

# Two New Disposable Bioreactors for Plant Cell Culture: The Wave and Undertow Bioreactor and the Slug Bubble Bioreactor

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Received 15 November 2005; accepted 15 August 2006

Published online 27 September 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21187

**ABSTRACT:** The present article describes two novel flexible plastic-based disposable bioreactors. The first one, the WU bioreactor, is based on the principle of a wave and undertow mechanism that provides agitation while offering convenient mixing and aeration to the plant cell culture contained within the bioreactor. The second one is a high aspect ratio bubble column bioreactor, where agitation and aeration are achieved through the intermittent generation of large diameter bubbles, “Taylor-like” or “slug bubbles” (SB bioreactor). It allows an easy volume increase from a few liters to larger volumes up to several hundred liters with the use of multiple units. The cultivation of tobacco and soya cells producing isoflavones is described up to 70 and 100 L working volume for the SB bioreactor and WU bioreactor, respectively. The bioreactors being disposable and pre-sterilized before use, cleaning, sterilization, and maintenance operations are strongly reduced or eliminated. Both bioreactors represent efficient and low cost cell culture systems, applicable to various cell cultures at small and medium scale, complementary to traditional stainless-steel bioreactors.

Biotechnol. Bioeng. 2007;96: 914–923.

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**KEYWORDS:** disposable bioreactor; cell culture; slug bubble; secondary metabolites; *Nicotiana tabacum*; *Glycine max*

## Introduction

Plant cell cultures are often cited as a possible alternative source of secondary metabolites (Alfermann and Petersen, 1995; Fu et al., 1999; Misawa, 1994; Mulabagal and Tsay, 2004; Stafford, 2002; Stöckigt et al., 1995; Wu and Zhong, 1999; Zhong, 2001). They have also been used in several recent studies as a possible mean for the production of various recombinant proteins such as antibodies, enzymes,

vaccines, and blood factors (Choi et al., 2003; Doran, 2000; Gao et al., 2004; Girard et al., 2004; Hellwig et al., 2004; McDonald et al., 2005; Soderquist and Lee, 2005; Sorrentino et al., 2005; Verdelhan des Molles et al., 1999). Plant cell cultures allow fully controlled production and downstream processing, without any risk of foreign gene dissemination in the environment or contamination by mammalian pathogens, and a good public acceptance.

Different well-known culture systems have been used for industrial development such as airlift, stirred-tank, or rotating drum bioreactors (Doran, 1993; Fu et al., 1999; Misawa, 1994; Sajc et al., 2000). In spite of the interest in this technology, and numerous scientific and technical advances, there are very few examples of economical production of metabolites, such as the red dye shikonin (Takahasi and Fujita, 1991), ginseng cells (Hibino and Ushiyama, 1999), berberine (Matsubara and Fujita, 1991) and more recently Taxus-derived drugs (Venkat, 1998). The limited development of the technology at an industrial scale is due to well-known drawbacks, such as the low growth rate (often below  $0.7 \text{ day}^{-1}$ , minimum doubling time around 20 h), low productivity and/or instability of the productive cell strains and the high cost of the traditional bioreactor technology (stainless steel). The potential instability of the cell strains can be overcome through cryopreservation such as for *Peganum harmala* (Courtois et al., 1988), *Glycine max* (Federici et al., 2003), or *Nicotiana tabacum* cell cultures (Schmale et al., 2006). However, using batch production in traditional bioreactors means operating no more than 15 runs per year with plant cell cultures in expensive equipment.

Traditional culture systems are generally composed of a rigid container (glass or stainless steel) having a means for

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aerating and mixing the culture (air sparger, impeller). Industrial equipment and support facilities associated with aseptic bioprocessing are expensive. More than 60% of the production costs is due to the fixed costs, especially high depreciation costs of the fermentation equipment. The initial capital expenditure may also be a limitation to the scaling up of this technology (Yoshioka and Fujita, 1988). The running costs are high, due to low yields and the need to clean and sterilize the bioreactor after each culture cycle. In order to decrease the production costs, a few alternatives to traditional stainless-steel bioreactors have been developed (Curtis, 1999; Hsiao et al., 1999; Singh, 1999). Singh (1999) developed a disposable bioreactor with an original agitation apparatus, using an inflated bag placed on a rocking mechanism that moves the bag inducing a wave-like motion to the liquid contained therein. This system is mainly used for animal cell cultures. Few articles have been published in plant cell domain, and only with small working volumes (0.5–1.0 L) (Bentebibel et al., 2005; Eibl and Eibl, 2006; Palazon et al., 2003).

In the present article, we describe two new flexible plastic disposable bioreactors. The first one is based on the principle of a wave/undertow mechanism providing convenient mixing and aeration to the plant cell culture (“WU bioreactor”). The second is a new bubble column bioreactor that allows an easy increase of working volumes (up to several hundred liters) with the use of multiple units.

The cultivation of tobacco and soya cells producing isoflavones is described using 70 and 100 L working volumes for the SB and the WU bioreactors, respectively.

