

## MASS TRANSFER STUDIES ON *CYNARA CARDUNCULUS* & *CENTAUREA CALCITRAPA* SUSPENSION CULTURES. THE INFLUENCE OF OXYGEN-VECTORS.

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### SUMMARY:

Volumetric oxygen mass transfer coefficients  $-k_{La}$  were measured at several agitation-aeration conditions in a 2-l stirred bioreactor, to study the effect of hydrodynamic stress on plant cell suspension cultures of *Cynara cardunculus* (cardoon) and *Centaurea calcitrapa*. Cellular oxygen yields were correlated with mixing, aeration and cell metabolism. High oxygen transfer ( $k_{La} = 14.00 \text{ h}^{-1}$ ) inhibited *C. cardunculus* L. cell growth, while a lower  $k_{La}$  value ( $6.07 \text{ h}^{-1}$ ) led to the best productivity ( $0.496 \text{ g D.W. l}^{-1} \text{ day}^{-1}$ ).

Initial  $k_{La}$  presented linear correlations with aeration and agitation and decreased with cell density in both cell cultures.

One of the main bottlenecks of the aerobic fermentations is the low accessibility of the oxygen in the broth. Butyric acid was successfully used as oxygen-vector to improve oxygen transfer rates during bioreactors experiments, in a batch system. It was demonstrated that the use of 1.85 % of butyric acid enabled a two fold increase of the  $k_{La}$  coefficient. The use of 3.75 % (v/v) of butyric acid did not evidenced a better oxygen mass transfer.

**Key words:** Plant cell suspension; mass transfer;  $k_{La}$ ; oxygen-vectors *Cynara cardunculus*.

## INTRODUCTION

Mass cultivation of plant cells in a fermenter depends on bioreactor type and size, and process parameters. Stirred (Drapeau *et al.*, 1986; Scraag *et al.*, 1987; van Gulik, 1989, 1992; Schlattmann, J.E., 1995), magnetic (Hegarty *et al.*, 1986) and air-lift fermenters (Alfermann *et al.* 1985) have been used for biomass and metabolites production. Stirred fermenters have shown advantages for high plant cell densities (Ulbricht *et al.* 1985; Tanaka *et al.* 1981) but the cell damage caused by hydrodynamic stress can be a limitation of this type of bioreactors. Nevertheless, Leckie *et al.* (1991), and van Gulik (1993) could obtain successfully *C. roseus* growth at high agitation speeds (300-1000 r.p.m.).

Process parameters, such as, initial inoculum, metabolic stage of the cells and nutrient effects have been mainly studied in shake flasks. Oxygen transfer, mixing and shear, which have been reported for cultures in bioreactors (Bader *et al.*, 1987; Allan *et al.* 1988; Meijer *et al.* 1989), are also important technological parameters for plant cell production.

Cell suspension culture growth of *Cynara cardunculus* has been previously reported in shake flasks and continuous culture (Lima Costa *et al.*, 1987, 1996). The present work correlates the oxygen mass transfer of *C. cardunculus* and *C. calcitrapa* cell cultures grown in a 2-l stirred fermenter, at different hydrodynamic conditions, and discuss the sensitivity of this cell culture type to shear stress.

Aeration is one of the limitations of conventional technology when operating aerobic fermentation. Limitations of oxygen transfer in fermentation can be solved using additive compounds (oxygen-vectors) with a higher oxygen solubility than water (Rols and Goma, 1989).

## MATERIAL AND METHODS

### Materials

The oxygen-vectors used were from Riedel-de Haen.

## Cell Growth and Production Medium

Cell suspension cultures of *Cynara cardunculus* were initiated by adding 2 g wet weight of friable *calli* to 100 ml of Gamborg B5 medium (Gamborg et al., 1968), supplemented with 2% sucrose, 1 mg/ml 2,4 D and 0,1 mg/ml benzyladenine. The cultures were maintained in 500 ml erlenmeyer flasks, at 25° C in orbital shaker (150 r.p.m.) for a 16 h photoperiod and subcultured weekly, by inoculating the cell suspension into fresh medium .

Fermentation runs were performed in a 2-l mechanical stirred fermenter, at several aeration-agitation parameters (30, 60 and 100 r.p.m.; 100, 200 and 400 cm<sup>3</sup>/ml) at 25° C with an inoculation ratio of 20%, initial pH 5.5 and an air-flow between 0.1 and 0.4 v.v.m.

### Plant Bioreactor characteristics:

Total volume	2.0 l
Working volume	1.5 l
Height	20.0 cm
Liquid height	13.0 cm
Diameter	12.0 cm

The bioreactor is equipped with a double pitched blade turbine of 6.0 cm diameter. Each blade has 1.9 cm width and 7.0 cm length.

