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Fractal morphology of *Beta vulgaris* L. cell suspension culture permeabilized with Triton X-100®**

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A b s t r a c t. In this work, morphology of *Beta vulgaris* L. cells permeabilized with 0.7 mM of Triton X-100® was evaluated using digital image processing and concepts of fractal dimension (perimeter- area relations). Important morphometric changes were found when the contact-time with chemical agent was increased. The size of cells decreased, the cells lost the roundness and their shape was more sinuous; this behaviour was a result of a probable shrinkage caused by the excess of exposure with the permeabilization agent. Morphology of *B. vulgaris* cells after permeabilization, exhibited a fractal nature since the slope of the ratio of the logarithm of the perimeter vs logarithm of the area was higher than unit. Fractal geometry of the cell morphology was affected as a result of the exposure to Triton X-100®. Those changes can be attributed to the loss of turgor and structure of the cell wall.

K e y w o r d s: fractal dimension, morphometry, Triton X-100®, suspension cell culture

INTRODUCTION

The cell suspension culture is a useful tool for developing efficient metabolite production systems. It has numerous advantages, including more homogeneity and efficiency than traditional technologies for chemical, pharmaceutical and other commercial products. Product recovery is of crucial importance as downstream processing often accounts for 50 to 90% of the production costs (Rito-Palomares, 2008). Since many secondary metabolites are stored in vacuoles, it is necessary to improve the excretion of these substances into the culture medium, using a non-lethal method. Permeabilization with chemicals is one of the most useful methods to obtain secondary metabolites produced by plant cells cultures (Boitel-Conti *et al*., 1996). Initial trails were targeted in this sense; nevertheless, most of the treatments lead to loss of cell viability (Brodelius, 1988; Park and Martinez, 1992; Thimmaraju *et al*., 2004). During permeabilization, the evaluation of the overall process becomes relevant for numerous parameters such as, cell growth and viability, consumption of nutrients and production of the desired secondary metabolite as well as visual aspect of the culture and cell morphology.

The methodologies used to characterize cell morphology are subjective and require intensive labor (Ibaraki and Kenji, 2001). Recently, digital image processing (DIP) has been applied to cell cultures. This technique is simple, non-destructive or invasive and has a potential to automation. DIP analyzes the features of an object from visual data and allows visualization of living-cells to monitor their growth and production (Trejo-Tapia *et al*., 2007). In addition, DIP measures different morphometric characteristics and permits a three-dimensional reconstruction from serial section images. In fact, some applications of image processing to cell cultures have been reported (Pepin *et al.*, 2000; Rodríguez *et al*., 2004; Trejo-Tapia *et al.*, 2007). Most of these studies include size and shape factors of the cells in the culture. However, also is important to understand the effect of culture conditions on viability, changes in shapes and production of secondary metabolites as well as on cell morphology. This is often difficult to achieve since cells often make complicated and irregular shapes.

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Recently, fractal geometry has been attracting attention as a quantitative analytical method to characterize many kinds of irregular shapes in nature, when their areas are similar. The fractal concept, proposed by Mandelbrot in 1977, has been applied to analysis of irregular lines and surfaces observed in nature. Using this concept, the degree of irregularity can be estimated by a fractal dimension (Yokota *et al*., 1999). In this study, the degree of irregularity of red beetroot (*Beta vulgaris* L.) permeabilized cells was evaluated by means of the fractal concept using a digital image processing.

MATERIALS AND METHODS

Non-differentiated cells from the phenotype cellular line FRP (purple red) of *Beta vulgaris* L, 'Crosby Egyptian' variety were used as models for the present study. The culture and incubation conditions have been reported by Trejo-Tapia *et al.* (2007). Four grams of fresh cells were inoculated into flasks containing 50 ml of B5 culture medium (Gamborg *et al.*, 1968) added with 30 g l^{-1} of sucrose, 0.1 mg l^{-1} of 2,4 dichlorofenoxiacetic acid, and 0.01 mg l^{-1} of kinetin. Cultures were incubated at 25 ± 3°C, under a photo period of 16 light hours at an intensity of 4000 lx and constant orbital agitation of 100 min^{-1}.

