed for the impeller at the beginning of the culture, but at the end of the culture (day 8) this value was increased to 45 rpm. The minimal level of oxygen in the medium for this culture was established at 40% according to the saturation value (mass flow controllers, Tylan, Mykrolis, MA, FC 260). The culture was started under the previous condition with an oxygen and air mix flow of 200 ml/min (50% maximum in oxygen), and at the end of the culture (day 8) the flow mix was raised to 400 ml/min.

3.2.3 Medium recirculation and perfusion strategies

One bioreactor culture was conducted in batch mode during 4 days to establish adaptability of the culture at bioreactor conditions. Complete MS medium was used in this experiment.

In a second culture, a batch period of 4 days was followed by a perfusion period (day 4 to 9 at a perfusion rate of 1.0 l d^{-1}) until day 9. In this culture, IgG1 present in the perfused medium was extracted using an affinity chromatography column. Complete MS medium was also used. Figure 2 b. shows cellular growth for this culture.

A third and final culture strategy was also defined, divided into three periods.

One batch culture period with complete MS medium (day 0 to 4 without perfusion).

One perfusion step (day 4 to 8 at a perfusion flow of 0.5 litters per day), during this step the phosphate concentration was one fifth of the complete MS medium and the medium did not contain ammonium nor nitrate sources. This step was used in order to increase the concentration of carbon skeletons that are used in the synthesis of amino acids.

A final perfusion step (day 8 to day 12 at a perfusion flow of 0.5 litters per day), in which the nitrate and ammonium concentration was one fifth of the complete MS medium without phosphate sources. Phosphate limitation was chosen to diminish cell growth.

Perfusion values were chosen for each case based on previous results of sedimentation velocity and practical procedures used to determine the sedimented cell volume (Boivin, Jolicoeur, unpublished data).

3.2.4 Analytical

Culture samples (60-80 ml) were taken at every hour during the first 5 hours of culture to determine the cellular phosphate capture rate. After 5 hours of culture, samples were taken every 12 hours approximately. The volume removed by sampling was replaced with a similar volume of sterile MS culture medium.

The following intra- and extracellular concentration measurements were taken: cations (NH_4^+, K^+, Na^+, Mg^{2+}, Ca^{2+}), anions (Cl^-, NO_3^-, PO_4^{2-}, SO_4^{2-}), glucose,

total soluble proteins, IgG1 produced and free amino acids. Fresh weight, dry weight biomass and the medium pH were also measured.

Fresh weight biomass was measured upon filtering (Whatman filter, Fisher Scientific, 09874-48) 10 ml of the homogeneous culture sample. The previous fresh weight sample was placed in a stove at 60°C for 24 hours before measuring dry weight. Fresh biomass was rinsed twice with 30 ml of deionised water and crushed in a chilled mortar using liquid nitrogen. Crushed biomass (3 g approximately) was kept in 1 ml cryovials (Fisher Scientific, 03-374-21) and conserved in liquid nitrogen for posterior analysis. The culture medium (approximately 12 ml) obtained after culture medium filtration was stored at -20°C for posterior analysis.

Intra- and extracellular IgG1 concentration measurements were done by ELISA

Before glucose and ion analysis, frozen cells (1 ml vial of crushed cells) were freeze-dried 48 hours under a vacuum.

3.2.4.1 Ions

Ten milligrams of lyophilised biomass was extracted with 1.0 ml of cold trichloroacetic acid (5% w/v). The mix was vortexed and then centrifuged 5 minutes at 16000 g. The supernatant was filtered (0.45 µm, Millipore nitrocellulose filters) and used to measure the concentration of intracellular ions by HPLC as described in Lamboursain and Jolicoeur (2005). Samples of

extracellular culture media were filtered at 0.45 µm and also used to quantify ions concentration by HPLC.