### Analytical Methods

Fresh weight (F.W.), dry weight (D.W.) cell number/ml, respiratory rate, phenol accumulation were measured and used to quantify cell growth.

**Fresh weight** was determined after placing 10 ml of well mixed suspension culture into preweighed centrifuge tubes. The samples were centrifuged during 10 min at 4500 r.p.m. After removing the supernatant, the fresh weight was obtained. **Dry weight** was determined after drying the cells for 24 h at 80° C.

**Phenol content** was quantified in supernatant, by a direct spectrophotometric method at  $\lambda=270$  nm. (Anselmo *et al.*,1985).

An amperometric Ingold oxygen electrode was used to measure the **dissolved oxygen** in the culture medium.

The initial volumetric oxygen transfer coefficient **-k<sub>L</sub>a-** in the fermenter was determined by the dynamic gassing out method (Leckie *et al.*,1990).

The addition of the oxygen-vectors compounds (sterilized apart by filtration) to the fermentation medium occurred before the culture became limited by the oxygen supply, at the end of exponential phase. The dispersion was produced by mechanical agitation at 90 rpm for 10 s.

**Cell number** was determined as previously described (Lima Costa *et al.*,1987).

**Cell viability** was determined using fluorescein diacetate as described by Larkin, 1976.

### **Oxygen yields**

The **true biomass yield**  $Y'_{x/O_2}$  and maintenance coefficient for oxygen ( $m_{O_2}$ ) were determined, based on the Pirt model (1975) without product formation.

**Cellular oxygen yield** was obtained according to Wilson (1967). The total mass of oxygen consumed per unit of work volume, during a period of no aeration, can be calculated using a trapezoidal integration approach. The apparent oxygen yield was determined from the slope of biomass concentration curve against total oxygen consumed.

## RESULTS AND DISCUSSION

To study the influence of oxygen transfer in suspension cultures of *Cynara cardunculus* and *Centaurea calcitrapa* fermentation runs were performed at several agitation-aeration parameters (30, 60 and 100 r.p.m.- 0.1, 0.2 and 0.4 v.v.m.) expressed by the volumetric oxygen mass transfer coefficient  $-K_La$ . Low values of agitation were chosen to prevent a shear stress effect on cells, as reported by Berlin *et al.* (1985) (60-80 r.p.m.) for a potato cell fermentation with a six- flat bladed turbine impeller and by Pareilleux and Vinas (1983) for *Catharantus roseus* cells fermentation with a double pitched blade turbine. According to Leckie *et al.* (1991), that used high agitation speeds 100-700 r.p.m. with *C. roseus* cell culture, they could obtain an increase in the fresh and dry weight biomass.

### Effect of $K_La$ on *Cynara cardunculus* growth

The main growth parameters are shown in Table1. The best results for biomass production were obtained for  $k_La$  values between 6.07-7.40  $h^{-1}$  respectively with  $m_g = 0.59 \text{ day}^{-1}$  and  $\mu_g = 0,98 \text{ day}^{-1}$  (Fig.1), and the best biomass productivity was obtained for  $k_La=6.07 \text{ h}^{-1}$ . For apple cell culture 4L fermentation, it was reported, by Pareilleux and Chaubet (1980) optimum  $k_La$  value of 7  $h^{-1}$ .

Tab.1-Growth parameters of *Cynara cardunculus* L. cell suspension fermentation for several initial  $K_La$  values.

$k_La$ ( $h^{-1}$ )	Initial cell.Nº. $10^4$ /ml	Maximum cell Nº. $10^4$ /ml	Maximum Mass Inc F.W. (g/l)	Productivity (g D.W./ $l^{-1}day^{-1}$ )	$m_g$ ( $day^{-1}$ )	Maximum. O <sub>2</sub> uptake rate (nmoles $min^{-1}g^{-1}$ D.W.)
6,07	7,12	12,80	52,6	0,496	0,590	203,5
6,50	4,71	11,22	53,9	0,319	0,420	117,5
7,40	4,82	13,00	57,6	0,343	0,980	220,5
9,44	7,58	18,20	92,0	0,318	0,340	204,8
10,70	9,58	13,51	52,8	0,32	0,259	105,0
12,57	7,68	16,2	20,7	0,096	0,100	-
14,00	13,37	11,96	26,3	0,0-	0	-
15,52	12,47	12,47	0.0	0,0	0	-
24,00	10,11	10,20	0.0	0.0	0	-

For the stirred fermenter used in this work, there is cell growth inhibition ( $\mu_g = 0$ ) for initial  $k_La$  values higher than  $12.6 \text{ h}^{-1}$  (Table 1), which suggests that *C. cardunculus* cells are shear sensitive. It was also reported by Tanaka *et al.* (1981), that cell suspensions cultures of *Cudrania tricuspidata*, which have high sensitivity to hydrodynamic stress show a growth decrease for high  $k_La$  values.

