The Expression of the 14D9 Catalytic Antibody in Suspended Cells of *Nicotiana tabacum* Cultures Increased by the Addition of Protein Stabilizers and by Transference from Erlenmeyer Flasks to a 2-L Bioreactor

Patricia L. Marconi and María Alejandra Alvarez
Cátedra de Farmacobotánica y Farmacognosia, Carreras de Farmacia y Bioquímica, Facultad de Ciencias de la Salud, Universidad Maimónides, Ciudad de Buenos Aires, Argentina

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The effect of two protein stabilizers (polyvinylpyrrolidone [PVP] and gelatine) on growth and 14D9 yield of Nicotiana tabacum *cell suspension cultures (Ab-KDEL and sec-Ab) was analyzed. The addition of PVP at a concentration of 1.0 g L^{-1} produced the highest total 14D9 yield (biomass + culture medium) in the Ab-KDEL line (4.82% total soluble protein [TSP]). With the addition of gelatine, the highest total 14D9 yield (2.48% TSP) was attained in the Ab-KDEL line at 5.0 g L^{-1} gelatine. When the Ab-KDEL suspended cells were cultured in a 2-L bioreactor, the highest 14D9 yield was 8.1% TSP at a 5% w/v inoculum size, which was the best 14D9 yield so far obtained in the platforms tested (*E. coli, N. tabacum *leaves and seeds,* N. tabacum *hairy roots, and cell suspension cultures).* © 2014 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:1185–1189, 2014
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Introduction

The increasing demand for recombinant antibodies by the pharmaceutical industry has triggered the development of alternative production platforms (transgenic animals, transgenic plants, insect cell cultures, and so forth) to the traditional ones (mammalian cell and bacterial cultures). Among them, plants offer particular advantages such as the reduced costs of the process, the ability to perform post-translational modifications typical from eukaryotes, and the absence of human and animal pathogens, toxins, oncogenes, or prions.[1] When the plant platform corresponds to *in vitro* cultures, there are additional advantages such as the development of the whole process in controlled conditions and, as a consequence, the ability to produce the recombinant protein under good manufacturing and good laboratory practices as is required by the pharmaceutical industry.[2–4]

Amid the antibodies of interest, the catalytic antibodies are of particular significance given their ability to catalyze chemical reactions with a high regioselectivity and stereoselectivity.[5] Furthermore, they can catalyze chemical transformations on demand, even in the case of reactions where no natural enzyme are available, making their production attractive to the chemical industry.[6] Several strategies have been implemented to produce catalytic antibodies such as chemical synthesis, semisynthesis,[7,8] and the expression of recombinant antibodies in bacteria,[9] yeasts,[10] or plants.[11]

The antibody 14D9 is one of the fastest and most practical catalytic antibodies to date, being the first example of an enantioselective gram-scale synthesis with a catalytic antibody.[12,13] 14D9 is an IgG_1-type murine antibody obtained by immunization against a piperidinium hapten[14] that catalyses the highly enantioselective (>99% *ee*) protonation of enol-ethers. Its potential use for the release of an essential growth factor, for example, biotin, from a specifically synthesized substrate is under study.[9] As a recombinant protein, 14D9 was expressed in *E. coli* as a single-chain fragment (scFv) fused to the N-utilization substance protein A (scFv-NusA) and as the chimeric fragment (Fab), and in *Nicotiana tabacum* plants as the whole antibody.[9,11]

In a previous work, we have established *N. tabacum in vitro* cultures that express the functional 14D9. Besides, we have also demonstrated the beneficial effect of the ER-retention signal KDEL on the expression level of the functional whole antibody both in cell suspension cultures and in hairy roots (HRs).[15,16] The aim of this study is to analyze the effect of the addition of two protein stabilizers to the culture medium (gelatine and polyvinylpyrrolidone [PVP]) as a strategy to increase antibody yields. In addition, we also performed the scale-up of suspended cells growing in Erlenmeyer flasks to a 2-L bioreactor and compared its performance with that of cell suspension cultures growing in agitated flasks. Finally, we compared 14D9 yield and productivity in *E. coli*, *N. tabacum* seeds and leaves, and *N. tabacum in vitro* cultures (cell suspension and HRs).

