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MONITORING OF BIOFILM DEVELOPMENT AND CHARACTERIZATION OF IMMOBILIZED YEAST CULTIVATIONS

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Abstract: A mathematical model is proposed to describe the growth dynamics of an immobilization system formed by calcium alginate pellets and the yeast *Saccharomyces cerevisiae*. The system was used for the production of the alcoholic beverages tequila and beer. Scanning electron microscopy is employed to monitor the interactions caused by biomass growth and distribution inside the polymeric matrix support. It was found that the growth of immobilized yeast can be described by first order kinetics in the aerobic phase and by a power-law kinetic model in the anaerobic phase.

1. INTRODUCTION

The production processes of ethanol have evolved from rudimentary vessels and "wild" micro-organisms, used in the early days, to fully controlled bioreactors and genetically "engineered" micro-organisms employed nowadays. In the search for alternative methods to produce alcoholic beverages and energy from complex media and renewable sources, the use of immobilized systems has been established as viable alternative to improve the overall yield of the fermentation processes (Nagashima et al., 1983; Fumi et al., 1997; Wheals et al., 1999; Alegre et al., 2003). The immobilization offers some advantages over the traditional cultivation with free micro organisms, since they increase the cell density per reactor volume, ease the handling of both, the biological and abiotic phases, reduce inhibition effects of substrates and products, and allow continuous product extraction (McGhee et al., 1982; SivaRaman et al., 1982; Doran and Bailey, 1985; Van Haecht et al., 1985; Galazzo and Bailey, 1988, 1990a, 1990b; Gilson and Thomas, 1995; Norton et al., 1995; Sanches et al., 1996; Balci et al., 2002). However, even when the use of immobilized systems has demonstrated to yield better bioreactor operation, it is still a current challenge to correlate their performances with the intrinsic growth kinetics of the immobilized species.

In this work, the use of Scanning Electron Microscopy (SEM) to monitor and to analyze the interactions caused by the growth of *Saccharomyces cerevisiae* trapped in calcium alginate pellets is demonstrated. The immobilization system was used to produce two alcoholic beverages, tequila and beer. The work focused exclusively on microbial growth under non-limiting substrate conditions in the cultivation media, i.e. during exponential cell proliferation, since this kind of growth imposes a maximal mechanical stress to the support matrix. The fermentations were carried out in a repeated-batch fluidized bed bioreactor and the distribution of immobilized yeasts was monitored as function of space and time in the support matrix through out the whole fermentation process. Finally, a simple model was established to describe the biofilm development in both, aerobic and anaerobic cultivation phases. The model considers the volume changes of the immobilization matrix as function of microbial growth and characterizes the breakage of the matrix support.

2. MATERIALS AND METHODS

2.1 Fermentation media, strains, immobilization and cultivation process.

In a first stage, the yeasts were cultivated in Erlenmeyer flasks and allowed to grow in their respective propagation media for 24 h. Afterwards they were centrifuged and separated from the supernatant and the separated biomass was used as source for yeast immobilization.

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Propagation medium for tequila. 2.0% hydrolyzed and filtrated "agave azul tequilana" extract (Nobel, 1987, 1998) consisting of 50 g/L total reducing sugars and 15°Bx, according to the official tequila norm (NOM-006-SCFI, 1994), 0.2% yeast's extract (Difco, Inc.), 1.0% mineral salts (0.2 g/L KH_2PO_4, 0.17 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L $(NH_4)_2SO_4$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.1 g/L NaCl), 0.1% trace elements, 96.7% water.

Propagation medium for beer. 3.0% anhydrous dextrose (Sigma Aldrich Inc.), 0.3% yeast's extract (Difco, Inc.), 0.35% malt extract (Difco, Inc.), 1.0% mineral salts (0.2 g/L KH_2PO_4, 0.17 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L $(NH_4)_2SO_4$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.1 g/L NaCl), 0.1% trace elements, 95.25% water.

Both media were first autoclaved at 121°C and after cooling the solutions to room temperature, they were inoculated in 100 mL flasks.

Yeast strains. An industrial strain of *S. cerevisiae* (classified under code AMAT-2) was used for the tequila fermentation and a wild type strain of Baker's yeast for the beer fermentation.

Immobilization procedure. After propagation and separation of the biomass, *S. cerevisiae* was trapped in spherical pellets made of calcium alginate following the method of King and Zall (1983). Pellets were continuously and gently agitated at 9°C (tequila) and at 5°C (beer) in a sterilized calcium chloride solution until curing of the polymeric material was complete (approximately 24 h).