3.2.4.2 Glucose

Fifteen milligrams of frozen dried biomass were extracted 3 times with 0.5 ml of cold ethanol (80% v/v). The mix was vortexed and then sonicated for 5 min. The supernatant obtained after centrifugation (16000 g x 5 min) was frozen in liquid nitrogen for further intracellular glucose concentration measurements. Culture medium samples were also thawed to measure extracellular glucose concentration. Glucose determination was done in both cases using an enzymatic kit (Glucose Assay Reagent, Sigma G3293-20ML).

3.2.4.3 Soluble proteins

The Bradford method (Compton and Jones, 1985) was used for to measure the concentration of total intracellular soluble proteins and of total soluble proteins in the culture medium. Proteins were extracted from ~300 mg FW of cells using a buffer solution prepared by mixing 1M tris-HCl pH 8.0 (5 ml), 5 M NaCl (6 ml), Triton X-100 (100 µl), deionised water (89 ml) and protease cocktail inhibitor (100 µl) (Sigma P8849-1ML).

Biomass was extracted twice (2 x 400 µl) using a chilled micromortar. The solution obtained was centrifuged (21000 g x 5 minutes) and the supernatant was used to quantify total soluble proteins and intracellular IgG1 concentration.

The same protocol was used to measure the soluble protein concentration in the extracellular medium.

3.2.4.4 IgG1 antibody

Intra- and extracellular measurements of C5-1 were accomplished by Enzyme-Linked ImmunoSorbent Assay (ELISA) for all cultures.

The following buffers were used in antibody measurements: (1) phosphate-buffered saline (PBS: 10 mmol L^{-1} sodium phosphate, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, pH 7.5) for the dilution of antibodies and the preparation of standard solutions; (2) the coating buffer was 0.1 M carbonate buffer with pH 9.6; (3) the blocking buffer was 1% casein (m/v) in PBS; (4) a PBS buffer containing 0.1% Tween-20 (v/v) was used for washing; (5) peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, 1:10,000 diluted in blocking solution) was used as a conjugated antibody solution; (6) TMB Microwell Peroxidase substrate solution (Sigma, T0440-100ML); (7) the enzymatic reaction was stopped with 1 M HCl.

Direct ELISA was carried out as follows: (1) Goat anti-mouse IgG1 heavy chain specific (Sigma M-8770) was diluted to 2.5 μg ml^{-1} in the coating buffer. 50 μl $well^{-1}$ of coating solution was added into a microtiter plate (Immulon 2HB, Thermo Labsystem 3655) and incubated at 4 °C overnight. (2) Plates were washed three times using 200 μl $well^{-1}$ of the washing buffer. Then, 50 μl $well^{-1}$ of antibody standard solutions and intra- and extracellular sample solutions were

added. Murine IgG1 (Sigma, M-9269, 1mg ml^{-1}) was diluted with blocking solution to prepare the standard antibody solutions of 0, 1, 2, 3, 4, 5, 7 and 10 ng ml^{-1}. Intra- and extracellular samples were diluted in blocking solution at 200x and 50x respectively to prepare sample solutions. (3) After incubation for 1 h at 37 °C, the unbound compounds were washed away. (4) 50 µl $well^{-1}$ of peroxidase-conjugated goat anti-mouse IgG was added. (5) After incubation for 1 h at 37 °C, the unbound compounds were washed away. (6) 50 µl $well^{-1}$ of TMB Microwell Peroxidase substrate solution was added. (7) After incubation for 8 minutes at room temperature the enzymatic reaction was stopped upon addition of the stopping solution (50 µL well−1) and then absorbance at 450 nm was measured.

3.3 Results and discussion

3.3.1 Cell growth

Growth data for the three bioreactor cultures performed are shown in Figure 2. These data show a batch period of about 4 days (specific growth rate 0.23 $\pm$ 0.07/day), ie. the time required by the cells to start growing at an exponential rate. In this phase, plant cells reform aggregates with a size distribution that enables medium perfusion with a minimal cell entrainment. Repeated cultures prove reproducibility of the growth data for the batch phase.