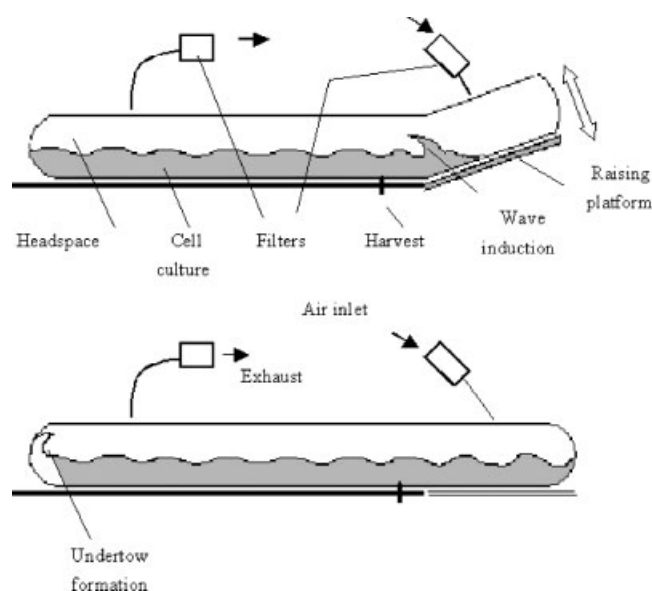
Both systems could be applied to the production of de novo metabolites, recombinant proteins, biotransformation or multiplication of biomass, undifferentiated or embryogenic cells and organ cultures, through batch culture but also continuous or fed-batch processes.

## Principle

### Description of the WU Bioreactor

The Wave and Undertow (WU) bioreactor is shown diagrammatically in Figure 1. It consists of a large flexible plastic container partly filled with medium and inflated with air. Agitation and oxygenation are achieved through wave and undertow induction.

The system is located on a horizontal table of variable length, and equipped on one side or each side with a platform raised periodically. The intermittent rising movement of the platform to the rest point, and down/descending movement back to initial position enables continuous mixing and aeration through the wave/undertow motion: the platform ascension leads to the wave formation; the wave propagates through the bag, bounces off at the extremity, creating an undertow which returns to the initial point. This action is repeated, creating a new impulse to ensure persistence of flow within the WU bioreactor.



**Figure 1.** Schematic diagram of the WU bioreactor.

For larger volumes and higher system lengths, or to improve the wave and undertow creation, both platforms, one at each side of the table, can be activated in strict opposition to improve the wave/undertow mechanism efficiency.

Wave/undertow induction provides liquid culture mixing and bubble-free aeration. The oxygen transfer is accomplished by transport from the headspace air to the liquid culture and enables growth without apparent shear stress.

Platform movements are simply achieved by pneumatic jacks located under the platform; the time needed to allow for the platform to raise and stay up ( $T_1$ ), and the time necessary for the platform to descend and stay down ( $T_2$ ) can be adjusted easily. Other parameters are adjustable (See Materials and Methods).

The system is pre-sterilized and has been designed to allow for medium introduction, inoculation, and sampling. Table I presents different sizes.

### Description of the Slug Bubble Bioreactor

The Slug Bubble (SB) bioreactor is shown diagrammatically in Figure 2. It consists of a vertical flexible plastic cylinder filled with medium up to circa 80% of its height. Agitation and aeration are achieved through the intermittent generation of large cylindrical single bubbles at the bottom of the system that rise to the top of the cylinder (see Materials and Methods). These bubbles are comparable to “Taylor bubbles,” or “slug bubbles” (Davies and Taylor, 1950; Nicklin et al., 1962; Sousa et al., 2005), that is bubbles

**Table I.** WU bioreactor volumes and dimensions.

Total volume ( $V_t$ )	Length ( $L$ )	Width ( $W$ )	$L/W$	Working volume ( $V_w$ )	Filling level (%)	Specific area ( $L \times W/V_w$ )
$L$	cm	cm	—	$L$	%	$\text{cm}^2/L$
60	175	35	5	20	33	300
				30	50	200
200	280	55	5	70	33	220
				100	50	150
750	390	75	5	250	33	120

currently encountered in a two-phase flow known as slug flow. Taylor bubbles can be described as long bullet-shaped bubbles, which nearly occupy the entire cross-section of a pipe. Between the bubble and the pipe walls flows a thin film of liquid; the bubble moves upward at nearly constant speed while the liquid flows downward as a falling film. The nose of the slug is a very stable region; on the contrary, the rear of the Taylor bubble is a region characterized by strong mixing,

where all transfer processes are enhanced. Mixing and oxygen transfer are therefore achieved at the same time.

A few examples of bubble columns under a slug flow regime have been described (Kantarci et al., 2005; Kurz, 1971); in all these cases, the gas velocity and the column diameter are such that the air flow has naturally turned from a homogeneous bubbly flow to a slug flow. Slug flow generally appears for high gas velocity (0.10 m/s) in small diameter column (less than 15 cm). In the present apparatus the slug flow regime is artificially produced by intermittent gas supply, using a solenoid valve and compressed air: the valve relieves a pre-determined quantity of air at the given frequency. The quantity of air can be adjusted by changing the inlet pressure ( $P$ ), the valve opening duration ( $T_1$ ), or the bubble frequency ( $f$ ). The usual inlet pressure is from 0.03 to 0.05 MPa for 10 to 70 L (working volume) reactor. The corresponding averaged flow rates vary between 0.1 and 0.5 vvm, which is consistent with values usually encountered with plant cell culture (Misawa, 1994).