Cultivation of immobilized yeast. The experimental runs took place in a system consisting of a 600 mL glass tubular bioreactor (VICOSA, Guadalajara, México). Previous to operation, the bioreactors were disinfected with a 70% ethanol aqueous solution circulating for 30 minutes; subsequently they were washed with bi-distillated, de-mineralized and sterilized water. After loading in the bioreactor the inoculated pellets, the operation conditions were fixed at a temperature of 30°C with a recirculation flow ranging from 246-480 mL/min, dependent on the pellets load.

The broth used for tequila production was the same medium utilized for propagation, while a blend of malt, hop and water was used as media in the beer production (Engan, 1981).

The fermentation process consisted of two phases, one aerobic and one anaerobic. In the aerobic phase the increment of the immobilized biomass was the main objective. The time period of the aerobic phase was fixed to 15 h for the tequila fermentation, and to 40 h for beer fermentation, respectively. The fermentation broth was oxygenated by injecting sterile air directly through a glass fringe at the bottom of the bioreactor.

In the anaerobic phase, the fermentation broth was changed at regular intervals until the carbon source was exhausted. One additional indication of this depletion was the diminishment of the gaseous CO_2 evolving from the pellets as well as a slow collapse of the fluidization bed.

Sampling was performed by removing some pellets through a bioreactor port at regular time intervals.

2.2 Scanning electron microscopy.

All samples were first dehydrated by sequential immersion in t-butyl alcohol aqueous solutions (Sigma Aldrich, Inc.) with increased alcohol concentrations of 50, 70, 80, 90 and 100%. Each dehydrating step lasted 30 minutes. Afterwards the samples were allowed to rest for 24 h in pure t-butyl alcohol. Before being lyophylized, the pellets were quickly frozen to avoid the growth of alcohol crystals and to ease alcohol vacuum removal. The freeze-drying process was carried out in a glass apparatus as reported by Kingsley (1991). The samples were completely dried after 3 h in the apparatus by -10°C.

Finally, the pellets were gold-coated in a Samsputter-2a automatic sputter-coating apparatus (Tousimis Research Corp., Rockville, Md., USA). A Jeol JSM-5400LV Scanning Electron Microscope (Jeol Techniques, Ltd., Tokyo, Japan) was used. All observations were done at 20 kV accelerating voltage with 15-20 mm distance work and low vacuum mode (10^{-2} Pa).

2.3 Digital image analysis.

SEM micrographs were digitalized at a working resolution of 400 dpi with 8-bit gray depth and a standard size of 540×480 pixels. Afterwards, a background homogenization with artifact removal and noise reduction was performed manually on each image (Russ, 1995), since the electronic micrographs were usually heterogeneous due to several factors difficult to control like electronic beam instabilities, sample treatment, etc.

After homogenization, binary images (1-bit) were obtained by means of a threshold-procedure obtaining the bi-dimensional projection of the pellet. The threshold value was determined manually as well. Based on the binary images, area-based equivalent diameters, D_A were evaluated using:

$$D_A = \sqrt{\frac{4A_P}{\pi}} \qquad (1)$$

A_P is the projected area of the pellet.

2.4 Parameter identification and microbial distribution.

The equivalent diameters were used to calculate pellet equivalent volumes. The estimated volumes were then employed in the estimation of the microbial kinetic model.

The kinetic parameters were adjusted by minimizing the sum of the squared residuals of the estimation/model pellet volumes by means of a simplex algorithm. The process models were implemented in the SIMULINK-Matlab environment (MathWorks, Inc.). The systems of differential equations were integrated using a variable-step routine (Dormand and Prince, 1980).

The evaluation of the microbial distribution was performed by direct counting of individual yeasts over a fixed, prespecified zone on the micrograph. No viability criterion was used to classify them at the different stages of the yeast growth. Microbial density was reported as the total number of present microorganisms per tested area.