The perfusion strategy proposed in the experiment shown in Figure 2.a. shows an exponential growth phase from day 4 to 9 (specific growth rate 0.29 $\pm$ 0.11/day) reaching a maximum DW of 14.9 g l^{-1} at day 11. Oxygen transfer became a limiting factor for growth after day 8 (below 10%, data not shown), which could explain the decreasing growth phase observed from this day on (specific growth rate -0.048 $\pm$ 0.004/day). This situation (Figure 2.a.) was corrected by increasing the maximum flow (400 ml/min) of the oxygen-air mix furnished to the bioreactor to a dissolved oxygen value within a desirable value of around 40% oxygen dissolved in the medium. According to these results, *N. tabacum* suspension cell lines can be successfully grown in the bioreactor with a recirculation loop. The perfusion strategy and sedimentation columns were highly efficient in enabling a complete separation of the extracellular medium from the cells. No significant cell entrainment was detected under the operation conditions assayed in this work. Finally, the addition of Pluronic and anti-foam to the medium resulted in minimal foam formation during culturing, thus minimizing cell loss in the bioreactor head space. This observation is important because cells entrained within the foam show necrosis, a phenomenon which can result in the release of toxic compounds in the culture and affect both cell growth and productivity.

3.3.2 The recombinant mouse antibody IgG1 can be continuously extracted

Figure 3 shows the IgG1 production for each perfusion bioreactor cultures. Results obtained by ELISA were reproducible, with a variation coefficient of less than 5% for all the measurements taken.

In figure 3 a., three well defined periods can be distinguished in relation to IgG1 production. The first period goes from day zero to day three. In this phase intra- and extracellular IgG1 contents range at around 0.34 mg and 0.22, respectively. A second step goes from day three to day eight (0.81 to 3.37 mg of total IgG1), with an exponential increase in IgG1 production in parallel to cell growth (specific growth rate 0.29 + 0.11/day). A final plateau production (at around 3.49 mg) can be observed between days 8 and 12. At day 12, a marked diminution in protein production can be observed. This could be explained by the limiting oxygen conditions previously stated. Hypoxia has deleterious effects such as a decrease in cellular energy charge, a drop in cytoplasmic pH and accumulation of toxic end products from anaerobic respiration and reactive oxygen species (ROS) (Liu, 2005).

The most important aspect in this culture is the maximum IgG1 quantity obtained, ~3.6 mg of antibody for the whole bioreactor, including cumulative outlet perfused media.

At the beginning of the culture, IgG1 extracellular titre was, in average, greater than the intracellular one. During the exponential growth phase, however, intracellular titre was superior to the extracellular one, indicating that the IgG1 rate of production was faster than the rate of secretion. This situation can be explained by the antibody size (146 kDa), considering that proteins of less than 30 kDa tend to be secreted into the culture medium, whereas larger proteins are normally retained (Hellwig, 2004). It is clear from these results that the release of the recombinant IgG1 antibody in the medium is not due to cell lysis since it occurred during exponential growth where cell viability is expected to be high (Lamboursain and Jolicoeur, 2006; Lamboursain and Jolicoeur, 2005). However, more work is required to identify by which phenomenon large proteins are released from viable cells.

Figure 3.b. shows intra- and extracellular IgG1 production for the bioreactor culture described in Figure 2.b., in which the perfusion rate was higher than for the culture showed in Figure 2.a. A higher perfusion rate led to a higher medium dilution and to a higher degradation of IgG1. Two steps concerning IgG1 production can be identified in this culture. A first step from day 0 to day 5 were an increase in antibody production is present (0.26 to 1.05 mg of total IgG1) and a second phase where the antibody concentration remains stable at about 1.5 mg of total antibody in the bioreactor.