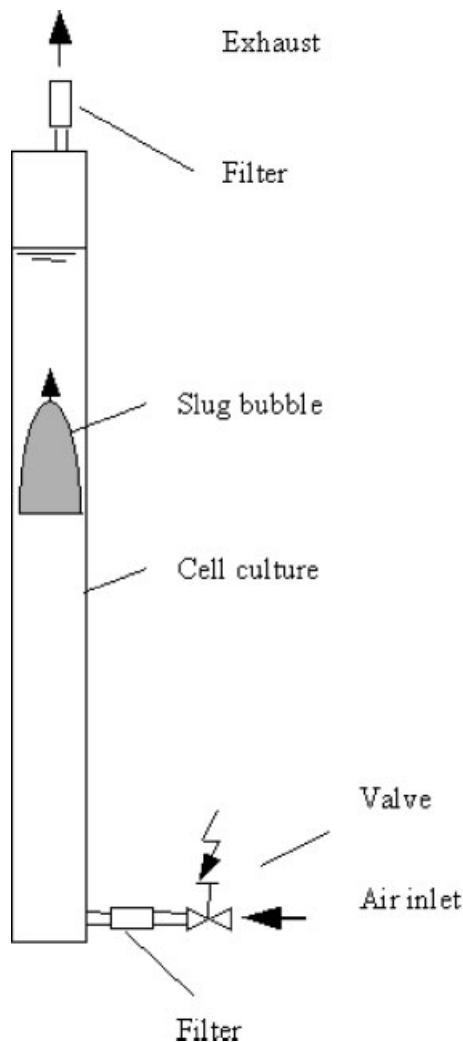
The system is pre-sterilized and has been designed to allow for medium introduction, inoculation, and sampling. A commercial rigid plastic tube (PVC) maintains the reactor vertical. A slot is cut up lengthwise (3–7 cm width) for the crossing of different inlets and outlets and the observation of the culture. Temperature control can be achieved by using a heating jacket inside the tube or in air-conditioned room. Table II presents different sizes.

## Materials and Methods

### Plant Material

#### *Soya Cell Strain*

A collection of cell strains of *Glycine max* (L.) Merr. was initiated in 1975 from one cultivar (Maple Arrow) and cultivated in Gamborg medium (Gamborg et al., 1968) supplemented with 30 g/L sucrose and 1 mg/L 2, 4-dichlorophenoxyacetic acid. pH is adjusted to 6.0 prior autoclaving (30 min at 115°C). The cell strain presently used (namely 13406) is subcultured in 250 mL Erlenmeyer flask (3 g/L fresh weight [FW] with 100 mL medium) every 2 weeks (Federici et al., 2003). The Erlenmeyer flasks are placed on a gyratory shaker (New Brunswick Scientific, Edison, NJ) at 100 rpm (shaking diameter 20 mm), at 25°C in darkness.

**Figure 2.** Schematic diagram of the SB bioreactor.

**Table II.** SB bioreactor volumes and dimensions.

Total volume	Working volume	Diameter ( <i>D</i> )	Floor surface	Height	Un aerated suspension height ( <i>H</i> )	Aspect ratio ( <i>H/D</i> )
<i>L</i>	<i>L</i>	cm	cm <sup>2</sup>	cm	cm	—
14	10	8.5	57	250	175	21
24	20	11	95	250	210	19
64	50	18	255	250	200	10
90	70	18	255	350	280	15

### Tobacco Cell Strain

*Nicotiana tabacum* L BY2 cell strain (Nagata, 2004) is currently grown in 250 mL Erlenmeyer flasks containing 100 mL medium on an orbital shaker (100 rpm) at 25°C in darkness and subcultured every 2 weeks at an initial density of 30 g/L FW (Verdelhan des Molles et al., 1999). The culture medium contains the standard MS components (Murashige and Skoog, 1962) with KH<sub>2</sub>PO<sub>4</sub> (270 mg/L instead of 170 mg/L), 0.2 mg/L of 2,4-dichlorophenoxyacetic acid and 30 g/L of sucrose, at pH 5.8. The medium is autoclaved for 20 min at 115°C.

**Culture in stirred-tank bioreactor.** A 14 L bioreactor (New Brunswick Scientific) equipped with a pitched blade impeller is used with the same medium and conditions of temperature and pH as mentioned above. The bioreactor containing 9 L of fresh medium is autoclaved for 40 min at 121°C. Fourteen day-old soy cells or seven day-old tobacco cells are harvested from two 1 L Erlenmeyer flasks (2 × 500 mL suspension) and are aseptically transferred to the bioreactor for inoculation. The stirrer speed is adjusted at 100 rpm. Dissolved oxygen (DO) is maintained at 30% by increasing or decreasing airflow rate. The bioreactor is equipped with a sterilizable oxygen probe (InPro 6110, Ingold Mettler Toledo GmbH, Greifensee, Switzerland), and a mass flowmeter.

**Culture in WU bioreactor (10, 20, 30, and 100 L working volumes).** The system is made of biopharmaceutical grade flexible PVC (Achilles, Everett, WA) and autoclaved (Fedegari, Albuzzano, Italy) for 40 min at 121°C. The culture medium is introduced through a 0.22 μm filter (Sartobran, Sartorius AG, Goettingen, Germany) into the system. For inoculation, 14 day-old soya cells or 7 day-old tobacco cells are aseptically transferred from Erlenmeyer flasks or from the stirred-tank bioreactor to the WU bioreactor via a sterile container. The inoculum was prepared in order to reach circa 30 g/L FW in the inoculated bioreactor. Inlet air is sterilized through membrane air filter (Sartofluor, Sartorius AG). The agitation intensity is adjusted depending on the batch volume and on the oxygen demand of the cell strain. Several parameters can be modified: the percentage of culture volume located on and lifted by the platform(s) (*v*), the platform raising angle (*α*), the rising and descending durations (*T*<sub>1</sub> and *T*<sub>2</sub>) and the air inlet flow rate (*Q*). When the bag is equipped with a sterilizable oxygen probe (InPro 6110, Ingold Mettler

Toledo GmbH, Switzerland or D100, Bradley James Corporation, Irvine, CA), the DO concentration is monitored using a recorder (Fuji Electric Instruments Co., Ltd, Tokyo, Japan). The room temperature is controlled at 25°C by air conditioner.