3. BIOFILM DEVELOPMENT MODEL

The first assumption for modeling is that single immobilized yeasts are incompressible entities having an ideal and standard size at any time in the fermentation operation. Furthermore, a group of budding yeasts is considered as a collection of various single ideal yeasts with standard size.
Taking these considerations into account, the dynamic model for the biofilm development of a single pellet is described by:

$$\frac{dV}{dt} = v_I - v_S \qquad (2)$$

v_I is the rate of volume increase and v_S is the rate of volume shrinkage.
The rate of volume increase includes the rate of volume change due to microbial reproduction, v_μ and the rate of individual cell volume increase, v_V:

$$v_I = v_\mu + v_V \qquad (3)$$

The rate of volume shrinkage is given by the rate of biofilm detachment, v_{BD} and the rate of microbial death, v_D:

$$v_S = v_{BD} + v_D \qquad (4)$$

However, for both cultivation phases, aerobic and anaerobic, it can be considered that the rate of individual volume increase is insignificant compared to the rate of microbial reproduction. Similarly, the rate of microbial death can be neglected considering microbial growth without limiting substrate, therefore:

$$v_V = v_D \approx 0 \qquad (5)$$

Under these considerations, Equation 2 reduces to:

$$\frac{dV}{dt} = v_\mu - v_{BD} \qquad (6)$$

4. RESULTS

3.1 Aerobic cultivation phase.

In the first phase, the aeration of the culture broth enables oxygen supply to the immobilized yeast. These conditions are well suited to induce microbial growth under respiratory flux conditions inside the pellet. Reproduction takes place all over the support volume forming small colonies or clusters, but particularly at the surface, where, as shown in Figure 1, there exists a great amount of budding yeast together.

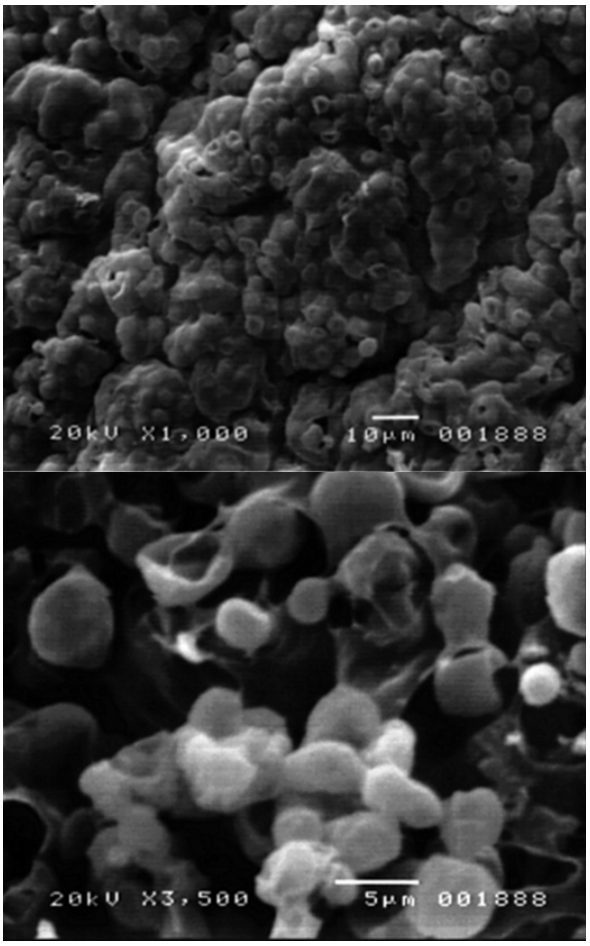

Fig. 1. SEM microphotography of the surface of a pellet from the aerobic cultivation phase showing the yeast clusters (upper image) and budding cells (lower image).

The trapped individual yeasts are initially homogeneously distributed all over the volume of the pellet. Each of these yeasts is a potential colony forming unit (CFU), which, depending on its radial position inside the pellet volume, disposes of a certain amount of essential nutrients. The availability of these substrates controls the microbial growth and the reproduction kinetics.
Due to the porous nature of the material support, the inner transport of nutrients is predominantly a diffusion phenomenon. These substrates, mainly dissolved oxygen and sugars, must diffuse through the spherical geometry of the support matrix thereby creating radial concentration gradients (Martinsen *et al*., 1992; Gilson and Thomas, 1995).
The initially homogeneously distributed CFU are then exposed to different microenvironments in the interior of the immobilization matrix and, by means of growth, generate compartments with microbial clusters in their interior. The microorganisms inside these compartments adapt by changing their growth rates according to the nutrients availability. Figure 2 shows a microphotography of immobilized yeast cells grouped in clusters. The emergence of compartments for each active growing CFU inside the matrix support is caused by the cell proliferation.