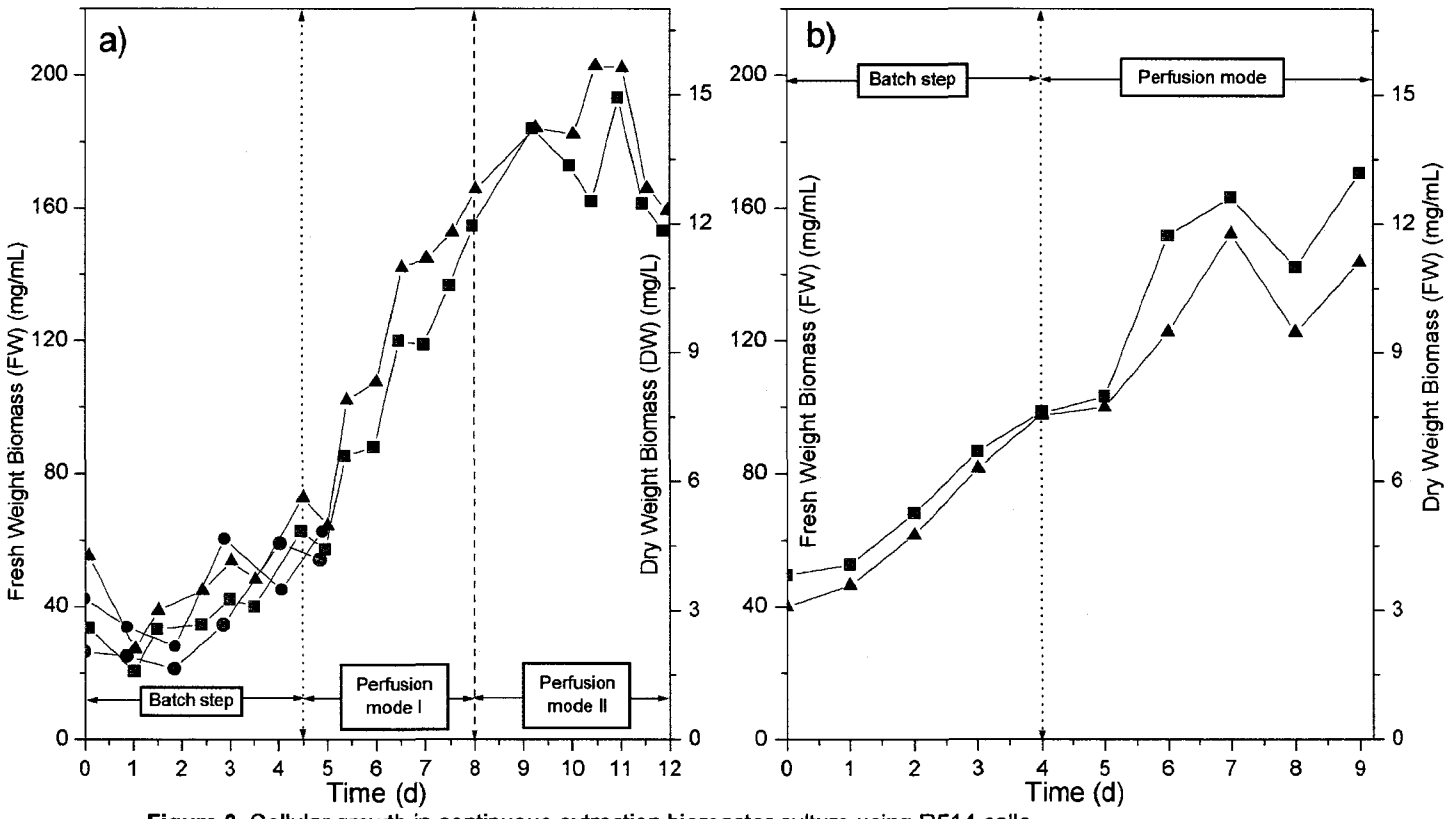

Figure 2. Cellular growth in continuous extraction bioreactor culture using R514 cells.
a) Three step culture Batch mode in KCMS complete medium. Perfusion mode I: 0,5 liters per day perfusing KCMS medium with 1/5 of phosphate and without nitrogen sources. Perfusion mode II: 0,5 liters per day of KCMS medium with 1/5 of Nitrogen and without phosphate sources. **b)** Perfusion culture done in two steps. Batch mode with KCMS medium and perfusion mode