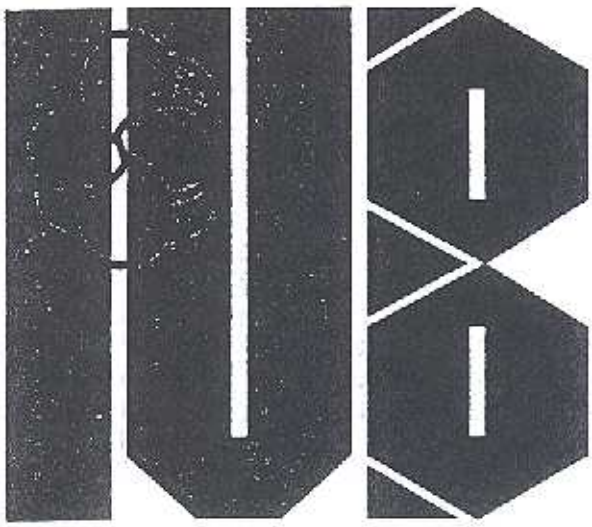

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PLANT CELL CULTURE AS A SOURCE OF PLANT PROTEASES

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SUMMARY

Cynara cardunculus cells, cultivated in bioreactors, present a specific growth rate $\mu g=0.435$ day-1 in batch conditions and a $\mu g=0.589$ day-1 in fed-batch conditions. These cells produce proteases with proteolytic activity 14 to 20 fold higher than those obtained in shake flasks. These proteases present clotting activity and can be used, free or imobilized as clotting enzymes. Suspended cells of C. cardunculus may constitute a good biomass source for production of plant proteases specially clotting enzymes.

INTRODUCTION

From more than two thousands of known enzymes, only about 1% are of industrial interest. Some industrial enzymes constitute enzymatic mixtures. The great majority of commercially used enzymes are presented in purified and normalized form. The first enzyme to be purified and commercialized was rennet.

Most of the enzymes with industrial interest are from microbial origin. Very few, such as rennet, trypsin and pepsin are from animal origin while ß-amylase, bromelain and papain are from plant origin.

The greater number of commercialized microbial enzymes may be due to the very high specific growth rate of microorganisms. However, some plant enzymes have to be produced either due to their specific characteristics or to the inability of microorganisms to produce their homologues.

From the plant enzymes with industrial interest, proteases appear as the most important due to their use either in food, pharmaceutical and detergent industries or in the preparation of leader and wool (1). Other enzymes from plant origin such as amylases, lipoxygenases and pectinases tend to be replaced by

their microbial homologues.

From the plant proteases with industrial use, the following may be considered by decrescent order of interest: PAPAIN (*Caryca papaya*), BROMELAIN (*Ananas comosus*) and FICIN (*Ficus glabra*). All these enzymes present cysteine and histidine as reactive center, a molecular weight ranging between 23000 and 26000, isoelectric point between 8.75 and 9.55, optimal pH 5.0-7.5 and critical temperature between 50 and 70 ° C. Other proteases (generally named as CYNARASES) are regionally important as clotting enzymes. Recently, three proteases (glycoproteins) have been isolated and partially characterized from the flowers of *Cynara cardunculus*. These proteins present proteolytic and clotting activity (2).

The use of "in vivo", spontaneous or cultivated, plants to obtain proteases present many disadvantages due to the heterogeneity of production from one region to the other, the seazonality of the production and other ambiental features. The establishment of plant cell cultures constitute a way to overcome some problems in the obtention of naturally occurring proteases with specific use. This report describes the interest of plant cell cultures in the production of proteases with clotting activity.

METHODS

Suspension cultures were initiated by shaking "calli" in TNO_3^- modified by Behrend and Mateles(3) and supplemented with kinetin 1mg l^{-1} and 2,4-D (2,4-Dichlorophenoxyacetic acid) (1mg l^{-1}) and in Gamborg B (4) medium supplemented with benzyladenine (0.1mg l^{-1}) and 2,4 D (1mg l^{-1}).

Incubation was performed in 500 ml Erlenmeyer flasks containing 100 ml - 200 ml medium.

Fermentations were performed in 1 l Gallencamp fermenter type (magnetic stirring) and 2 l Setric Genre fermenter, model SGI (mechanical stirring).

Optical density, cell dry weight, cell number and intracellular protein were determined as described before (5). Proteolytic activity was measured as previously described (5,6).

RESULTS AND DISCUSSION

In previous papers it has been reported the ability of *Cynara cardunculus* cells to grow and produce proteases in

erlenmeyer conditions either in Gamborg B_5 (5) or in TNO_3^- media (6). In both conditions, the specific growth rate depends on the inoculum density (Table 1).