**Culture in slug bubble reactor (10, 20, 50, and 70 L working volumes).** The system is made from biopharmaceutical grade polyethylene (CPL613; Charter Medical, Lydall Group, Winston-Salem, NC) and gamma-sterilized (Ionisos, Sablé-sur-Sarthe, France) or produced and pre-sterilized by Charter Medical using the same flexible plastic film. The culture medium is introduced through a 0.22 μm filter (Sartobran, Sartorius AG) into the reactor. For inoculation, the same procedure described for the WU bioreactor cell cultures is applied, with 7 day-old tobacco cells and 14 day-old soya cells. Agitation is achieved by aeration with compressed air sterilized through membrane air filter (Sartofluor, Sartorius AG). The aeration intensity [inlet pressure and valve (Asco Joucomatic SA, Rueil-Malmaison, France) opening duration and frequency (interval between two openings)] is defined according to the batch volume thanks to the programming device (Merlin Gérin, Rueil-Malmaison, France). When the bag is equipped with a sterilizable oxygen probe (InPro 6110, Ingold Mettler Toledo GmbH, Switzerland or D100, Bradley James Corporation), the DO concentration is recorded (Fuji Electric Instruments Co., Ltd). The room temperature is controlled at 25°C by air conditioner.

### Growth Measurements

Samples of the cell suspension are harvested regularly from flasks, stirred-tank bioreactor, and new cell culture system. FW is determined after filtration (nylon mesh 37 μm) and dry weight (DW) is determined after drying an aliquot of filtered cells (1 g FW) in an oven at 110°C overnight. The apparent growth rate (*μ*) is calculated as:

$$\mu = \frac{\ln(\text{final DW}/\text{initial DW})}{\Delta t}$$

during exponential growth phase.

Cell doubling time (*td*) is related to apparent growth rate by the expression: *td* = ln2/*μ*



The apparent conversion rate is calculated as:

$$Y_{X/S} = \frac{\text{max DW} - \text{initial DW}}{\text{sugar}_{\text{initial}} - \text{sugar}_{\text{max DW}}} \times 100$$

### Determination of Residual Sugar Concentration in Medium

The filtrate obtained after filtration of the culture (nylon mesh) is filtered again through a 0.45  $\mu\text{m}$  membrane filter before a 1,000-fold dilution in water. The analysis of sugars is performed by high performance anionic exchange chromatography (HPAE) using a Dionex machine (Sunnyvale, CA) equipped with a PA1 column (250 cm  $\times$  4.3 mm, Macherey-Nagel, Düren, Germany). Following injection of 20  $\mu\text{L}$  of sample, sugars were eluted isocratically at 1.5 mL/min in NaOH (40 mM) using pulsed amperometric detection (PAD) detector at 258 nm, scanning between 220 and 400 nm for peak identification. The analyses were conducted at room temperature. Standards (glucose, fructose, sucrose) were purchased from Sigma (St. Louis, MO). Retention times were 4.2, 5.1, and 8.3 for glucose, fructose, and sucrose, respectively.

### Determination of Volumetric Oxygen Mass Transfer Coefficient

The  $k_La$  of the Erlenmeyer flask, stirred-tank bioreactor, WU and SB systems were measured in duplicate by the dynamic gassing-out (air) method using a polarographic, temperature-compensated, DO probe. The liquid (water) in the system is deoxygenated by gassing nitrogen through the inlet filter; when the DO probe (Ingold) reached 0% saturation, aeration, and agitation were started at the conditions mentioned above and the rising DO is recorded as a function of time. The value of  $k_La$  is defined as:

$$\frac{dC}{(C^* - C)} = k_La \cdot dt$$

where  $C$ : oxygen concentration in the liquid at time  $t$  (mg/L)  
 $C^*$ : oxygen solubility in the liquid (mg/L).

For the Erlenmeyer flasks, the same procedure has been used, the only difference being that the  $\text{O}_2$  probe is a Clark oxygen electrode immersed in a chamber system, through which the liquid from the Erlenmeyer flask circulates along a closed circuit thanks to a peristaltic pump. This system avoids the perturbations provoked by the direct immersion of the probe in the Erlenmeyer flask.

### Extraction and Analysis of Isoflavones

Freeze-dried soya cell cultures (0.2 g) were ground and packed into a 15 mL plastic column. Ten milliliters of ethanol/water (80/10, v/v) were added to each sample. After 2 h, the mixture is centrifuged (centrifuge 3K18C,

Sigma-Aldrich) and the extract obtained is filtered through a 0.45  $\mu\text{m}$  membrane filter. Analysis of isoflavones is performed as previously described by HPLC (Hewlett Packard 1090) using a Nucleosil 100- $\text{C}_{18}$  column (250 cm  $\times$  4.3 mm, Macherey-Nagel, Germany) and solvent gradient (Federici et al., 2003). Analyses were monitored with a photodiode array detector at 258 nm, scanning between 220 and 400 nm for peak identification. Controls were daidzin, genistin, daidzein, genistein (Extrasynthèse, Genay, France), malonyldaidzin and malonylgenistin (purified from soybean extracts). The authenticity of the different isoflavones has previously been shown through HPLC-MS analyses (Federici et al., 2003).

### Statistical Analysis

An ANOVA test and a multiple range test (Newman-Keuls) (Statbox 6.5) were used to analyze data on growth.