Materials and Methods

Plant material

Cell Suspension Cultures. Soft, friable calli obtained from the leaves of *N. tabacum* plantlets expressing the

Correspondence concerning this article should be addressed to M. A. Alvarez at alvarez.mariaalejandra@maimonides.edu.

Table 1. Parameters of Growth and Antibody Yield in sec-Ab and Ab-KDEL *N. tabacum* Cell Suspension and Hairy Root Cultures

N. tabacum Line	μ (day^{-1})	t_d (days)	$Y_{X/S}$ (g DW g sucrose^{-1})	14D9 Yield (%TSP)	14D9 Specific Productivity (mg g^{-1} FW day^{-1})	14D9 Volumetric Productivity (μg L^{-1} day^{-1})
wt	0.086	8.05	0.51 ± 0.12	–	–	–
sec-Ab	0.069	10.07	0.42 ± 0.08	0.16 ± 0.01	0.6 ± 0.02	0.25 ± 0.03
Ab-KDEL	0.149	4.62	0.47 ± 0.04	0.62 ± 0.04	40.7 ± 1.86	17 ± 0.01
HR wt	0.105	6.60	0.36 ± 0.02	–	–	–
HR sec-Ab	0.06	11.55	0.31 ± 0.04	5.88 ± 0.24	213 ± 12.22	280 ± 18.42
HR Ab-KDEL	0.19	3.65	0.24 ± 0.07	7.82 ± 0.38	685 ± 13.28	954 ± 15.38

wt: *N. tabacum* wild-type cell suspension culture; sec-Ab and Ab-KDEL: cell suspension lines expressing the sec-Ab and Ab-KDEL 14D9 mutant, respectively; HR wt: hairy root wild-type line; HR sec-Ab and HR Ab-KDEL: hairy root lines expressing the sec-Ab and Ab-KDEL 14D9 mutant, respectively. μ, specific growth rate; t_d, doubling time; GI, growth index; TSP, total soluble protein; $Y_{X/S}$, biomass yield relative to substrate concentration. Each value is the mean of three replicates ± SD (adapted from Refs. 15 and 16 and from M. A. Alvarez, unpublished data).

secreted (sec-Ab) or KDEL-tagged (Ab-KDEL) mutant of the monoclonal antibody 14D9[16] were transferred to 150-mL Erlenmeyer flasks containing 40 mL of MSRT liquid medium (Murashige Skoog culture medium with Khana and Staba vitamins)[17] with 2.0 mg L^{-1} naphthalene acetic acid (NAA) and 0.2 mg L^{-1} kinetin (Kin) as plant growth regulators. Cultures were kept in an orbital shaker at 100 rpm, 24°C ± 2°C and a 16-h photoperiod (1.8 w m^{-2} seg^{-1}).

Effect of Additives on Growth and 14D9 Yield. For studying the influence of the additives gelatine and PVP, different concentrations of gelatine (1.0, 5.0, or 9.0 g L^{-1}) or PVP (1.0, 1.5, or 2.0 g L^{-1}) were added to the culture medium at the first day of culture. Samples were taken by triplicate at the 22nd day of culture to measure fresh weight (FW), dry weight (DW), and antibody accumulation both in the biomass and in the culture media. The culture conditions were the same as described above.

Performance of the N. tabacum *Cell Suspension in a Bioreactor.* Ab-KDEL cell suspension (inoculum size 1 or 5%, by w/v) was transferred to a 2-L stirred-tank bioreactor (Minifors, Infors HT®, Switzerland) containing 1 L of MSRT culture medium plus 2.0 mg L^{-1} NAA and 0.2 mg L^{-1} Kin as plant growth regulators. Mechanical agitation (100 rpm) was provided by a marine propeller and bubble aeration (0.1 vvm) by a porous metal sparger. The temperature was 24°C ± 2°C. The relative partial O_2 pressure and pH were monitored on-line. Samples by triplicate were taken every 2 days during a culture period of 15 days for measuring FW, DW, and 14D9 concentration.

