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New Tool To Monitor Biofilm Growth in Industrial Process Waters

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ABSTRACT: A new online methodology based on a continuous process video microscopy and image analysis has been developed to study the effects of enzymes on the formation of biofilm. This research consists of two parts: (1) the monitoring of the growth of a biofilm formed with the axenic culture isolated from the process waters of a recycling paper mill, aiming at determining the most appropriate way to quantify the biofilm growth from the obtained images; and (2) the study of the effects of three new enzymatic products on biofilm formation involving the natural flora of microorganisms present in the process water of the paper mill. The results demonstrate that the new methodology based on image analysis allows monitoring of the formation of biofilm and selecting the most efficient biofilm control product. The study also shows that enzymatic products could be an alternative for biocides on biofilm prevention and control.

1. INTRODUCTION

A bacterial biofilm is an organized community of microorganisms attached to a solid surface under submersion or in contact with water. Biofilm is constituted mainly by microorganisms, exopolysaccharides^{1,2} of microbial origin, and water. The properties of each biofilm depend on the environment in which it develops, this being characterized by various factors such as surface material, nutrient availability, hydrodynamics, source of microbiological contamination, and species distribution, etc.³ The presence of biofilm in an industrial system causes biocorrosion and/or biofouling due to the formation of deposits.

Paper mills suffer very often biofouling and biocorrosion problems, especially recycling paper mills, because along with the recovered paper used as raw material a vast array of microorganisms^{4–6} enters a warm and nutritious environment.⁷ In addition, the trend toward closing water circuits drives paper mills to work at process conditions that favor the growth of microorganisms, since the reuse of process water implies an increase in temperature and in the concentrations of dissolved organic material. These problems affect both paper machine operability and final product quality.^{8,9}

Biocides are usually added to the process water to prevent and control biocorrosion and biofouling in papermaking and other industrial systems. However, the use of these chemicals can reduce the efficiency of the biological treatment of the mill effluents and increase the environmental impact of the industrial process. These drawbacks can be reduced or even avoided by replacing biocides with enzymes, which do not destroy the cells but act as biofouling control agents. Nevertheless, the optimization of any antimicrobial or antifouling program depends on the information obtained from the monitoring of biofilm formation and growth.¹⁰ Ideally, this information should be acquired online, in situ, in a nondestructive manner and in real time to be representative of the actual processes. Furthermore, the technique should be easy to handle and robust.^{4,10–17}

The methods used to assess the efficacy of biocides on biofouling control can be classified in two categories: (1) techniques involving the partial or total destruction of the sample analyzed and (2) nondestructive techniques for online biofouling

monitoring. Among the destructive techniques, the most frequently used are as follows: dry weight and plate counts, techniques based on microscopy (epifluorescence, confocal laser-scanning, transmission electron, and scanning electron microscopy), enumeration of sessile bacteria, nucleic acid quantification, and the measurement of microbial activity.^{18–26} The aforementioned destructive techniques can provide detailed information about the biofilm, but they are very time-consuming and not suitable to large screening experiments at industrial scale. In industrial systems, the most frequently used monitoring methods are based on the analysis of the deposits that occurred on coupon systems and on control points within the process after their scrapping,¹⁹ although biofilm formation usually takes place in areas with complicated access.

The nondestructive techniques are typically focused on measuring the effects of fouling deposits, without evaluating their biological side, or on the direct measurement of microorganisms' accumulation. The most usual techniques from this group consist of optical methods (differential turbidity¹² and optical fiber sensors^{27,28}), methods based on the measurement of changes in pressure drop and/or heat- or electrical-transfer resistance due to biofilm accumulation²⁹ and electrochemical techniques (impedance³⁰ and capacitance³¹). These methods are not able to detect the initial stages in the attachment of microorganisms because they require biofilm thickness to be over a minimal detection threshold.²⁹ Most optical fiber sensors are based on the measurement of the light intensity received by a detector not taking any actual image.