TABLE 1

Comparison of doubling time of *C. carduncullus* cells in fonction of the culture medium and of the inoculum concentration. (B5 - Gamborg B_5 medium; TN TNO_3^- - Tulecke medium)

	INOCULUM CONCENTRATION (Cell n.º x 10^4/ ml)	DOUBLING TIME (td) (h)
B_5	6. 9 15. 6	144 33.6
TN	1.19 1.22 2.80	147.2 105.3 86.2

The best specific growth rate (µg) obtained for B_5 medium was 0.49 day-1 while for TNO_3^- was 0.193. This value of µg could not be improved probably due to the small concentration of nitrogen salts in this medium. When the innoculum densities used in erlenmeyer conditions are repeated in bioreactors conditions, an increase in the specific growth rate was observed in cultures performed in both media. These results suggest that specific growth rate of *C. carduncullus* is influenced by aeration (Table 2).

Table 2

Comparative growth of *C. carduncullus* cells on Gamborg B_5 medium (B5) and on TNO_3^- medium (Tulecke) (TN) in fonction of inoculum density and in batch and fed-batch conditions.

	CELL N.º (x $10.^4$)	BATCH (0.4 v. v. m)			CELL N.º (x $10.^4$)	FEB-BATCH (0.8 v.v.m)		
		µg (day-1)	td (h)	µg (prot.)		µg (day-1)	td (h)	µg (prot.)
B 5	3.30	0.435	38.2	0.149	6.18	0.589	28.2	0.055
TN	1.58	0.300	55.5	0.050	-----	------	-----	-----

In bioreactors conditions, values of µg= 0.38 day^{-1} were

obtained for *Catharanthus roseus* (7), μg = 0.21 day^{-1} for *Dioscorea deltoidea* (8) and μg = 0.53 day^{-1} for *Malus communis* (9).

Considering the values obtained in Gamborg B_5 medium (0.435 and 0.589 day^{-1} in batch and fed-batch respectively) and in TNO_3^- (0.30 day^{-1}), we can consider that B_5 medium is much more effective for biomass production than TNO_3^-. The greater content of nitrogen salts in B_5 medium may be responsible for the effectiveness of B_5 medium. This may indicate that growth in TNO_3^- is nitrogen limited. The role of nitrate in primary metabolism was previously described (10).

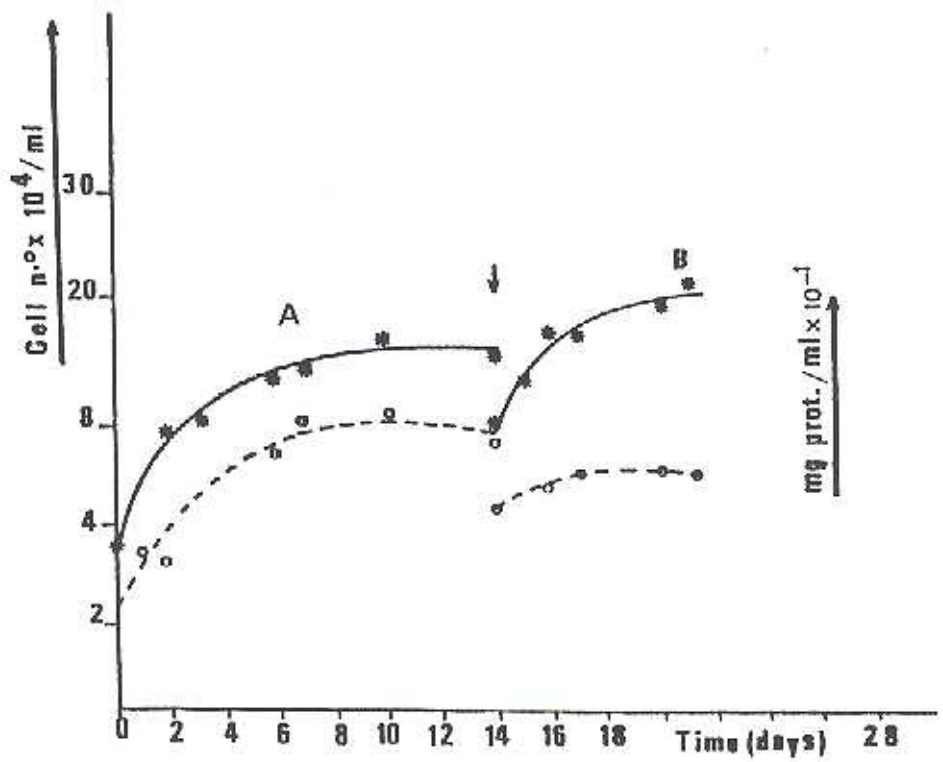

Fig. 1. Growth of *C. carduncullus* on Gamborg B_5 medium expressed in terms of cell no (*) and mg protein/ml$\times 10^{-1}$ (o). A (Batch system); B (Fed-batch system)

In batch fermentation on Gamborg B_5 medium, the cells of *C. carduncullus* present a specific growth rate μg=0.435 day^{-1} while in fed-batch system values of μg=0.589 day^{-1} were obtained (lable 2, fig. 1).