of 1.0 l/day of KCMS medium and extractive step by affinity chromatography. (—▲—) Fresh Weight biomass, (—■—) Dry Weigth Biomass, (—●—) Dry Weight in batch mode (adaptability test), (—●—) Fresh Weight in batch culture (adaptability test).

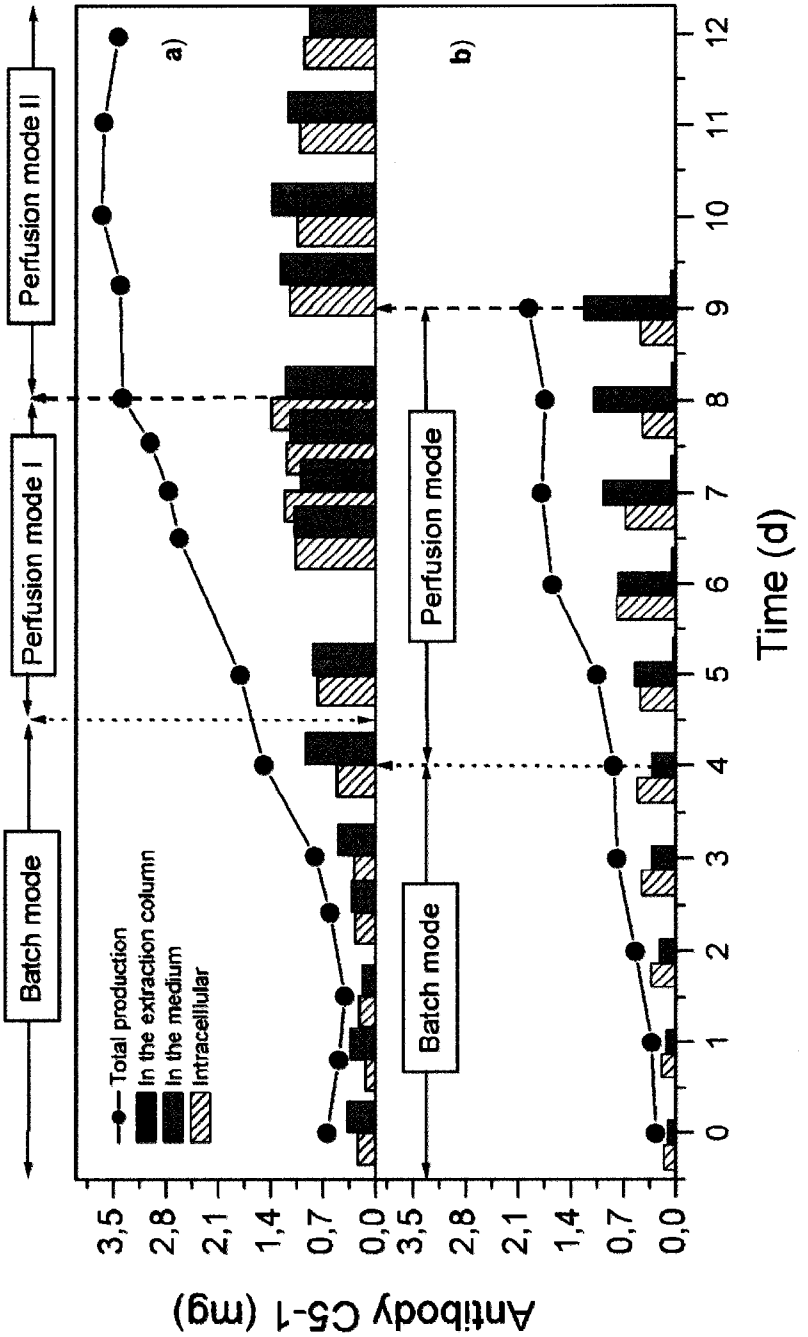

Figure 3: ELISA IgG1 concentration determination for two cultivations in continuous extraction bioreactor using R514 cells. **a**) Culture described in figure **2 a**). **b**) Culture described in figure **2 b**).