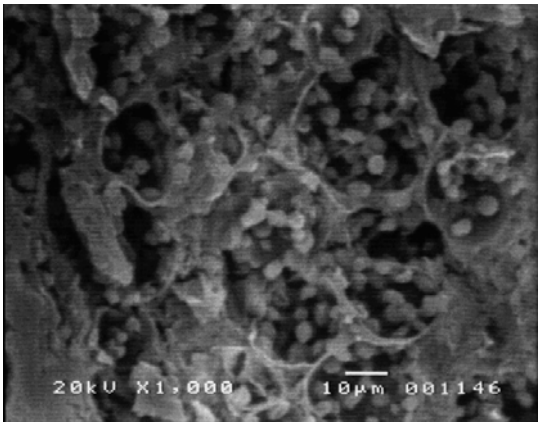

Fig. 2. Yeast clusters and compartments inside the immobilization matrix.

Due to spatial constriction and the mechanical resistance of the supporting material, microbial saturation takes place after a short period of time. As result of this saturation and the elastic nature of the calcium alginate pellets, the biofilm weakens mainly the outer part of the pellets. The most evident effect of this biofilm growth is the increase of the apparent pellet volume. The magnitude of volume change is a function of the radial position too, presenting a maximum at the outer shell and a minimum at the center of the pellet. Figure 3 shows a pellet cut where the microbial distribution can be seen throughout the sample radius: the darker the zone, the higher the microbial concentration.

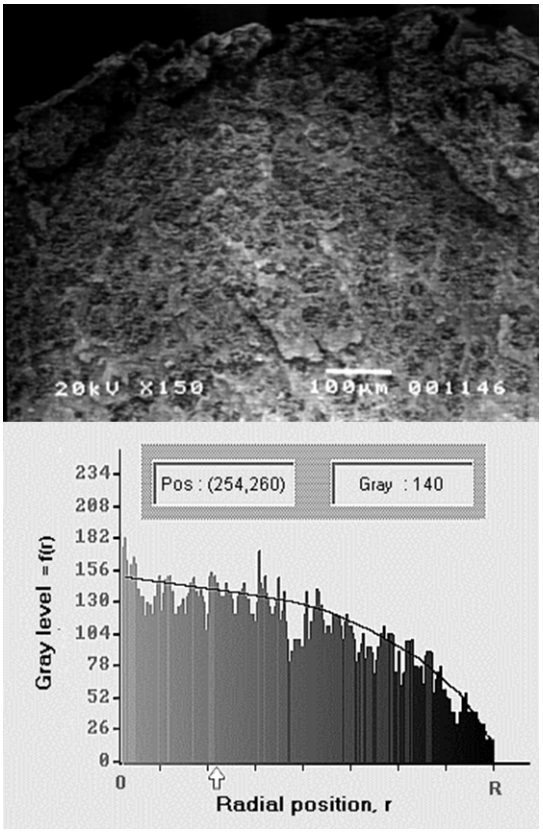

Fig. 3. Cell cluster distribution along the pellet radius: the concentration of the biomass is correlated to the gray tonality: the darker the zone the higher the biomass concentration.

Even when the support matrix was weakened by the microbial growth, no evidence of biofilm detachment was noticed during the aerobic cultivation phase, that is, the rate of biofilm detachment can be neglected:

$$v_{BD} \approx 0 \qquad (7)$$

Under these conditions, the number of immobilized yeasts is proportional to their volume. In case of no substrate limitation, i.e. non-limited growth, it can be assumed that the rate of volume change due to microbial reproduction, v_μ is proportional to the microbial volume, which is proportional to the apparent pellet volume, that is:

$$v_\mu = kV \qquad (8)$$

Considering that at the initial time $t = 0$ the apparent volume of the pellet is given by $V\ (t = 0) = V_0$ the solution of the differential Equation 6, considering Equations 7 and 8 and the proportionality constant k, Equation 9 describes the volume variation of the pellet as function of time:

$$V(t) = V_0 \exp(kt) \qquad (9)$$

Figure 4 presents the estimated and modeled pellet volume as function of time for beer fermentation. Normalized with the initial value V_0, the variable exhibits a linear dependency in the logarithmic scale. This fact validates the simple assumption taken for the microbial reproduction rate, which leads to a first order kinetics for the aerobic phase.