FRP cells were permeabilized with Triton X-100® (Sigma) according to the method developed by Trejo-Tapia *et al.* (2007). Cell suspension cultures of 6 day old (exponential grow cells) were inoculated in 50 ml erlenmeyer-flasks contained 10 ml of culture media in a 10% ratio (w/v). The following conditions were tested: 10, 15, 30 and 60 min contact time at a concentration of 0.7 mM of Triton X-100®.

Photomicrographs (4X) of permeabilized cells were captured with a digital video camera (Dage MTI DC-330, Japan) using the Metamorph software (V 6.1, Universal Imaging Corporation, USA). The camera was coupled with a light microscope (Nikon Eclipse i80, Japan) and interfaced to a computer. All images were captured in RGB colour (640 x 480 pixels) and stored in tagged image file format (*.tif).

The resulting micrographs were processed according to the method proposed by Chanona *et al.* (2003). The Corel Photo Paint software (Corel Draw V11.0, Corel Corporation, USA) was used for this purpose. Firstly, images were transformed to a 256 gray scale of 8 bits (*.bpm). Then, intensity, brightness and contrast were adjusted in order to achieve a precise definition of the cellular perimeter (border). Afterwards, the silhouettes of cells were cut as individual objects and pasted into a new template of 640 x 480 pixels. In order to evaluate the morphometry of each individual cell, the delimited objects were filled with one colour and binarized. Fifteen micrographs were captured for each permeabilization treatment, and at least five objects were processed, resulting in a minimum of 75 for each treatment, as recommended by Martínez-López, *et al.* (2002). This method reduces typical error to less than 10%.

Individual cells were analyzed with the Sigma Scan Pro software (SPSS, 1999), and the following morphometrical parameters were evaluated:

- Feret diameter (Fer), which is the equivalent theoretical diameter of the largest longitude;
- perimeter (P), which are the pixels delimiting the object border;
- area (A);
- shape factor (Sf), given by $(4\pi)/(P^2)$; note than a perfect circle will have a Sf of 1;
- compactness factor (Cf), defined as P^2/A, where a circle formed by pixels is 4π (approximately 12.57); this factor gives useful information on the sinuosity of the perimeter, and therefore on the irregularity of an object (SPSS, 1999).

Fractal dimension (FD) was evaluated with the relationship P vs A, which has been previously used to measure FD in ice crystals (Hagiwara *et al.*, 2002). As reported by these authors, perimeter (P) and area (A) of cells may be related by the Potency law, as follows:

$$A \propto Pmax^{(2/FD)} \quad (1)$$

Morphometric data obtained from the analysis of the micrographs were used to draw logarithmic curves of P vs A, and FD was calculated from the value of the slope.

A one way ANOVA ($\alpha = 0.05$) was applied in order to evaluate the statistical differences for each permeabilization treatment; also standard deviation were calculated for a minimum of 75 objects (cells). This was made using the Sigma Stat software (V 3.0, SPSS Inc., USA).

RESULTS AND DISCUSSION

In general, the results indicate that permeabilized cells underwent relevant morphometric changes as compared to the non-permeabilized (blank) cells. The results tend to follow one change pattern from the probable least damaging conditions (10 min) to the most extreme (60 min). Table 1 shows the results of the different morphometric parameters related to the different permeabilization treatments. For example, the area experimented a significant decrease (more than 50%) when the cells were in contact with Triton X-100® for 60 min; while the reduction of area was only 8% when the cells were permeabilized for 10 min; the difference with the control was not significant. The perimeter also decreased, but the difference was significant until 30 min of exposure time to the permeabilizing agent. Nevertheless, it is possible to observe that the values of the measured perimeters in all cases were higher than the theoretical perimeters, as if the cells were round.