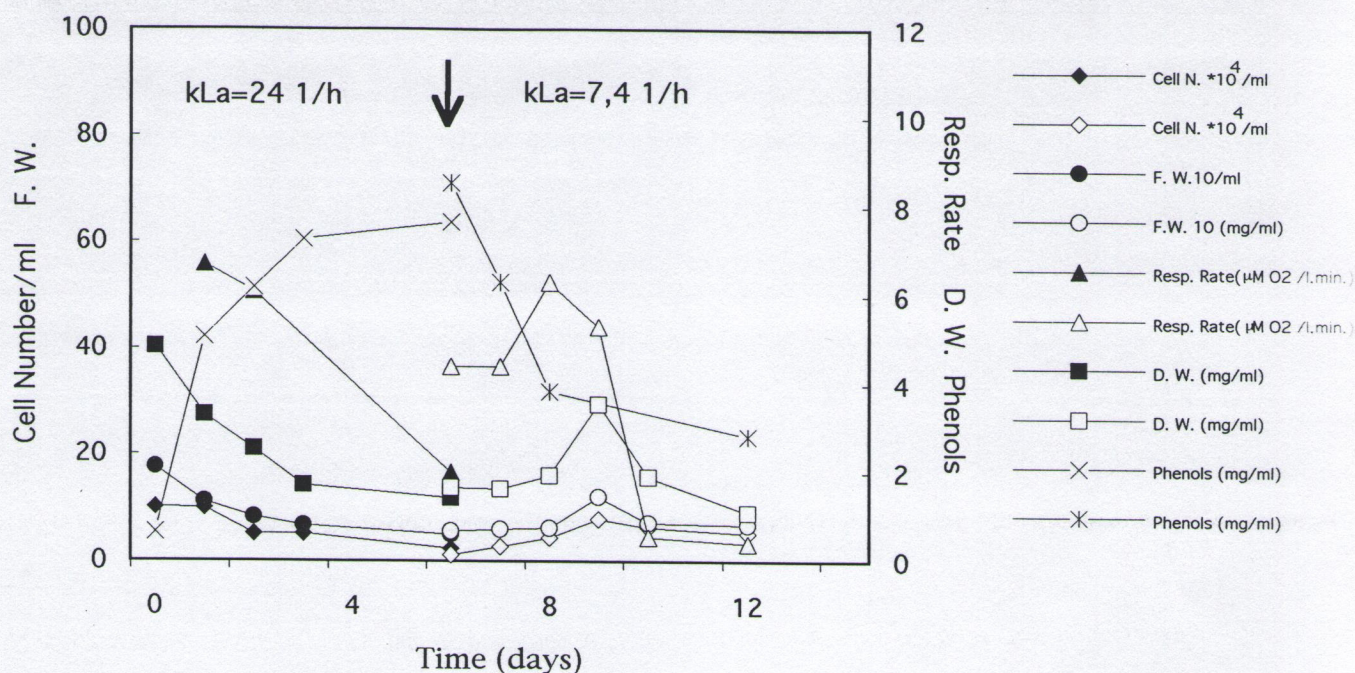


Fig. 1 - Cell suspension growth of *Cynara cardunculus* L. in a batch fermenter at an initial  $k_La = 24.0 \text{ h}^{-1}$ . The arrow means change of oxygen volumetric mass transfer conditions to  $7.4 \text{ h}^{-1}$ .

In batch fermentations performed at high initial  $k_La - 24 \text{ h}^{-1}$  (100 r.p.m.; 0.308 v.v.m.), cell growth was not observed and productivity was zero (Fig 1). After 6 days, only 24% of the initial cell concentration survived. Meanwhile, phenols were accumulated, which suggests hard stress conditions. On the 6<sup>th</sup> day of fermentation, the aeration rate was decreased from 0.308 v.v.m. to 0.077 v.v.m. and the stirred rate was maintained constant. Under these new conditions the surviving cells could recuperate the growth, with a very high specific growth rate ( $\mu_g = 0.93 \text{ day}^{-1}$ ) and an increasing on the cell viability (75%). According to Pareilleux and Chaubet (1981) the absence of cell growth for 0.03 v.v.m., was probably due to the loss of  $\text{CO}_2$  in the fermenter .