## Results and Discussion

### Volumetric Gas-Liquid mass Transfer Coefficient

Oxygen mass transfer coefficients measured in the WU bioreactor (Table III) were lower than those observed in a traditional stirred-tank bioreactor, but comparable to or higher than those encountered in Erlenmeyer flasks or other known flexible disposable cell culture systems such as the Wave Bioreactor (Eibl and Eibl, 2006; Singh, 1999).

Table IV shows that, for a given working volume, four parameters can be used to modulate oxygen transfer capabilities of the WU bioreactor; the most influential factor is the percentage of volume located on the rising platform. It should be stressed that this factor cannot be modified during the culture and therefore must be chosen prior to the culture, especially if the cell line is highly oxygen demanding; the three remaining parameters (platform rising angle, airflow rate, platform rising ( $T_1$ ) and descending ( $T_2$ ) durations) have a less significant influence than the previous factor and can be easily modified at any time during the culture.

The Slug Bubble bioreactor showed good oxygen transfer capacities. Oxygen transfer coefficients (Table III) were comparable to coefficients for traditional culture systems such as Erlenmeyer flasks and traditional bioreactors in the range of agitation and flow rates compatible with the oxygen demand and low shear stress required for plant cell cultures. As it might have been expected, these results also show that increasing the valve opening time (bubble size) or the opening frequency (bubble frequency), both leading to the increase of the average gas flow rate, resulted in higher  $k_La$  values. More surprisingly, Figure 3 shows that, for a given flow rate, the volumetric mass transfer coefficient is higher for the 50 L bioreactor than for the 20 L bioreactor. This result suggests that mass transfer capabilities are strongly

**Table III.**  $k_{L,a}$  (volumetric mass transfer coefficient).

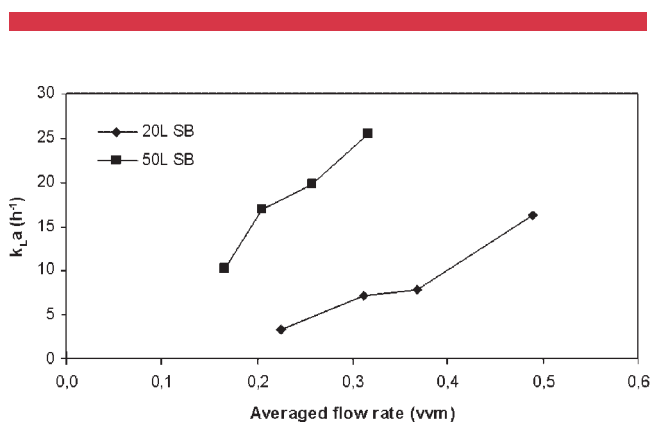
	Working volume	Agitation/aeration				$k_{L,a}$ ( $h^{-1}$ )
250 mL Erlenmeyer flasks	100 mL	100 rpm				5
14 L stirred-tank bioreactor	10 L	0.04 vvm; 150 rpm				3
		0.25 vvm; 200 rpm				15
		0.5 vvm; 100 rpm				29
		$\alpha$	$\nu$	Q	vvm	
60 L WU	20 L	15	30	2.5	0.12	10
60 L WU	30 L	15	30	2.5	0.18	9
		P	T1	T2	vvm	
24 L SB	20 L	0.04	0.5	7.0	0.31	7
			0.5	4.0	0.49	16
64 L SB	50 L	0.05	0.3	7.0	0.17	10
			0.5	7.0	0.21	17

$\alpha$ , Platform raising angle ( $^{\circ}$ );  $\nu$ , percentage of culture volume located on and lifted by the platform (%); Q, airflow rate (L/min); vvm, airflow rate (vvm); P, air inlet pressure (MPa); T1, valve opening duration (s); T2, time interval between two successive valve openings (s).

**Table IV.** Influence of different parameters on the  $k_{L,a}$  (volumetric mass transfer coefficient) in the WU bioreactor.

	Reference	Influence of									
		$\nu$	$\alpha$	T1 and T2		Q	$V_w$				
$\nu$	%	30	20	40							
$\alpha$	$^{\circ}$	15		10	20						
T1	s	1			1	2					
T2	s	1.5			1	2					
Q	L/min	2.5					1	5			
$V_w$	L	20						30			
$k_{L,a}$	$h^{-1}$	10.1	6.4	12.9	8.6	10.9	8.0	7.5	9.4	11	8.9

$\nu$ , Percentage of culture volume located on and lifted by the platform (%); T1, platform raising duration (s); T2, platform descending duration (s);  $\alpha$ , platform raising angle ( $^{\circ}$ ); Q, airflow rate (L/min);  $V_w$ , working volume (L).

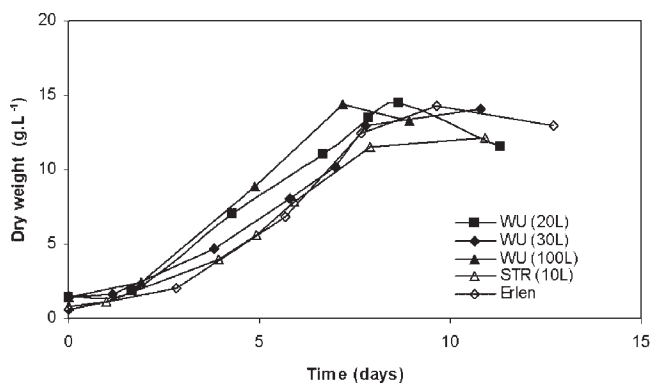
**Figure 3.** Volumetric oxygen transfer coefficient versus averaged flow rate in 20 and 50 L (working volumes) SB bioreactors.