This value is 1.4 fold higher than that obtained for batch cultures in the same medium. The best μg obtained in fed-batch conditions may be correlated with the high inoculum concentration in spite of the strong O_2 limitation (Table 3).

TABLE 3

Oxygen consumption in batch and fed-batch conditions at aeration rates of 0.4 v.v.m and 0.8 v.v.m respectively.

TIME (d)	BATCH dO_2 (%)	FED-BATCH dO_2 (%)
0	93.2	18
1	69.3	10
3	60.5	13
6	49.5	17.5
7	45.0	----
9	37.5	----
12	78.0	----

Fed- batch fermentations were performed at 0.8 v.v.m. which allows, probably, higher O_2 transfer. Some authors have postulated that the increase of (Kl a) promotes the continuous decrease in biomass of *Cudrania tricuspidata* (11). With high density cell suspensions (biomass concentration higher than 2%) the O_2 transfer coefficient (Kl a) is small due to the deficient homogenization of the culture medium. In this case, controlled agitation and high aeration rate may be efficient (12) and overcome problems in O_2 transfer. This may be the case of *C. carduunculus* suspension cultures in fed-batch system that have been performed at high aeration rate (0.8 v.v.m) with a very high increase in biomass production.

The increase in biomass observed in fed-batch conditions , when compared with erlenmeyer flasks and batch fermenter conditions may be correlated with higher mass transfer due to the increase of aeration rate. Similar results have been reported for cell suspension cultures of *Medicago sativa* (8). According to these authors, biomass also increased with aeration rate.

The experiments in erlenmeyer flasks and those performed in bioreactors (batch or fed-batch conditions) allowed the production of proteases. This production, assayed by the correspondent proteolytic activity, revealed that these proteins are produced in the exponential phase (5).

The proteolytic activities of *C. cardunculus* cells cultivated in NO_3^{-1} in batch conditions (1.060 Δ D.O.g^{-1} h^{-1}) are about three fold higher than those obtained in erlenmeyer flasks. When batch fermentations are performed using Gamborg B medium and keeping all the other conditions constant, a sixteen fold increase of proteolytic activity was obtained when compared with the NO_3^{-1} batch cultures .

This value can be increased when fed-batch cultures in B are performed (Lima-Costa *et al.*, unpublished data).

The cell suspension cultures of *C. cardunculus* present clotting activity (fig.2) and can be used either free or immobilized for milk clotting and cheese making.

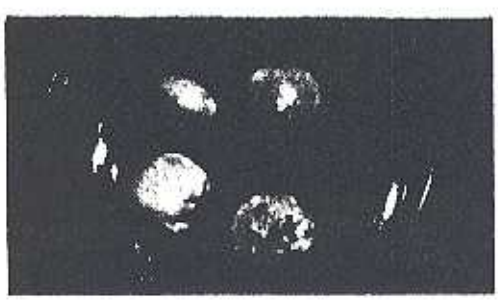

fig. 2, Aspect of small cheeses produced after milk clotting with free suspended cells.

When the clotting activity of the protein extract of both dried flowers of *C. cardunculus* and cultured cells are compared, it can be seen that cultured cells present a higher clotting activity than dried flowers. The clotting activity / proteolytic activity ratio is also higher in cultured cells than in dried flowers (Table 4).

TABLE 4

Clotting actvity (ζ .C) and ratio clotting activity / proteolytic activity determined for the protein extracts of dried flowers and cultured cells. (ζ = clotting time; C = ml of protein extract, biomass dry weight or , for purified enzymes, ng of units for enzymatic activity).

	CLOTTING ACTIVITY	CLOT. ACTIV. /PROT. ACTIV.
Dried flowers	0.085	17.2
Cultured cells	0.113	22.9

This lower clotting activity / proteolytic activity ratio observed in dried flowers extracts may be responsible for different organolleptic properties from cheese made using proteases either from flowers or from cultured cells.

The cultured cells can also be used in continuous milk clotting either free or immobilized in alginate or polyurethan foams. The experiments performed with cells entrapped in calcium alginate revealed that clotting time and residence time in the reactor are directly correlated with the plant cell concentration in the reactor (13). When the efficiency of *C. cardunculus* cells, measured in terms of residence time and of clotting time, is compared with the same parameters assayed for a purified enzyme such as "rennilase" (13) it could appear that *C. cardunculus* suspended cells are less effective. It is necessary to consider that the increased residence time and clotting time of immobilized cells must be due to the necessary permeabilization of the protease through the cell wall.

The experiments reported in this paper show that biomass production of *C. cardunculus* may constitute a good process to produce specific plant proteases.

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