The change in size and, therefore in volume, may be a consequence of the excretion of betanins into the culture medium, due to an increase in permeability caused by a larger contact time with Triton X-100® (Trejo-Tapia *et al*., 2007). These changes in cell dimensions result in changes in the

T a b l e 1. Morphometric properties* of *B. vulgaris* permeabilized cells whit Triton X-100®

Contact time (min)	Area (μm^2)	Perimeter (μm)	Feret diameter (μm)	Shape factor	Compactness factor
0	3 497 ± 213a	229 ± 9.7a	59.3 ± 2.3a	0.81 ± 0.04a	15.9 ± 1.2a
10	3 195 ± 262a	224 ± 10.9a	59.1 ± 2.0a	0.77 ± 0.05b	17.2 ± 1.1a
15	2 900 ± 276b	219 ± 13.3a	56.9 ± 4.6b	0.76 ± 0.04b	18.2 ± 1.1b
30	2 598 ± 239b	204 ± 11.23b	53.4 ± 3.5b	0.70 ± 0.05b	20.1 ± 1.2b
60	1 761 ± 184b	193 ± 13.2b	51.0 ± 4.6b	0.59 ± 0.04b	23.7 ± 1.0b

*Results obtained with at least 75 replicates ± the standard deviation. The same letters in columns indicate that non-significant differences were obtained ($\alpha = 0.05$).

geometry of Sf and Cf, since both parameters depend on the ratio between perimeter (maximum and average) and area. Also, the shape of permeabilized cells showed relevant changes as compared to the blank. According to Sf values, cells tend to lose their rounded shapes when was in contact with Triton X-100® for longer times, while the value of Cf increased when the contact time with the detergent increased too. Both parameters are directly affected by the square value of the perimeter (maximum and average). Sf is only decreased as a function of perimeter, since the value of 4π is a constant. On the other hand, the increase in the area of an object may not affect the value of Cf if P is not substantially modified. The opposite takes place when the cellular border is very sinuous (SPSS, 1999).

The distribution of Sf is shown in Fig. 1. It was observed that over 70% of unprocessed cells (blank) had a Sf value between 0.8 and 0.9. When the contact time with Triton X-100® was increased, it was observed that the initial Sf mean value diminished. Also, the distribution of this parameter changed as a function of the contact time *eg* after 30 min approximately 55% of the cells had a Sf≅0.5 and only 3% presented a Sf above 0.8. These values indicate an important loss in the roundness of cells, which tend to adopt an irregular shape.

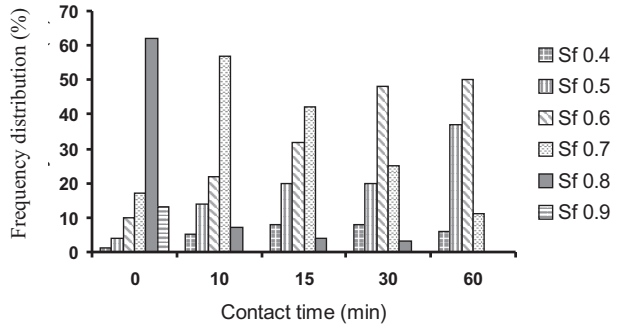

Fig. 1. Sf frequency distribution (%) related with contact time (min) in permeabilized cells with Triton X-100®, 0.7 mM.

Finally, the compactness factor (Cf) presented a similar tendency to Sf; higher values of Cf were observed as result of the increase of the irregularity caused by the permeabilization process. Another reference of the irregularity degree would be the Feret diameter (Fer), because this factor is an equivalent diameter of the object, if it were circular in shape. In this sense, the experimental area of the cells was 1 761 μm^2 for processed cells during 60 min and 3 497 μm^2 for the blank.

Then, as if the cells were round, the perimeter should be approximately 149 and 209 μm respectively and, therefore, Cf≅12.58; nevertheless according to the values of Fer, the perimeter should take values of 167 and 186 μm, respectively. In other words, the perimeter of the permeabilized cells was very roughness and kept a lower area. The relevant differences between theoretical and experimental values of these parameters reflect the changes in both, shape and irregularity of the cells, as a consequence of the permeabilization process. This can be observed in the micrographs presented in Fig. 2. It is possible to observe that, while in the control the cells are more regular in shape and size, the permeabilized cells become more irregular and smaller as the contact time is increased.