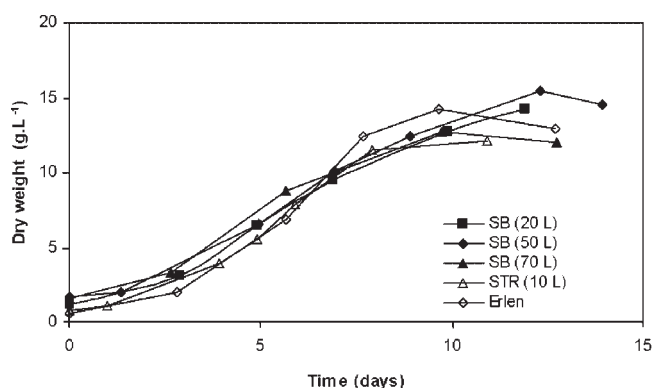
dependant on the column diameter. This point will be discussed later (scale up and volume increase).

Finally, it has been verified that the probe position does not affect  $k_{L,a}$  values: two probes located 50 cm away from the bottom of the column, and 50 cm away from the unaerated liquid at the top of the column, have shown similar oxygen transfer coefficients (difference inferior to 5% for six successive measurements on a 20 L working volume column).

### Tobacco Cell Culture

Figures 4 and 5 give an example of the growth kinetics currently obtained in the different culture systems. Tobacco cells were cultivated in four different volumes in the WU bioreactor and in the SB bioreactor. The results obtained were similar to those observed in traditional systems (Erlenmeyer flasks and bioreactor). This was confirmed using data collected from several independent experiments

**Figure 4.** Growth of tobacco cell cultures in Erlenmeyer flasks, stirred-tank bioreactor, and WU bioreactor.



**Figure 5.** Growth of tobacco cell cultures in Erlenmeyer flasks, stirred-tank bioreactor, and SB bioreactor.

(Table V). These results clearly establish that cultivation in WU bioreactor with a 100 L working volume can be used instead of a traditional stainless-steel stirred-tank bioreactor. For the SB bioreactor, cultivation up to 70 L working volume has been demonstrated.

Interestingly, 70 L SB bioreactor cultures are growing as well as the others except in the latest phase of growth (Table V and Fig. 3): they show the lowest maximum dry matter (12.9 g/L), which can be correlated to the lowest biomass yield based on the consumption of sugars (38.8%). Therefore, the culture conditions do not seem to be optimal at the end of the culture cycle. Nevertheless statistical analysis of the maximum DWs shows no significant difference for all the systems described in Table V, except at 10 L scale, where DW values are slightly higher.

### Soya Cell Culture and Isoflavone Production

In a previous publication (Federici et al., 2003), we have shown that it is possible to select high-producing isoflavones soya cell strains from a collection of 25 year-old strains. In

the present report, the ability to grow this soya cell strain in traditional bioreactor or WU and SB bioreactors has been demonstrated using batch cultures (see Table VI). The results are different from those obtained with tobacco cells: the stirred-tank bioreactor is the less efficient culture system (only 30% sugar conversion rate). Soya cell suspensions are much more aggregated (clump formation) than tobacco suspension. This result could be due to a higher shear stress sensitivity of the cells due to mechanical impeller agitation. The cultivation in WU and SB bioreactors is similar to cultivation in Erlenmeyer flasks. No differences were found between the different systems through variance analysis.

### Isoflavones Content

A large variability is observed between experiments, whatever the culture system. Most of the standard deviations were rather large, showing that an optimal control of the culture conditions for the production of isoflavones in large volumes is not reached in any culture system (Table VII). Federici et al. (2003) have shown that the production of isoflavones in Erlenmeyer flasks is highly linked to the physiological state of the cell culture, with an accumulation at the end of the growth phase but before the stationary growth phase. The same pattern was observed with soya cell cultures in both WU and SB bioreactors; as an example, Figure 6 shows isoflavone production of a 30 L (working volume) soya cell culture in a WU bioreactor. The difficulty in precisely controlling the production phase might be the reason for the observed variability in isoflavone content in all the culture systems. New culture systems are not detrimental to the production of isoflavones, but further investigation is required to identify the key parameters linked to the biosynthesis and accumulation of isoflavones.

### Scale Up and Volume Increase

#### Scale Up and Volume Increase for the WU Bioreactor

In the present study, we first tried to maintain the WU system geometrically similar: constant bag length over bag

**Table V.** Growth parameters of tobacco cell cultures in Erlenmeyer flasks, 10 L stirred-tank bioreactor, WU and SB bioreactors.