14D9 Antibody Extraction and Analysis. For antibody extraction, samples were grinded using a cold mortar and pestle in cold PBS (0.24 g L^{-1} KH_2PO_4, 1.44 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KCl, 8 g L^{-1} NaCl, pH: 7.0–7.2) with 10 μg mL^{-1} leupeptin and then centrifuged at 14,000*g* for 20 min at 4°C; antibody was estimated in the supernatant. Antibody in the culture medium was determined after the addition of leupeptin (10 μg mL^{-1}), as protease inhibitor. The concentration of antibodies possessing both heavy (γ) and light (κ) chains was determined by sandwich enzyme-linked immunosorbent assay (ELISA) using goat anti-mouse antibodies specific for γ and κ chains and a mouse IgG as standard (Sigma Chemical). Only antibodies assembled into γ–κ chain complexes were detected.

The functionality of the antibody was verified by competitive ELISA with bovine serum albumin (BSA) coupled to the 14D9 haptene.[11] Western blot analysis was performed with goat anti-gamma mouse chain-conjugated peroxidase (1:1,000; Southern Biotechnology). The immune complexes were detected after incubation with Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical).

Growth index (GI) was calculated as the ratio of the final to the initial weight.[18] FW was determined after filtering the plant material through a Whatman filter paper under vacuum; residual medium was removed by washing three times with distilled water. Then, the cells were transferred to pre-weighted dishes, and the total mass was measured. DW was estimated by transferring the harvested biomass to a drying oven (60°C) until constant weight. Specific productivity is defined as the amount of antibody produced per gram of biomass per day of culture (mg g^{-1} FW day^{-1}). Volumetric productivity is defined as the amount of antibody produced per liter of culture media per day of culture (μg L^{-1} day^{-1}).

The protein concentration was determined according to the method proposed by Bradford,[19] and proteases and sucrose according to Ref. 16.

Statistics

Three replicates were used in all analytical determinations. Analysis of variance was performed for each test.

Results and Discussion

In Table 1, we review the parameters of growth and antibody yield in sec-Ab and Ab-KDEL *N. tabacum* cell suspension and HR cultures, which are the background levels we seek to improve.

Effect of additives on antibody yield

A strategy to improve the stability of foreign proteins is the use of protein stabilization agents or protease inhibitors such as gelatine, BSA (for protein-based stabilization), mannitol (to regulate the osmotic pressure of the medium), PEG, and other polymers (for protecting proteins from denaturing agents released by plant cells into the medium).[20–23]

Figure 1 shows the effect of PVP and gelatine on 14D9 yield, expressed as a percentage of the total soluble protein content (%TSP), in sec-Ab and Ab-KDEL cell suspension cultures (S). In the biomass of the Ab-KDEL cell suspension culture, antibody increased from 1.4 to 4.8% TSP and 2.99% TSP with PVP at 1.0 and 1.5 g L^{-1} concentration, respectively. In the biomass of the sec-Ab line, the increase was from 0.17 to 1.16% TSP with a PVP at 1.5 g L^{-1} concentration. No significant increase was measured in the cultures at the other PVP concentrations tested ($P < 0.05$). As for the amount of antibody in the culture medium, it increased from