These results could be confirmed by another fermentation run at a lower initial  $k_{La} = 14 \text{ h}^{-1}$  (30 r.p.m.; 0.308 v.v.m.) (Fig. 2). After 6 days fermentation running, the specific growth rate was approximately zero. When aeration rate was decreased to 0.308 v.v.m. to 0.077 v.v.m. in the same fermentation, cell growth could be recovered, with a specific growth rate of  $0.98 \text{ day}^{-1}$ , and high viability (80 %), suggesting that the decreasing on the hydrodynamic stress influences positively the cell growth. A very deep decrease in oxygen uptake rate was also observed, meaning a lower oxygen transfer (Fig. 2). This may be a consequence of broth viscosity (14 cp). In fact the oxygen transfer time for high cell densities of *C. cardunculus* is 6000 s and is much higher than the oxygen consumption time ( $t_{o/c} = \text{oxygen saturation} / \text{maximum respiratory rate}$ ) - 1315.8 s -, calculated for the 10<sup>th</sup> day of fermentation. These results show that, in this phase of cell growth, there exists an oxygen limitation in the cell metabolism. It has also been demonstrated (Rols and Goma, 1991; Leckie *et al.*, 1991) that shear rate is affected by medium viscosity.

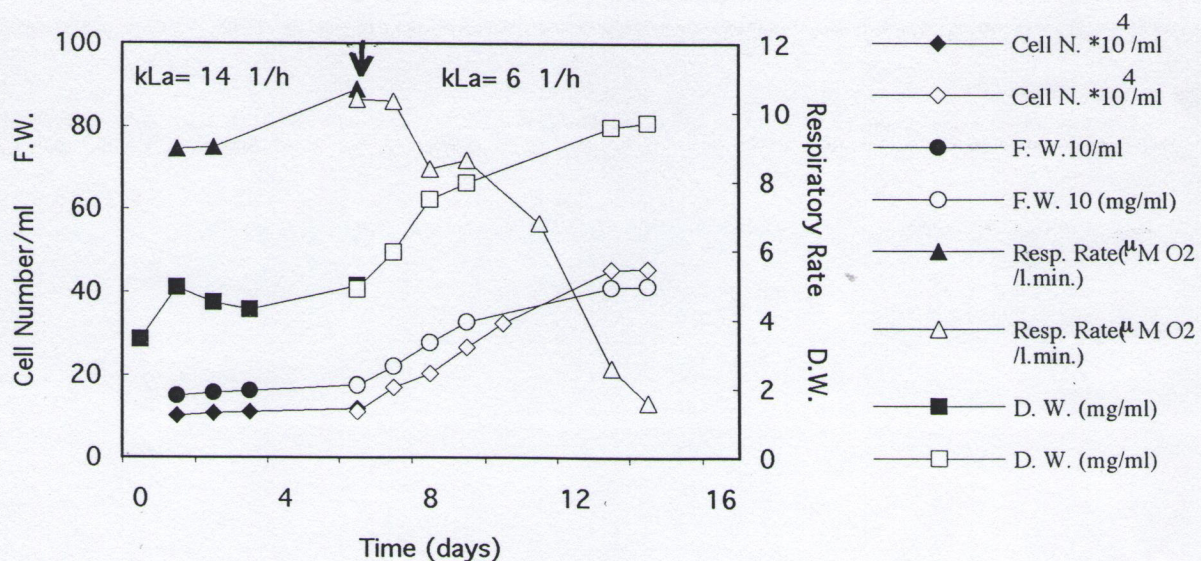


Fig. 2 - Cell suspension growth of *Cynara cardunculus* L. in a batch fermenter at an initial  $k_{La} = 14.0 \text{ h}^{-1}$ . The arrow means change of oxygen volumetric mass transfer conditions to  $6.1 \text{ h}^{-1}$

The batch assays performed for *C. cardunculus* cell culture at high  $k_{La}$  values suggest that the growth inhibition can be attributed to hydrodynamic stress, oxygen excess or deficiency on carbon dioxide.

In order to clarify these hypothesis, fermentation runs were made at constant  $k_La=14h^{-1}$ , but changing aeration and agitation parameters from 30 r.p.m. / 0.308 v.v.m. to 135 r.p.m. / 0.166 v.v.m. on the 6<sup>th</sup> day fermentation. Although the decrease on aeration and subsequently increase on  $CO_2$ , *C. cardunculus* growth could not be observed during the batch process, indicating that the hydrodynamic stress is the dominant reason for the observed inhibition cell growth.