Type of system (working volume)	Number of independent experiments	Max dry weight (g/L)	Apparent growth rate ( $\text{h}^{-1}$ )	Doubling time (days)	Apparent conversion rate (%)
Erlenmeyer flask <sup>a</sup>	3	13.9 ± 0.5	0.34 ± 0.07	2.1 ± 0.4	44.5 ± 1.5
STR (10 L) <sup>b</sup>	3	14.4 ± 2.1	0.38 ± 0.07	1.9 ± 0.3	50.0 ± 1.8
WU (10 L)	2	13.6 ± 0.0	0.31 ± 0.03	2.3 ± 0.1	41.5 ± 1.0
WU (20 L)	4	12.8 ± 1.9	0.31 ± 0.05	2.3 ± 0.4	42.8 ± 4.4
WU (30 L)	3	12.6 ± 1.3	0.34 ± 0.03	2.0 ± 0.2	44.8 ± 2.1
WU (100 L)	5	13.0 ± 1.1	0.31 ± 0.05	2.3 ± 0.3	41.2 ± 5.0
SB (10 L)	2	17.2 ± 0.6	0.34 ± 0.3	2.0 ± 0.2	53.5 ± 1.7
SB (20 L)	5	13.7 ± 0.6	0.34 ± 0.03	2.1 ± 0.2	46.1 ± 2.8
SB (50 L)	3	14.2 ± 1.2	0.36 ± 0.07	2.0 ± 0.4	48.8 ± 2.7
SB (70 L)	2	12.9 ± 0.3	0.29 ± 0.01	2.4 ± 0.1	38.8 ± 2.3

<sup>a</sup>Erlenmeyer flask: 250 with 100 mL medium, 100 rpm on a gyratory shaker, 26°C.

<sup>b</sup>10 L stirred-tank bioreactor: New Brunswick bioreactor, 100 rpm, 0.25–0.5 vvm, 26°C.

**Table VI.** Growth parameters of soya cell cultures in Erlenmeyer flasks, 10 L stirred-tank bioreactor, WU and SB bioreactors.

Type of system (working volume)	Number of experiments	Max dry weight (g/L)	Apparent growth rate (h <sup>-1</sup> )	Doubling time (days)	Apparent conversion rate (%)
Erlenmeyer flask <sup>a</sup>	3	14.6 ± 0.4	0.25 ± 0.05	2.8 ± 0.6	40.8 ± 5.0
STR (10 L) <sup>b</sup>	3	12.9 ± 4.7	0.22 ± 0.08	3.5 ± 1.5	30.0 ± 4.9
WU (10 L)	1	14.3	0.28	2.5	48.2
WU (20 L)	5	13.8 ± 1.8	0.22 ± 0.04	3.2 ± 0.7	44.0 ± 9.0
WU (30 L)	2	16.5 ± 0.5	0.29 ± 0.02	2.4 ± 0.2	49.9 ± 3.9
WU (100 L)	2	15.5 ± 0.1	0.32 ± 0.01	2.2 ± 0	55.6 ± 8.6
SB (20 L)	6	13.9 ± 0.9	0.25 ± 0.03	2.8 ± 0.3	45.3 ± 5.4
SB (50 L)	3	14.7 ± 2.0	0.27 ± 0.06	2.7 ± 0.5	46.8 ± 4.9

<sup>a</sup>Erlenmeyer flask: 250 with 100 mL medium, 100 rpm on a gyratory shaker, 26°C.

<sup>b</sup>10 L stirred-tank bioreactor: New Brunswick bioreactor, 100 rpm, 0.25–0.5 vvm, 26°C.

width ratio (here this ratio is equal to 5) and ensure an efficient bag utilization, that is keeping the proportion of the volume occupied by the suspension (filling level) between 33 and 50%. This second objective was set up to try to keep the system compact and avoid designing very large bags and therefore large tables. This approach has one major drawback: it leads to a decrease of the specific area (that is the bag area over working volume ratio) or to an increase of the culture medium height (when the system is not under agitation). The minimum specific area, below which the system may not be viable, has to be determined.

For large culture volumes, the system could be adapted to enable the inoculum and the batch to be grown in the same bag. Some preliminary trials have been performed partitioning a WU bioreactor during the first part of the culture at about 3/5th of its length; the bag structure (other than its length) and location are unchanged (same width, same port location); the surface area is reduced to grow the inoculum; the system goes back to its initial shape when medium is added to grow the entire batch. This promising technique would reduce the risks of contamination through successive inoculations and decrease scale up time. Nevertheless, as several parameters (specific area, headspace volume, filling level, etc.) are different from one phase to the other, the influence of these differences as well as hydrodynamic parameters in the first phase of the culture, such as  $k_{La}$ , have

to be studied more in-depth to validate this improvement. Figure 7 shows the successful growth of a tobacco cell culture obtained in two successive steps (10 and 100 L working volumes) in the same bioreactor.

#### Scale Up and Volume Increase for SB Bioreactor

SB bioreactors (10, 20, and 50 L working volumes) have first been designed as 2.5 m high columns (with unaerated liquid height close to 2 m). Column diameters have subsequently been chosen to fit that criterion. This leads to quite a large range of aspect ratios (from 10 to 20), but did not prevent all systems from growing comparable and viable cultures. However, the column diameter is a significant parameter in slug flow. In the case of gas slugs rising through stagnant liquids, slug formation is dependant on wall shear forces and becomes more difficult for large diameter columns. Slug bubble velocity is also highly dependant on diameter (it increases with the square root diameter) (Nicklin et al., 1962). Finally, the flow pattern in the wake of a slug, which is likely to directly affect mixing, also depends on the column diameter as well as other parameters such as the liquid properties (viscosity and density) (Campos and Guedes de Carvalho, 1988). This information suggests that the bioreactor characteristics may have to be carefully studied for each column diameter. They may also account for the