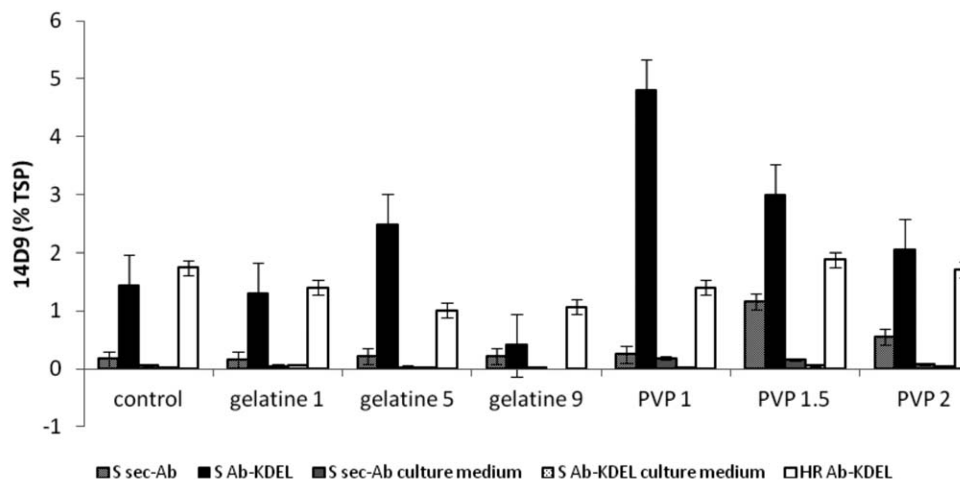

Figure 1. The effect of gelatine and PVP on 14D9 expression level (% antibody per TSP) in sec-Ab (S sec-Ab) and Ab-KDEL (S Ab-KDEL) cell suspension cultures and in Ab-KDEL hairy root cultures (HR Ab-KDEL).

The height of each bar represents the average of three individual identical experiments (± SD).

0.02 to 0.049 and 0.035 with 1.5 and 2.0 g L^{-1} PVP, respectively, for the Ab-KDEL line, and from 0.06 (control) to 0.18% TSP and 0.15% TSP with 1.0 and 1.5 g L^{-1} PVP, respectively, for the sec-Ab line. Concerning the influence of gelatine, at a concentration of 5.0 g L^{-1}, the antibody yield increased in the Ab-KDEL biomass from 1.43 to 2.47% TSP. No significant influence ($P < 0.05$) on 14D9 concentration was found in the biomass of the sec-Ab or in the culture medium of both cell suspension lines. On the other hand, protease concentration in the biomass was 1.0 U g FW^{-1} h^{-1}, a value that was maintained along the culture, whereas in the media, the protease activity increased after the 10th day in culture to a maximal level of 0.16 U g FW^{-1} h^{-1} at the end of the culture.

In Figure 1, we have also included the results obtained with HRs[15] to compare the performance of both production platforms. When compared with the HRs, cell suspension cultures appeared more influenced by the presence of the additives being the highest total 14D9 yield (biomass + culture medium) achieved in Ab-KDEL cell suspension cultures with 1.0 g L^{-1} PVP (4.82% PTS). The beneficial effect of PVP is attributed to its excellent complexing, stabilizing, and colloidal properties, whereas it is metabolically and physiologically inert, or for being a sacrificial substrate for protease activity. Moreover, PVP would be the preferred additive in a production process for being a nonhuman and nonanimal source.

Our results are partially in agreement with those reported by other authors[23,24] expressing the secreted version of the monoclonal antibody Guy's13 in *N. tabacum* cell suspensions and HRs. However, they did not find a significant influence of PVP on antibody accumulation in the biomass of cell suspensions and have established that PVP and gelatine diminishes antibody content in HR biomass. Conversely, they have reported an increase of total antibody accumulation (biomass + culture medium) both in cell suspension as in HRs when PVP or gelatine were added at a concentration of 1.5 or 9.0 g L^{-1}, respectively, due to an increase in antibody accumulation in the culture medium.

Table 2 shows the influence of the additives tested on growth, expressed as GI. In a previous work, we have established that dimethyl sulfoxide resulted toxic to cell suspension and HR cultures at all the concentrations tested (1.5, 2.0, 2.5, 5, and 10%, v/v). Also we analyzed the effect of the addition of mannitol (as an osmotic agent) and found that it also has a negative influence on cell growth but increased 14D9 yields to 1.96 mg L^{-1} in line sec-Ab and to 2.31 mg L^{-1} in line Ab-KDEL.[15,16]

Table 2. Effect of PVP and Gelatine on Growth Index (GI) of *N. tabacum* Hairy Roots (AB-KDEL line) and Cell Suspension Cultures (Ab-KDEL and sec-Ab lines)