This behaviour may be attributed mainly to the structural change of the cell wall caused by the permeabilizing agent (Meiners *et al.*, 1991; Bassetti *et al.*, 1995; Trejo-Tapia *et al*., 2007), rather than the shear stress caused by agitation. On this regard, Sowana *et al.* (2002), reported that the non-ionic detergent Pluronic F68® has a protective effect over the cell wall and cell membranes of *Daucus carota*, while dimethyl sulphoxide at a concentration of 0.5% (v/v) damaged the cell wall structure, causing cellular rupture and therefore a loss of viability in *Coleus blumei* (Park *et al*., 1992). Rodríguez *et al*. (2004), studied cultures of *Solanum chrysotrichum* and reported that the elliptical shape was maintained along the fermentation process, in both the flasks and a stirred tank bioreactor, in spite of the different shear stress levels given by each culture system. Therefore, it can be considered that the morphological changes observed in different vegetables may be due to the particular characteristics of each species (growth dynamics, phenotype, cell-cell and

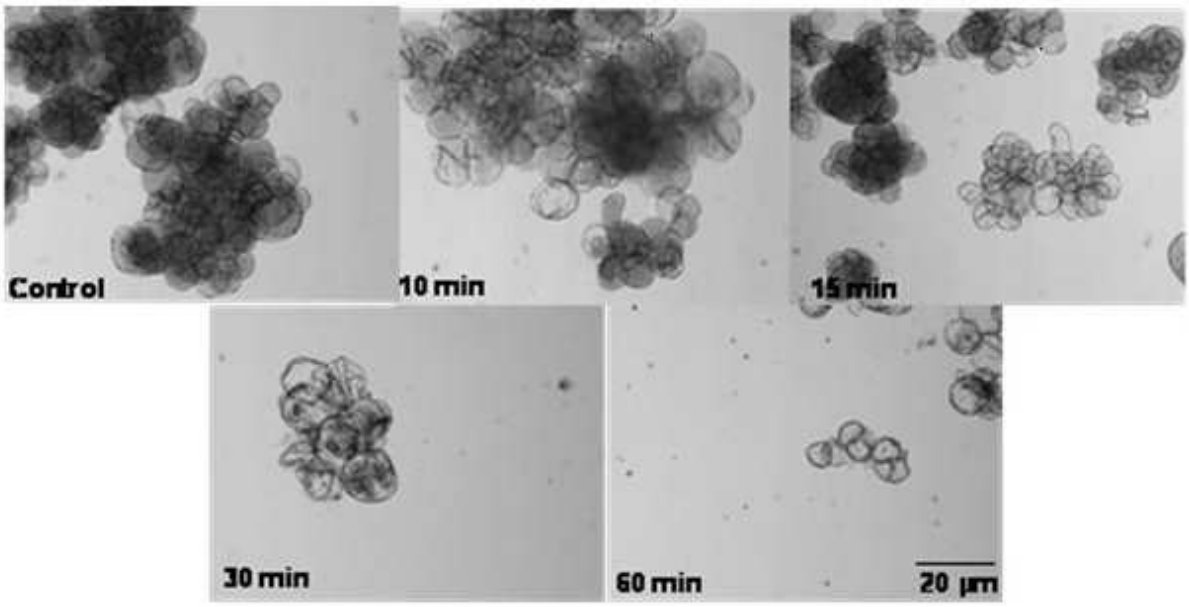

Fig. 2. Representative digital micrographs of *B. vulgaris* cells permeabilized with Triton X-100® at different contact time.

cell-culture medium interactions, *etc*.), as well as to the particular permeabilizing agent and the contact time (Waliszewski and Konarski, 2001; Verpoorte *et al*., 2002).