**Table VII.** Isoflavone production.

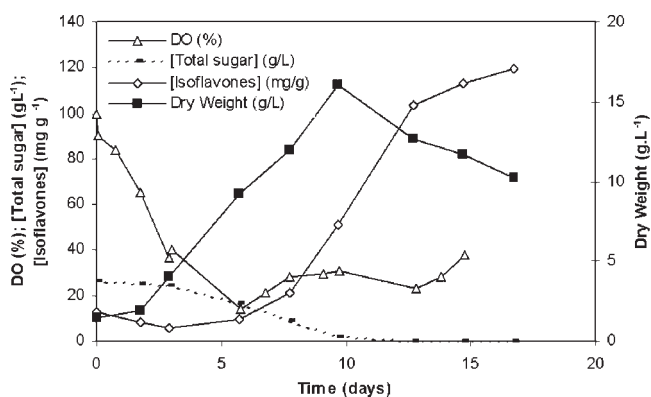
Type of system (working volume)	Number of experiments	Max. isoflavone concentration (mg/g DW)
Erlenmeyer flask <sup>a</sup>	6	61 ± 35
Stirred-tank bioreactor (10 L) <sup>b</sup>	3	28 ± 20
WU (10 L)	1	17
WU (20 L)	5	39 ± 39
WU (30 L)	1	120
WU (100 L)	1	ND <sup>c</sup>
SB (20 L)	6	23 ± 11
SB (50 L)	3	48 ± 34

<sup>a</sup>Erlenmeyer flask: 250 with 100 mL medium, 100 rpm on a gyratory shaker, 26°C.

<sup>b</sup>10 L stirred-tank bioreactor: New Brunswick bioreactor, 100 rpm, 0.25–0.5 vvm, 26°C.

<sup>c</sup>Not determined.





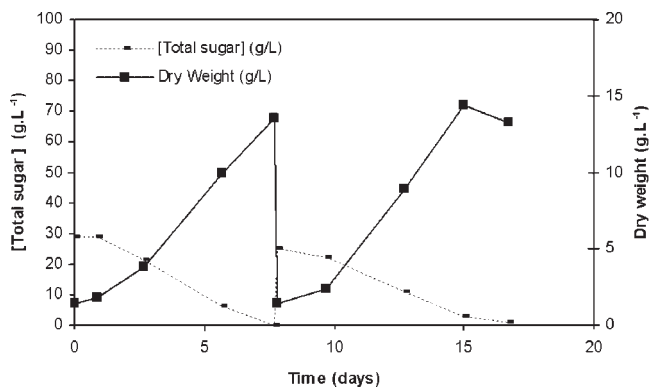
**Figure 6.** Growth of soya cell culture, dissolved oxygen (DO) evolution, sugar concentration, and isoflavone production in a 30 L (working volume) WU bioreactor.

different oxygen transfer capacities of the 20 and 50 L working volume SB bioreactors at a given flow rate. Subsequent bioreactors (50 and 100 L scales) will be designed in order to maintain a constant aspect ratio ( $H/D = 20$ ), which is going to give a maximum volume for a given column diameter.

Finally it can be noted that running multiple experiments in parallel or increasing production volumes can be rapidly achieved having several culture systems close together (or even connected) in a small area.

### High Cell Density and Shear Rate

Relatively high oxygen mass transfer obtained in relation to the aeration rate (vvm) suggests that the SB bioreactor might be used for cultures with high cell density, which is an issue



**Figure 7.** Growth of tobacco cell culture in two successive steps (10 and 100 L working volumes) in a 200 L WU bioreactor.

for plant cell cultures as well as animal cells, requiring high aeration rates.

Nevertheless, possible shear stress is an issue that needs to be addressed to improve our understanding of the system. However, the actual mechanism of shear damage to plant cells is not clear. Studies with animal cells (Handa-Corrigan et al., 1989; Wu and Goosen, 1995) have indicated that cell death in sparged systems is mainly due to interaction of cell with bursting bubble at the air-liquid interface. It is also suggested that smaller bubbles, more efficient for oxygen transfer, were also more detrimental to cells than larger bubbles. Cherry and Hulle (1992) have given an estimation of shear stresses observed after bursting of very small bubbles: 200–300 N/m<sup>2</sup>. If this is also applicable to plant cells, this could be an advantage of the SB bioreactor compared to traditional bubble columns. James et al. (2004) described a less violent phenomenon during slug burst: upon arrival at the surface, the slug nose flattens out as liquid drains rapidly to form a membrane. This membrane quickly collapses leading to a small pressure step (less than 10 N/m<sup>2</sup> for a 40 mm diameter column). However, rapid deceleration of the annular film back to stationary after slug burst may generate a pressure increase. Further studies are needed to better understand the mechanism involved. In the case of the WU bioreactor, the absence of air sparging should eliminate damage due to gas disengagement while the absence of mechanical mixer eliminates zones of high shear in comparison with stirred-tank bioreactors. Again, further studies are needed to ensure that the constant flow of liquid at the wall of the vessel is not detrimental to the cells, whatever the culture conditions and volumes.

### Conclusions

Several plant cell species have been successfully cultivated in two new flexible plastic-based disposable bioreactors: *Nicotiana tabacum*, *Glycine max* described in the present article as well as *Pilocarpus sp.* cell cultures (data not shown). Both systems rely on very different mechanisms to provide oxygen and ensure proper mixing and culture homogeneity. They are operated through a very simple apparatus: an air piston and timer for the WU bioreactor, valve and timer for the SB reactor. The systems being disposable, they are easy to operate and do not require time for cleaning, sterilization, or maintenance. As a bubble column, the SB bioreactor can provide two other benefits: it is very compact, and mechanically robust and therefore easy to operate aseptically.

Due to their simplicity (low air pressure requirement, no mechanical apparatus needed for the agitation, no cleaning or maintenance operations), both culture systems described in the present article lead to the development of efficient, low cost cell culture systems applicable at small to medium scale and complementary to traditional stainless-steel bioreactors.

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