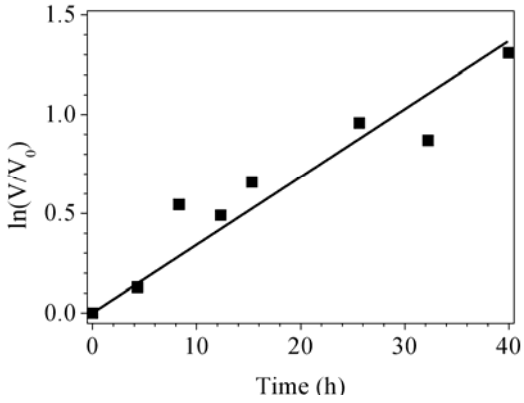

Fig. 4. Variation of pellet volume as function of the cultivation time in the aerobic phase (beer fermentation process). An approximate value of 21 h for the doubling time of the immobilized yeast can be estimated from the slope of the line ($0.033\ h^{-1}$).

3.2 Anaerobic cultivation phase.

After the aerobic phase, the old broth is removed and replaced with fresh broth but is not aerated any longer. This situation changes the micro-environmental conditioning of the immobilized yeasts drastically. Direct consequence of oxygen absence is the predominance of the fermentative flux over the respiratory flux, i.e. ethanol and CO_2 instead of biomass are predominantly produced.

As the anaerobic cultivation progresses more and more dissolved CO_2 is excreted by the immobilized yeast to the liquid microenvironment of the clusters. After the concentration of dissolved CO_2 reaches saturation levels, small bubbles of the gas form in the pellets and begin to emerge from their surface. Meanwhile, the fluidized bed rises due to the uploading force exerted by the gas trapped in the pellets. Finally, the combination of the increasing pressure of gaseous CO_2 inside the pellet, the force exerted by the still growing microorganisms and the permanent attrition in the fluidized bed prompt the breakage of the support. The pressure exerted by gas under the external surface of the pellet and the mechanical resistance of the support lead to the emergence of a hull surrounding the main core of the immobilization material. Under these conditions, the biofilm detaches from the pellet and cellular washout takes place releasing yeasts from the exposed internal surface. However, the volume of the support matrix reaches a pseudo-steady state after continuous renewal. Figure 5 presents a micrograph where the hull, the internal exposed surface of a pellet and the yeast on its surface are distinguishable.

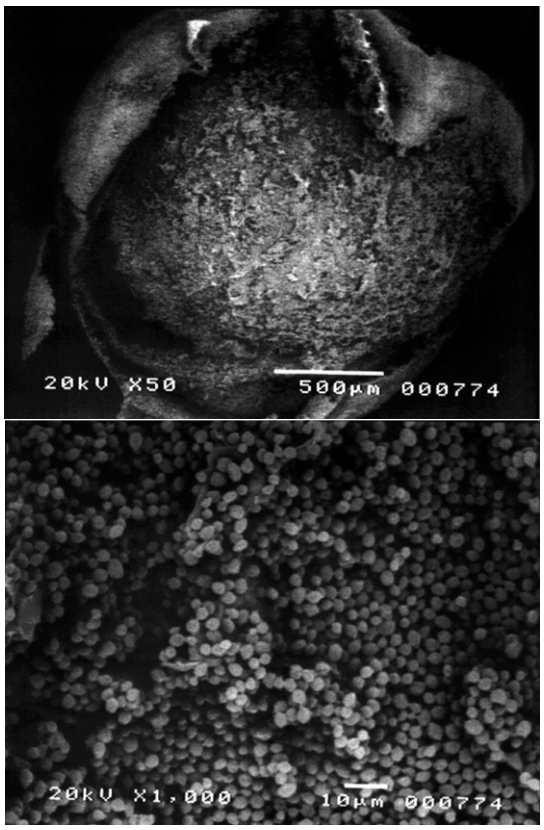

Fig. 5. SEM microphotography of a pellet from the anaerobic phase (tequila fermentation). The hull surrounding the still compact core is clearly visible and the surface of the support matrix core is saturated with yeast.