F.W./D.W. ratio was followed during 20 days fermentation, in which agitation parameters were changed from 30-60-100 r.p.m. Fig.3 shows the profile of F.W./D.W. increasing during cell growth for different agitation speeds, which suggests higher water content, hence bigger cell volume. Similar results were obtained by Snape *et al.* (1989) for *Catharantus roseus* cell fermentation. In addition, Dong-II Kim *et al.* (1991) found that the ratio F.W./D.W. increases during *Thalictrum rugosum* cell growth in shake flasks.

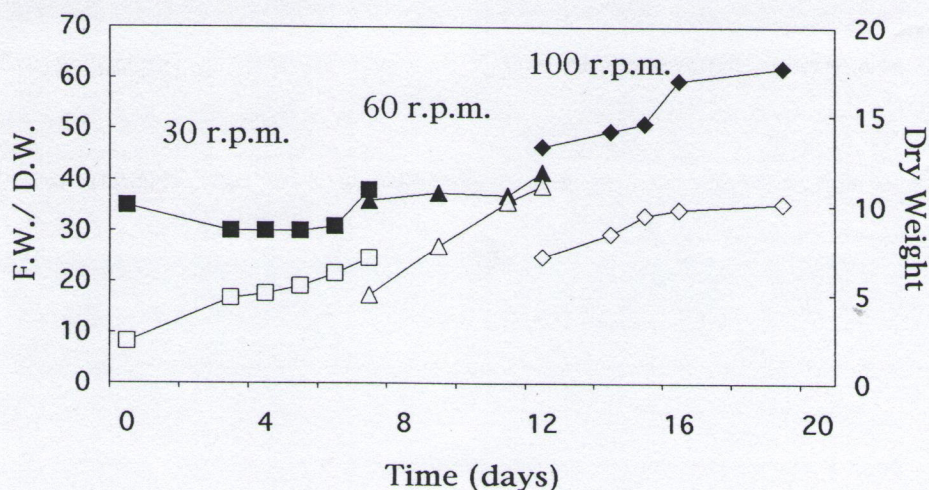


Fig. 3 - The fresh weight / dry weight for *C. cardunculus* cell culture at different mixing conditions.

To assess the correlation between oxygen consumption and initial  $k_La$  values, oxygen cellular yields were measured for different mass transfer conditions (Fig.4). The cellular oxygen yield highly decreased with increase  $k_La$ . This result agrees with the growth inhibition observed for high  $k_La$  values (Table 1). The highest oxygen yield (2.8 g D.W./g  $O_2$ ) obtained for *Cynara cardunculus* cells corresponds to the highest cell growth productivity, which suggests that the carbon source was primarily used for growth and a very low percentage for cell maintenance.

Values of 0.84 g D.W./g O<sub>2</sub> were reported for *Daucus carota* suspension cultures at low specific growth rate (0.28 day<sup>-1</sup>) (Wilson,1987). *Cynara cardunculus* cells growing at lower specific growth rate (0.1 day<sup>-1</sup>), presented values of oxygen yield of 0.2 g D.W./g O<sub>2</sub>.

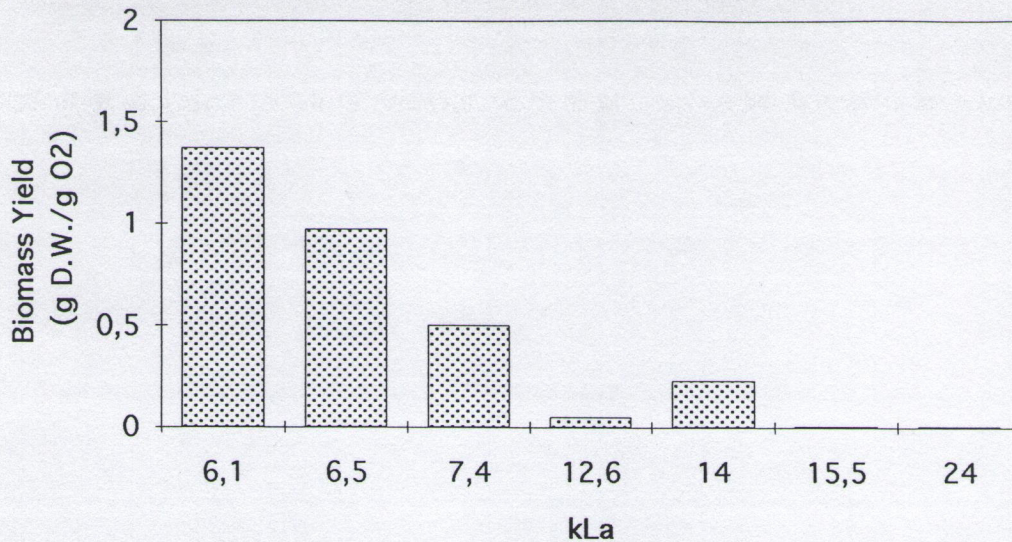


Fig. 4 - Influence of the oxygen volumetric mass transfer on the biomass yield determined as a function of oxygen consumption.