Additive (g L^{-1})	Ab-KDEL Hairy Roots	Ab-KDEL Cell Suspension Culture	sec-Ab Cell Suspension Culture
Control	3.11 ± 0.16	5.93 ± 0.19	5.07 ± 0.13
PVP 1	1.93 ± 0.02	1.72 ± 0.22	2.10 ± 0.08
PVP 1.5	3.24 ± 0.24	1.53 ± 0.24	4.40 ± 0.04
PVP 2	3.91 ± 0.15	2.37 ± 0.14	3.70 ± 0.22
Gelatine 1	3.64 ± 0.22	3.40 ± 0.24	3.12 ± 0.19
Gelatine 5	2.12 ± 0.17	5.66 ± 0.33	3.31 ± 0.24
Gelatine 9	2.82 ± 0.09	4.50 ± 0.31	3.86 ± 0.14

Control: Hairy root or cell suspension culture without the addition of PVP or gelatine. Values are the mean of three replicates ± SD.

Bioreactor culture

Table 3 shows the values of μ, maximal antibody yield, and antibody specific and volumetric productivity in Ab-KDEL *N. tabacum* cultures initiated with two different inoculum sizes, 1 and 5% w/v, in a 2-L bioreactor. No significant differences were found in μ between the cell suspension cultures growing in Erlenmeyer flasks (0.170 per day) and in the bioreactor with a 1% w/v inoculum size (0.15 per day), although it was significantly lower with a 5% w/v inoculum size (0.08 per day; Figure 2). The GI and the antibody content were higher with a 1% w/v inoculum size than with a 5% w/v inoculum size. Biomass yield with respect to the carbon source ($Y_{X/S}$) do not show significant differences ($P < 0.05$) between cell suspensions growing in Erlenmeyer flasks and in the bioreactor with an inoculum size of 1% w/v. On the contrary, $Y_{X/S}$ was significantly lower in the bioreactor with a 5% w/v inoculum size, probably for a nutrient limitation derived from the highest cell density in the culture.

Table 3. Influence of the Inoculum Size on the Parameters of Growth and Antibody Yield in Ab-KDEL Cell Suspension Cultures Growing in a 2-L Bioreactor Initiated with 1 or 5% v/v Inoculum Size

Inoculum Size (w/v)	μ (day^{-1})	GI	$Y_{X/S}$ (g DW g^{-1} sucrose)	Maximal 14D9 Yield (% TSP)	14D9 Specific Productivity (mg g FW^{-1} day^{-1})	14D9 Volumetric Productivity (μg L^{-1} day^{-1})
1%	0.150	7.08 ± 0.63	0.595 ± 0.026	8.10 ± 0.26	354 ± 23.8	39 ± 1.34
5%	0.08	2.40 ± 0.19	0.236 ± 0.004	5.58 ± 0.09	120 ± 15.24	33 ± 5.62

Values are the mean of three replicates ± SD. μ, specific growth rate; GI, growth index; TSP, total soluble protein; $Y_{X/S}$, biomass yield relative to substrate concentration.