To model the dynamics of volume development in the anaerobic phase, the assumption of volume increase due to microbial reproduction is made again. However, the rate of volume change is different from that in the aerobic phase because to the change in the metabolism:

$$v_\mu = k' V \qquad (10)$$

k' is the kinetic rate constant for the anaerobic phase. As stated before, the rate of biofilm detachment is mainly provoked by the gaseous CO_2 inside the pellet and the permanent attrition in the fluidized bed. The excretion rate of CO_2 is directly correlated with the number of producing yeasts and, therefore, can be correlated with the apparent pellet volume, i.e. with the active biomass. Similarly, it is assumed that the mechanical resistance of the support matrix and the attrition in the fluidized bed are correlated with the volume of the pellet as well. In order to describe these complex phenomena, a power-law approach was used, such that:

$$v_{BD} = k'' V^n \qquad (11)$$

In this approach, the k'' is a kinetic rate constant and n the kinetic order. Combining Equations 10 and 11 into Equation 6, the dynamic model for the biofilm development of a single pellet in the anaerobic phase is described by:

$$\frac{dV}{dt} = k' V \left[1 - \left(\frac{V}{V_\infty} \right)^{n-1} \right] \qquad (12)$$

In this equation, V_∞ is a constant and represents the asymptotic or final apparent volume of the pellet ($V_\infty^{n-1} \equiv k'/k''$).

Figure 6 presents the estimated and modeled volume variation in the anaerobic phase for a tequila-producing cultivation. Even when a clear asymptotic behavior was observed for the maximal size of the support matrix, the accuracy of the estimation of the pellet volume was severely affected by the presence of the external hull, as can be stated by the estimations of the last samples.

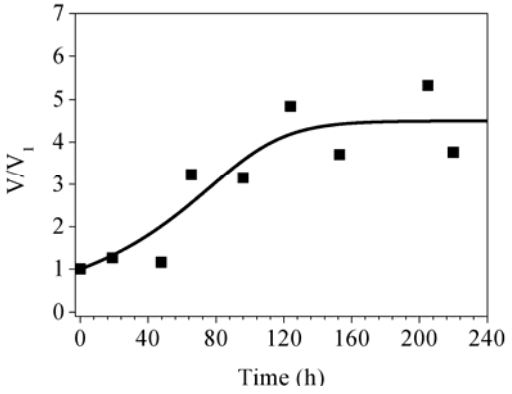

Fig. 6. Variation of pellet volume as function of the cultivation time in the anaerobic phase (tequila fermentation process). V_1 is the pellet volume at the beginning of the anaerobic phase.

As previously reported by Martinsen et al. (1992), the polymeration of the calcium alginate pellets is more effective at the surface, generating a material more resistant to mechanical stress. In the anaerobic phase the hull of the pellets was formed initially and

detached slowly, but was not regenerated anymore. An argument for the improved mechanical resistance of the material is presented in Figure 7, where further microbial growth is observed between the layers of the hull even after detachment.

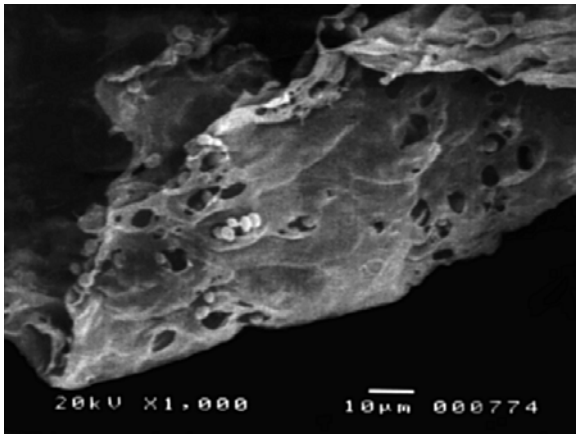

Fig. 7. Even after detachment, the pellet hull serves as immobilization support for the yeast.

Table 1 presents the values for the different kinetic parameters and their standard deviations obtained for both fermentation processes.

Table 1 Kinetic parameters for the biofilm development model.

Parameter	Value	Units
k	0.033 ± 0.001	h^{-1}
k'	0.013 ± 0.002	h^{-1}
V_∞	5.6 ± 0.3	µL
n	4.5 ± 0.5	-

5. CONCLUSIONS

The use of SEM to monitor and characterize the microbial growth, distribution inside a support matrix, and size development of immobilized yeasts clusters was demonstrated. Exemplified at the production of the alcoholic beverages beer and tequila, mathematical modeling of biofilm development was carried out successfully based on SEM observations of the interaction phenomena occurring between microorganisms and their support material. Furthermore, it was found that the growth of immobilized yeast can be described by first order kinetics in the aerobic phase and by a power-law kinetic model in the anaerobic phase.

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