With regard to the probable fractal nature of permeabilized cells, Fig. 3 presents the curves for the logarithmic relationship between P and A, for all the permebilization conditions as well as for the blank. In every case, it can be observed that there is a linear correlation between the parameters.

Therefore, under the experimental conditions of this study, it can be considered that the *Beta vulgaris* cells presented fractal geometry, since all the established restrictions were achieved (Martínez-López *et al*., 2002).

Table 2 shows the Richardson plots used to calculate FD. From the image analysis of the projected areas of the cells, it was observed that the relationship between P and A is higher than unity in every case. This further demonstrates that the suspended *B. vulgaris* cells, including those that were permeabilized, have fractal geometry. In spite of lacking previous knowledge of the fractal nature of vegetable cells grown in suspension in a liquid medium, Yokota *et al.* (1999), pointed out that callous tissue of *D. carota* grown in a semi-solid medium presented a fractal behaviour, with FD values between 1.05 and 1.11, and Kubik and Nozdrovicky (2005) obtained FD values between 1.3 and 1.6 for porous systems (soil). In this sense, FD can take different values as a result of the particular methodology used to its estimation (Buczkowski *et al.*, 1998). In the present work values of FD higher than 1.5 were found, because fractal dimension was calculated from area/perimeter relationships of the aggregates population. In a similar way, values of R^2 higher than 0.95 were obtained for every sample (except for the blank) with typical errors lower than 8%. This last parameter is among the intervals reported by other authors for the digital analysis of cell images (Hagiwara *et al*., 2002; Rodríguez *et al*., 2004). On the other hand, it was observed that the value of FD increased as a function of contact time with Triton X-100®, which indicates that the permeabilization process produced a significant increase in the irregularity of the cell morphology. This may be the result of a structural change and later of an irreversible damage on the cell wall and membranes that took place as a consequence of the release of the secondary metabolite (Boitel-Conti *et al*., 1996; Trejo-Tapia *et al*., 2007).

The increase in the values of FD and Cf together with the decrease in size and Sf indicate an increment in irregularity and roughness or sinuosity of the cell wall, as well as shrinkage and a change in the cellular shape. These changes may be due to the loss of cellular turgor (Thimmaraju *et al*., 2004) produced by the excretion of the vacuole contents, like betacyanins and other soluble components (in much lower concentrations). This complex system (cells aggregates – culture media and permeabilizating agent) is driven by two main factors, the turgor pressure and concentration differences between the vacuoles and the culture media (apoplast) in which the cells are immersed (Mukundan *et al.*, 1998). This can be analogous to the change in shape and size caused by the shrinkage and therefore, the increase in FD that certain biological materials undergo when dried (Chanona *et al*., 2003; Alamilla-Beltrán *et al*., 2005). It has been reported that one of the mechanisms of the permeabilization process is the formation of micropores or microchannels through the cell wall that allow the flow of metabolites (Lerner *et al.*, 1978; Brodelius, 1988).