3.3.3 Nutrient concentration changes

The feed strategy employed for the culture shown in Figures 2.a. and 3.a. was successful in maintaining a constant concentration of intracellular sugars. This strategy also resulted in a limitation of intracellular inorganic phosphate that can diminish growth rate and antibody production. Taken together, these results suggest that a certain modulation of metabolite pools is possible for this cell line (Figures 4 and 5).

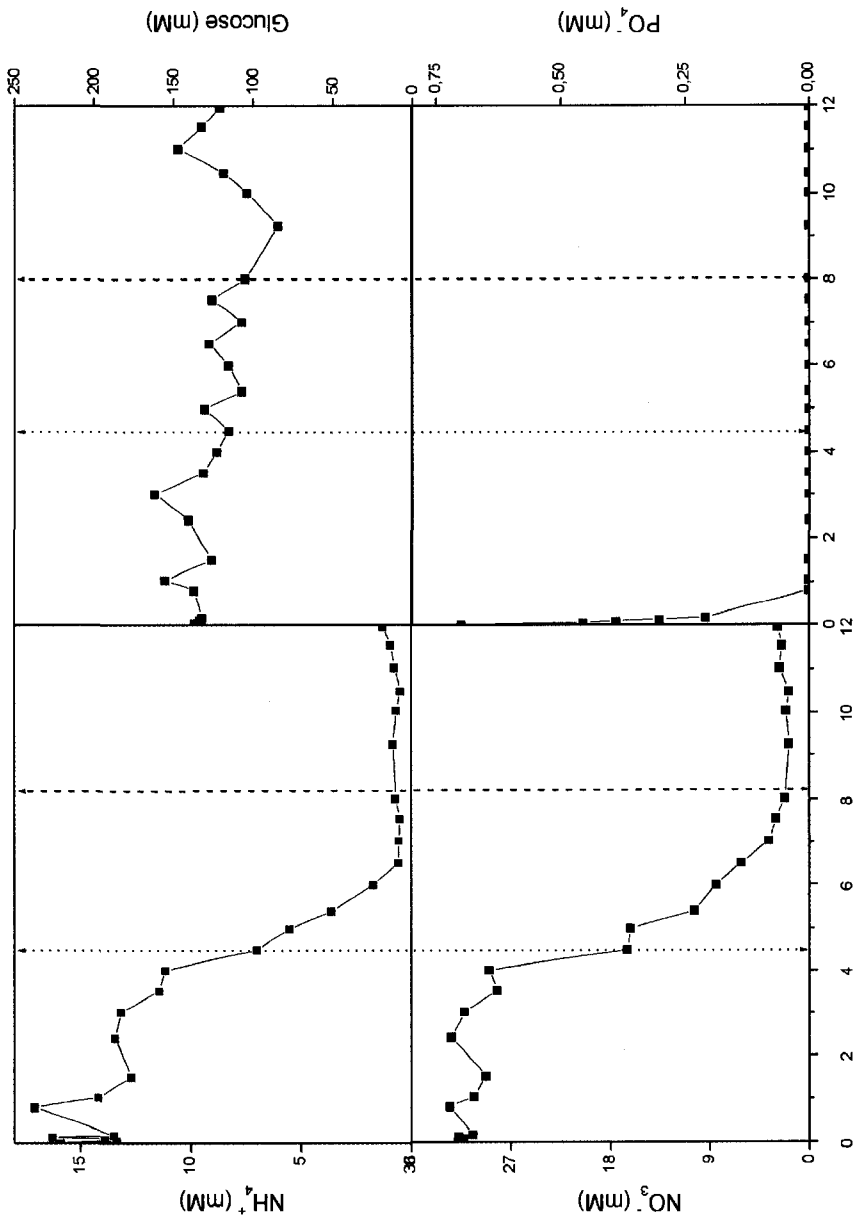

Figure 4: Extracellular nutrient concentration in continuous extraction bioreactor culture of R514 tobacco cells.