According to the Pirt model (1965) the true oxygen yield, for *C. cardunculus* suspended cells, was determined for different specific growth rates -  $Y'_{x/O_2} = 0.78$  g D.W./g O<sub>2</sub> -. It was found, in literature, 1.41 g D.W./g O<sub>2</sub> and 0.375 g D.W./g O<sub>2</sub> for *Catharantus roseus* cells in a 10 L fermentation (Drapeau *et al.*, 1986), suggesting that biomass yields depends on plant cell culture species and its growth conditions.

For *C. cardunculus* fermentations, grown at different cell concentrations, it was verified that volumetric mass oxygen transfer coefficient  $-k_La-$  decreases with the increasing in cell density, attaining extremely low values (1-2 h<sup>-1</sup>) which can be justified by the high viscosity values (16cp) found in the broth at the end of the stationary growth phase.

#### **Effect of oxygen-vectors on *Centaurea calcitrapa* growth**

In order to solve the critical problem of oxygen limitation for high densities cell cultures, experiments was done with several oxygen-vectors, soybean oil, n-dodecanol and butyric acid.

In the study performed in bioreactor without cells, the initial value of  $k_{La}$  increased at about 2.5 fold and approximately 3 fold by adding 1.85 % (v/v) and 3.85 % (v/v) of butyric acid (Table 2) respectively. It was observed that initial  $k_{La}$  varies linearly with aeration and agitation (Fig. 5). Identical aeration correlation was reported by Leckie *et al.* (1991) and Lima Costa (1994).

Table 2 - Values of  $k_{La}$  in a culture medium without cells, in the absence of butyric acid (\*) and in the presence of 1.85% (v/v) (\*\*) and 3.85% (v/v) (\*\*\*) of butyric acid for different aeration/agitation conditions.

Agitation (r.p.m.)	0.1 v.v.m.*	0.1 v.v.m.**	0.1v.v.m.***	0.4v.v.m.*	0.4v.v.m.**	0.4v.v.m.***
30	2.42	5.54	6.02	6.00	9.69	16.6
60	2.90	7.43	9.31	8.68	14.9	18.9
100	3.36	12.7	13.1	11.3	19.9	21.1

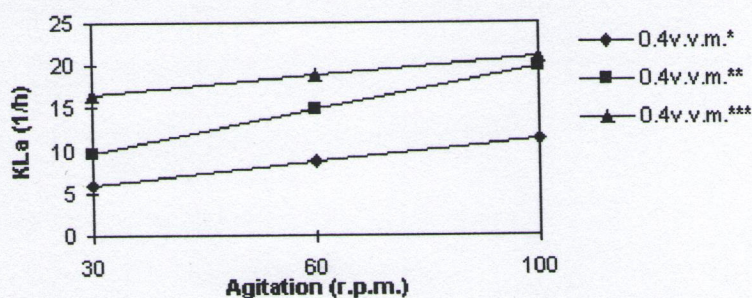
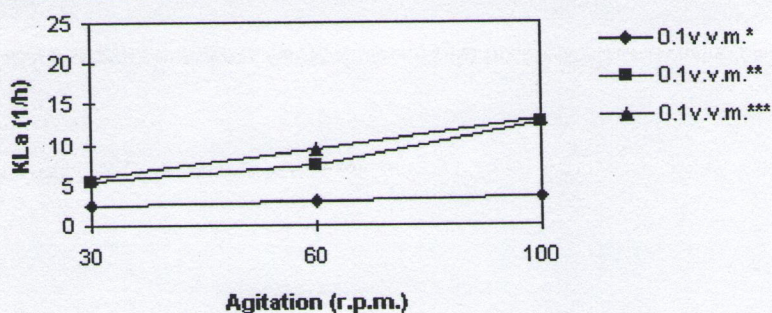


Fig. 5 - Variation of  $k_{La}$  with agitation for 0.1 and 0.4 v.v.m. of aeration, in a medium without cells. (\*) without butyric acid; (\*\*) with 1.85 % (v/v) of butyric acid; (\*\*\*) with 3.85 % (v/v) of butyric acid.

*Centaurea calcitrapa* suspended cell cultures fermentation were performed in the presence of butyric acid dispersed by mechanical agitation. With the addition of 1.85% (v/v) of butyric acid it was also observed an increase of approximately 2 times in the value of  $K_L a$  coefficient (Table 3). In this case, the use of 3.75% (v/v) butyric acid did not evidenced a better oxygen mass transfer, suggesting a constant saturation for the oxygen-vector (Fig. 5 and 6).