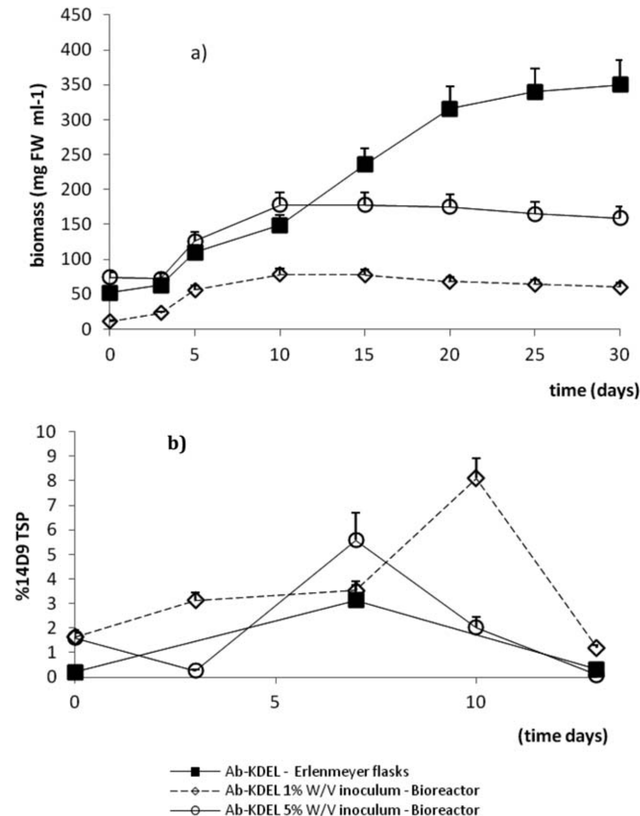

Figure 2. Time course of (a) growth and (b) 14D9 expression in cell suspensions growing on MSRT medium added by NAA:Kin (2:0.2, by mg mL^{-1}) in 250-mL Erlenmeyer flasks (S Ab-KDEL) or in a 2-L bioreactor with two different inoculum sizes (1 and 5%, w/v).

(—■—) Ab KDEL in Erlenmeyer flasks; (- - -◇- - -) Ab-KDEL, 1% w/v inoculum size in a 2-L bioreactor; (—○—) Ab-KDEL, 5% w/v inoculum size in a 2-L bioreactor. Each point represents the mean of three replicates ± SD.

Comparing the performance in Erlenmeyer flasks and the bioreactor, the antibody yield (% TSP, volumetric productivity) was significantly higher in the 2-L bioreactor, which is predictable considering the highest availability of nutrients and the earlier antibody peak production attained in the bioreactor (between Days 5 and 10 of culture) than in Erlenmeyer flasks (between Days 10 and 20 of culture; Figure 2b). In addition, antibody yield (as % TSP) was higher than in Ab-KDEL HRs in Erlenmeyer flasks. However, productivity was significantly lower ($P < 0.05$) than in HRs (Tables 1 and 3), probably due to the fast growth of the high-productive HR cultures.

Finally, we have compared 14D9 yield of cell suspension and HR cultures with those of leaves and seeds of *N. tabacum* plants and with the 14D9 single-chain fragment (scFv) and a chimeric Fab fragment yield of *E. coli* cultures (Table 4). The highest yields correspond to HRs and Ab-KDEL cell suspension cultures (in a 2-L bioreactor). Taking into account the genetic and biochemical stability and the high productivity of HRs, their culture in a bioreactor seems attractive; however, it is a challenge considering their branched growth that require of specific adaptations. Thus, cell suspension cultures will remain the preferred alternative considering the simplicity of their culture. Studies are being performed to improve yields in batch cultures in the bioreactor.

Table 4. 14D9 Yield in *E. coli* and *N. tabacum* Leaves and Seeds, Cell Suspension, and Hairy Root Cultures Growing in Erlenmeyer Flasks, and Cell Suspension Cultures Growing in a 2-L Bioreactor

Expression Platform	14D9 Yield	Reference
N. tabacum leaves	5.20% TSP	11
N. tabacum seeds	0.41% TSP	11
N. tabacum cell suspension (Erlenmeyer flasks)	0.62% TSP; 1.13 mg L^{-1} (Ab-KDEL)	Table 1 and text
N. tabacum hairy roots	7.80% TSP; 1.27 mg L^{-1} (Ab-KDEL)	Table 1 and text
N. tabacum cell suspension (2-L bioreactor)	8.10% TSP; 3.35 mg L^{-1} (Ab-KDEL)	Table 2 and text
E. coli	1.4 mg L^{-1} (14D9 Fab)	9
E. coli	0.5 mg L^{-1} (14D9 scFv)	9

TSP, total soluble protein.

Conclusions

Manipulation of the culture medium by the addition of different agents and the use of different plant systems are means of increasing recombinant protein production in *in vitro* plant cell cultures. In this work, we established that the accumulation of 14D9 in *N. tabacum* cell suspension cultures in Erlenmeyer flasks was enhanced by the addition of PVP at a 1% g L^{-1}. In addition, the scale-up of Ab-KDEL suspended cells to a 2-L stirred-tank bioreactor increased 14D9 yield (expressed as % TSP). However, 14D9 productivity remained higher in HR cultures. When compared with the heterologous systems tested to date, we conclude that *N. tabacum* cell suspensions cultured in a bioreactor and HR cultures are competitive platforms for producing the antibody 14D9 in higher yields.

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