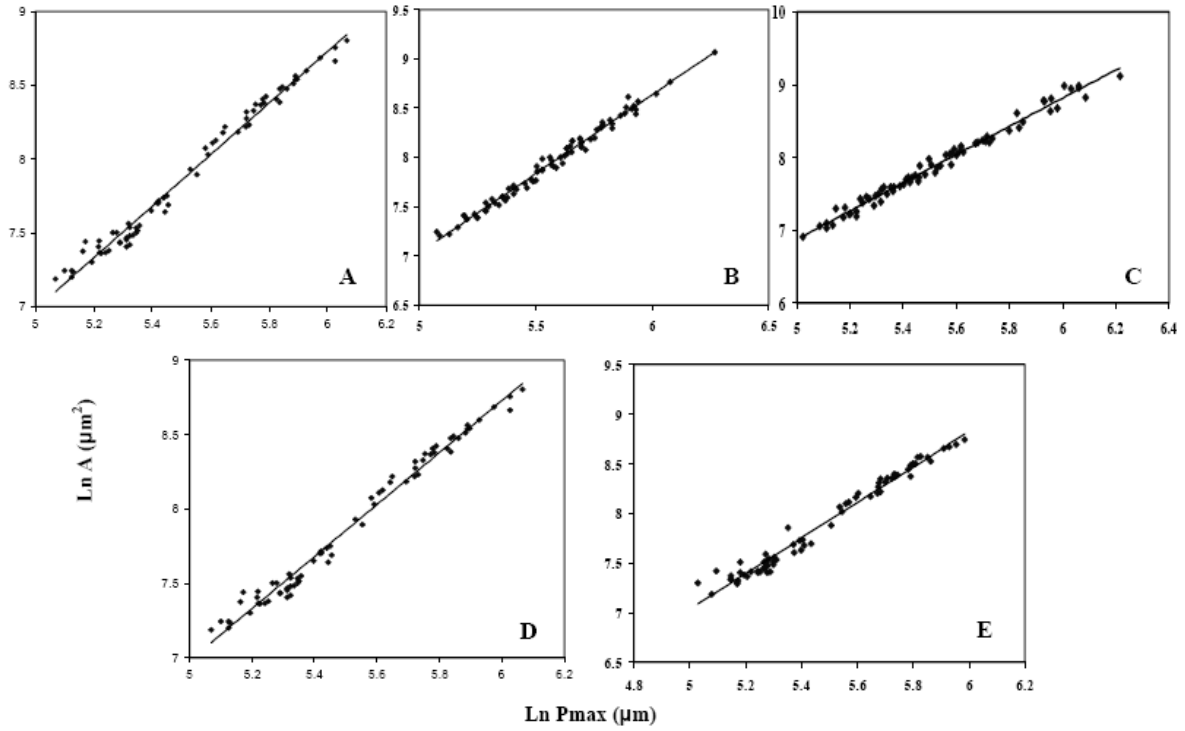

Fig. 3. Richardson plots for *B. vulgaris* L. permeabilized cells with Triton X-100®, at different contact time: A – 0 (blank), B – 10, C – 15, D – 30, and E – 60 min. The slope corresponds to fractal dimension values for cell aggregates.

T a b l e 2. Fractal dimension (FD) of *B. vulgaris* L. permeabilized cells

Contact time (min)	Number of objects analized	FD	Correlation factor	Standard deviation (%)
0	84	1.5355	0.9342	7.965
10	79	1.6063	0.9859	4.978
15	83	1.6906	0.9842	5.397
30	84	1.7273	0.9848	4.880
60	83	1.7881	0.9767	6.321

The quantity of micropores and their temporality are functions of the type, concentration and contact time of the permeabilizing agent. Trejo-Tapia *et al*. (2007) reported that a 0.7 mM concentration of Triton X-100® for 10 min is enough to produce the excretion of 40% of betacyanins in *B. vulgaris*. Also, these authors found that higher concentrations and contact times resulted in a significant decrease of the re-growth capacity and viability loss of the cells. This may be due to loss of turgor as well as irreversible changes and collapse on the structure of the cell wall, which is observed in the increase of the irregular morphology of the cell border and therefore in the FD value. As it happens in other types of cells, under these conditions they tend to aggregate, maybe as a protection and reparation mechanism against the damage caused by the formation of pores (Kang *et al.*, 2000). Further research is needed to corroborate those theories with both chemical evidence and image analysis, in which the evaluation of the FD of the cell wall surface may be involved, since this parameter has been particularly useful in other studies (Hagiwara *et al*., 2002; Ibaraki *et al.*, 2001).

CONCLUSIONS

1. *Beta vulgaris* L. suspension cells presented fractal morphology, since the ratio of area and perimeter was larger than the unity.

2. The fractal behaviour was more evident when cells were permeabilized, and fractal dimension increased significantly if the contact time with Triton X-100® increased.

3. Cell morphometry and fractal dimension values, evidenced loss in cell turgor and possible structure collapse in cell wall as a result of higher contact time with Triton X-100®.

4. Image analysis and the fractal dimension of the cells were useful tools in the evaluation of the morphometry of plant cells suspension cultures.

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