Table 3 - Variation of  $k_L a$  at stationary phase of *Centaurea calcitrapa* cell culture fermentation in the absence of butyric acid (\*) and in the presence of 1.85% (v/v) (\*\*) and 3.85% (v/v) (\*\*\*) of butyric acid for different aeration/agitation conditions.

Agitation (r.p.m.)	0.1 v.v.m.*	0.1 v.v.m.**	0.1v.v.m.***	0.4v.v.m.*	0.4v.v.m.**	0.4v.v.m.***
30	1.52	3.34	3.86	6.14	9.34	14.7
60	2.35	4.35	6.00	6.22	12.4	15.7
100	2.84	9.77	9.49	9.10	17.0	17.9

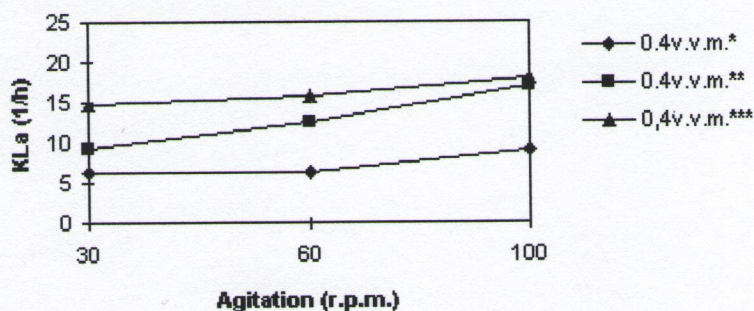
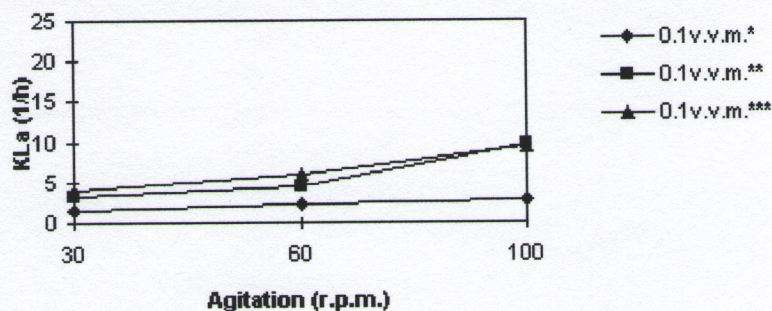


Fig. 6 - Variation of  $k_L a$  with agitation for 0.1 and 0.4 v.v.m. of aeration, in a *Centaurea calcitrapa* cell culture. (\*) without butyric acid; (\*\*) with 1.85 % (v/v) of butyric acid; (\*\*\*) with 3.85 % (v/v) of butyric acid.

The behaviour of these compounds is not yet well understood, but it is related with the spread coefficient of acid droplets in the gas-liquid interface (Rols and Goma, 1990). Oxygen solubility in the butyric acid is higher than in water what provokes an enhancement of the volumetric oxygen transfer coefficient.

Soybean oil and n-dodecanol were also tested as oxygen-vectors but evidenced technical troubles for getting an homogenised mixture, due to vector emulsification. Butyric acid showed advantages in *Centaurea calcitrapa* cell culture fermentation because it was possible to get a rather homogenised broth.

## CONCLUSIONS

The present work shows that the ability of plant cells to grow in a bioreactor is related with hydrodynamic stress. The experiments using a small scale bioreactor, at several volumetric oxygen mass transfer coefficient  $-k_{La}$ -, showed that for high  $k_{La}$  values, low cellular oxygen yields were obtained and cell growth could not be observed. Therefore, respiratory rate revealed to be an accurate and quick growth parameter compared with fresh weight or dry weight.

The F.W./D.W. ratio, for growing cells, increases for higher agitation speeds, indicating an increase in water content and bigger cell volumes for higher stress hydrodynamic.

These results suggest that *C. cardunculus* cells are shear sensitive as shown for *Cinchona robusta* and *T. divaricata* (Meijer et al. 1989). In contrast, *Nicotiana tabacum* and *Catharantus roseus* present cell wall and cell membrane shear resistance (Screag et al. 1991 and Meijer et al. 1989 ).

By the addition of butyric acid, there was a notable increase in the value of  $K_{La}$  coefficient for the two essays, in the presence and absence of cells. Butyric acid evidenced to be a

effective oxygen-vector when compared with other compounds reviewed by Rols and Goma (1989). one of the advantages of butyric acid is its solubility in the broth, without emulsion formation.