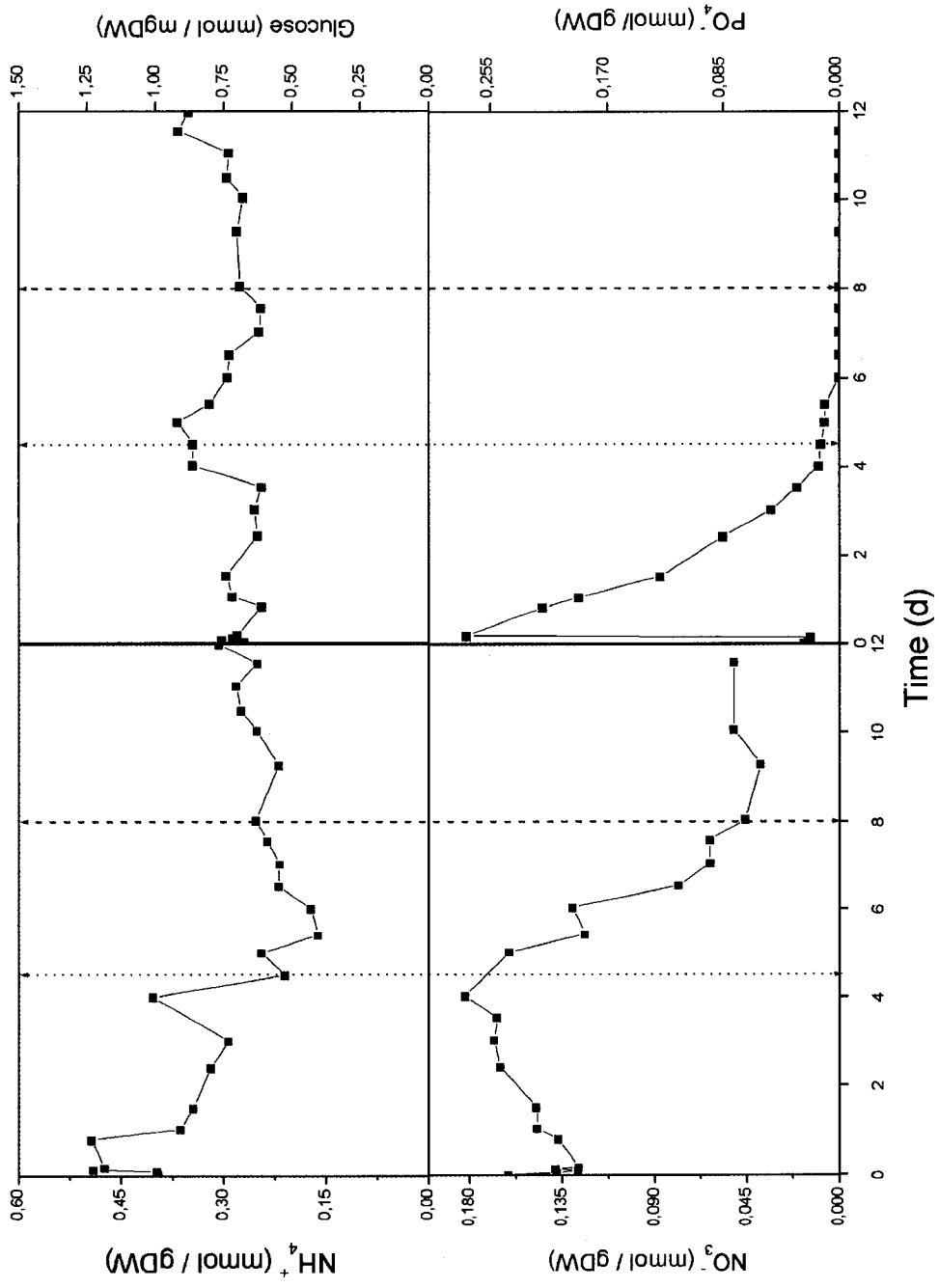

Figure 5: Intracellular nutrient concentration in a continuous extraction bioreactor culture of R514 tobacco cells

CONCLUSIONS

The transgenic plant R514 cells were successfully grown in a bioreactor operated in perfusion. This system permits the production of biologically active antibodies and enables the continuous extraction of proteins using an ultimate separation steps such as affinity chromatography coupled to the effluent system. The platform demonstrated its efficacy in secondary metabolite or recombinant protein production for different cell species including alfalfa (Jolicoeur, unpublished results), tobacco (present work) and California poppy (De Dobbeleer et al., 2006).

Improvements in the quantity of IgG1 produced and strategies for antibody recovery and satisfactory extracellular secretion of the antibody for the cell line will be desirable in order to improve high scale production of murine IgG1 in tobacco cells. Our results show how perfusion strategy together with changes in nutrients in the bioreactor feed can be a suitable way to increase antibody production and modulate internal metabolite pools.

This bioreactor allows cost reduction in downstream processing (DSP), which usually represents the major part of the production cost, as the product can be secreted into the extracellular medium thereby reducing processing steps.

Secreted proteins are sensitive to proteases or physical degradation and it is therefore imperative to collect them as quickly as possible to protect them. The

platform presented is a non-destructive system which allows fast harvesting of the protein produced, continuous production and extraction, and control of the medium composition during culture. In order to take advantage of a medium perfusion mode, the bioreactor is specially adapted for biomolecules with short half-lives since they can be extracted as fast as one hour after their secretion into the culture medium.

RECOMMENDATIONS

This project illustrates a series of aspects that deserve more study and improvement:

The recuperation of IgG1 using an ulterior separation step such as affinity chromatography still requires some work. The extraction process in this system consists in an adsorption affinity column containing a bed expanded absorption resin that separates IgG1 present in culture media, via affinity chromatography. The separation procedure presents a couple of aspects to be improved. The first consists in increasing the selectivity and affinity of the resin used in the expanded bed separation. This must be done to increase the low level of saturation observed in the results obtained (Figure 3.b.). Another aspect that must be studied is the hydrodynamic design of the absorption column. This design should allow most of the antibody produced to be extracted in the chromatography column separation step.

Also, the adaptation of a metabolic model for the R514 cell line could enable a more rational design of feeding strategies. For this last purpose, there are certain key points to consider. In the present project, a direct method was used in order to quantify Immunoglobulin gamma I (IgG1). Complementary methods for IgG1 measurement, such as surface plasmon resonance, should be explored in order to compare the quantity of antibody produced. A structural model

describing the production of IgG1 based upon intracellular amino acid pools must be included in the final model. In this part, identification and measurement of key amino acids pools must be done in a reliable way, preliminary results (Meneses, unpublished data) have shown that methods that include the formation of stable derivative compounds and posterior measurement by liquid chromatography coupled to mass spectroscopy present an affordable option for intracellular and extracellular amino acids measurement.

Carbohydrate measurements including hexose and pentose phosphate metabolites intermediaries are important. Preliminary results (Meneses, unpublished data) have shown that simple solvent extraction methods and subsequent quantification by Liquid Chromatography are suitable for these quantifications.

After the adaptation of a kinetic metabolic model, the design of a feeding strategy seeking to increase the production of IgG1 would eventually become an operational research problem.

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