### References

1. Allan E.J., Scraag A.H., Pugh, K. (1988) *J. Plant Physiol.* ; 132:176-183.
2. Alfermann A.W., Spieler H., Reinhard E. in Primary and Secondary Metabolism of Plant Cell Cultures (Deus-Neuman B., Barz W. and Reinhard E., eds.) Springer-Verlag, Berlin, 1985, pp. 315-322.
3. Anselmo A.M., M. Mateus, J.M.S. Cabral, J. M. Novais. (1985) *Biotech. Letters* 7 (12) : 889-894.
4. Bader F.G. (1987) *Biotechnol. Bioeng.* 30: 37-51.
5. Berlin J., Wray V., Forche E., Reng H-G, Schuler H., Luckinger H. and Muhlbach. (1985) *J. of Exp. Bot.* 36(173):1985-1995.
6. Dong-II Kim, G. Heon Cho, H. Pedersen, Chee- Kok Chin (1991) *Appl. Microb. Biotechnol.* 34 :726-729.
7. Drapeau D., Blanch H.W., Wilke C.R. (1986) *Biotechnol. Bioeng.* 28:1555-1563.
8. Ducos J. P., A. Pareilleux (1986) . *Appl. Microb. Biotechnol.* 25 :101-105.
9. Leckie F., Scraag A.H. and Cliffe K. C. (1991) *Biotechnol. Bioeng.* 37:364-370.
10. Esquível, G. (1987) M. Sc. Thesis, Faculdade de Ciências de Lisboa -Universidade de Lisboa-
11. Gamborg O.L., A. Miller, K. Ojima (1968) Nutrient Requirements of Suspension Cultures of Soybean Root Cells. *Exp. Cell Research* 50 : 151-154.
12. Kato A., S. Nagai (1979). *Eur. J. Appl. Microb. Biotechnol.* 7:219-225.
13. Kato A., Y. Shimizu, S. Nagai (1975) *J. Ferm. Technol.* 53 (10) :744-751.

14. Hegarthy, P. K., N. J. Smart, A.H. Scraag, M.W. Fowler.(1986) *J. of Exp. Bot.* 37 (185):1911-1920.
15. Larkin, P., (1976) *Planta* 128: 213 - 216.
16. Leckie F. , A.H. Scraag, K.C. Cliffe (1991). *Enzyme Microb. Technol.* 13:296-305.
17. Lima Costa E., Novais J., Pais M.S., Cabral J.M.(1987), Proceedings of A.S.I. on Plant Cell Biotechnology.
18. Lima Costa, E. ,Ph D (1994 ), Universidade do Algarve
19. Lima Costa, E., van Gulik, W., ten Hoopen H.J.G., Pais, M.S., Cabral, J.S. (1996) *Enzyme and Microbial Technology* 19: 493-500.
20. Meijer,J.J. ,Ph D Thesis (1989) , Biotechnology Delft Leiden, BDI Project Group Plant Cell Biotechnology.
20. Pareilleux A., N. Chaubet (1980). *Biotechnol. Letters* 2 :291-296.
21. Pirt S.J. (1975) The principles of microbe and cell cultivation. Ed. Blackwell Scientific , Oxford.
22. Robertson G.H. , L.R. Doyle, P. Sheng, A.E. Pavlath, N. Goodman. (1989) *Biotechnol. Bioeng.* 34 :1114-1125
23. Rols J.L., G. Goma (1991), *Biotechnol. Letters* 13 : 7-12.
24. Schiel O., Berlin J. *Plant Cell Tissue Org. Cultures* (1987) ; 8: 153-161.
25. Snape J.B., N.H. Thomas (1989), *Biotechnol Bioeng.* 34: 1058-1062.
26. Scraag A.H., Morris P., Allan E.J. , Bond P., Fowler M.W.(1987) *Enzyme Microb. Technol.* 9:619-624.
27. Tanaka H. (1981) *Biotechnol.Bioeng.* 23 :1203-1218.
28. Ulbrich, B., W.Wiesner, H. Arens(1985) in Primary and Secondary Metabolism of Plant Cell Cultures, Eds. Newman et al. (Springer-Verlag, Berlin, Heidelberg and New York ).
29. Gulig, W. van (1990) Ph D. Thesis. University of Delft.
30. Gulig, W. van ,M.J.J. Meijer, H.J. G. ten Hoopen, K. Ch. A.M. Luyben, K.R. Libbenga (1989). *Appl. Microb. Biotechnol.* 30:270-275.
31. Wilson P.D.G. (1987) *Biotechnol Tech* 1 : 151-156.