In this study a new online methodology based on the use of a continuous process video microscopy probe that takes biofilm images has been developed, tested, and used to study the effects of enzymatic products on the formation of biofilm in a simple system. In particular, the research comprised the study of the growth of biofilm formed by an axenic culture of *Enterobacter*

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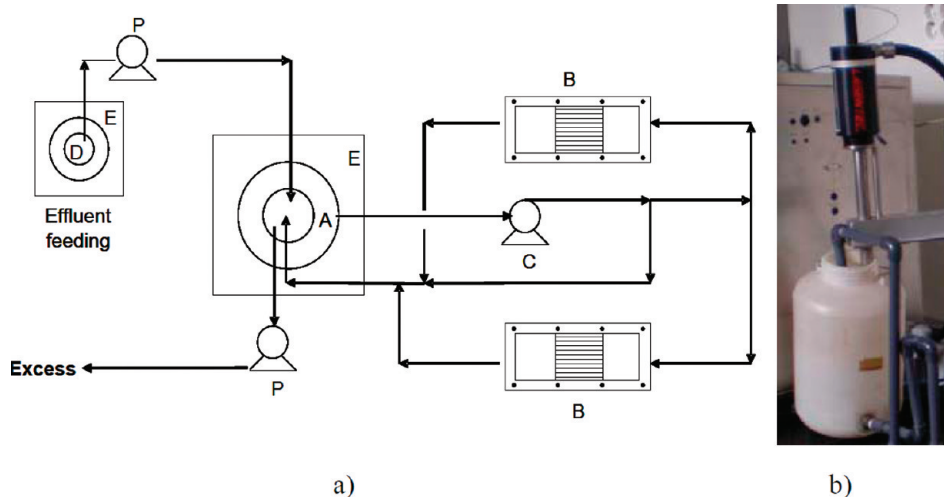


Figure 1. (a) Continuous flow system: Stirred tank (A), flow chamber with coupons (B), centrifugal pump (C), feeding tank (D), isothermic bath (E), and peristaltic pump (P). (b) Probe location in the system.

cloacae (*E. cloacae*) isolated from the process water of a recycling paper mill. Then, the methodology was used to monitor the biofilm formation in real process waters sampled from the forming wire section (a continuously moving wire screen where a slurry of cellulose fibers having a solids level of 0.3–1% is converted into a wet web, which has a solids level of about 15–22%, by draining the water through the wire pores using the gravity force and vacuum), and the results were compared with the ones obtained from the analysis of the biofilm deposited in coupons. Three different enzymatic treatments to prevent and control biofilm formation were tested.

2. MATERIALS AND METHODS

2.1. Particle Vision and Measurement. Particle video microscopy (PVM 800-10-T12-K) is a commercial in situ imaging system developed by Lasentec from Mettler Toledo AutoChem, for analyzing particles or droplets contained in process reactor vessels or pipelines at full process concentrations, having been used in suspensions with suspended solids concentrations as high as 60% (w). It can be used off-line but also online and even in-line in a mill pipe.^{32–34} The device can be inserted directly into a process stream with minimal disruption of the flow. The probe has two major sections: a frontal part being a probe tube which is closed at the distal slurry end by a sapphire window and a rear part being a probe housing which is generally not inserted in a process flow to be monitored. The probe tube and housing are connected to form a rigid sealed shell which contain all of the parts of the probe and protect them from the process environment. For this study, the probe was modified by replacing the sapphire window by a new one with two scratches to favor the attachment of the bacteria.

2.1.1. Image Collection. The PVM technique allows obtaining up to two images per second of the particle droplets, or objects suspended in a fluid medium in a plane parallel to the window's surface. The distance between this plane and the window's surface can be modified by the user. In this research, this distance was reduced to zero. Therefore, images correspond to particles attached to the window's surface. Because of this, the turbidity of the suspension did not affect the measurement. The probe generates a laser beam that focuses on the plane immediately

adjacent to the external window surface. The light reflected by the particles attached to the window is carried to the detector through an optic fiber cable. The detector generates a black and white image of a $850 \times 654 \mu\text{m}^2$ surface with an approximate resolution of $3\text{--}5 \mu\text{m}$.³⁵

2.1.2. Image Analysis. The analysis of the collected images can give quantitative information about the size of the deposits formed on the window surface. The analysis was carried out by means of the "image-analysis module chord 1", which is a fast and simple algorithm which calculates the chord length distribution based on a simple threshold method. This module is part of the "PVM statistic acquisition" software. Images are scanned horizontally from left to right in search of pixels with a white intensity that falls into the threshold range selected (particles appear in white over a black background). Any contiguous horizontal strand of these white pixels is taken to be a single chord. A transition from "not white" to "white" marks the beginning of a chord, and a transition from "white" to "not white" marks the end of that chord. The intervening length is the chord length in pixels, which is then converted, via the image-scaling information, to a chord length in micrometers. Many chords are measured from each image, and a chord length distribution is obtained every 4 h from all the chord lengths obtained during the analysis of the 10 images collected during 5 min.

2.2. Experimental Setup. The experimental setup, which is based on the one developed by Pedersen³⁶ in 1982 to study the microbial biofilm formation in water systems, consists of a feeding open tank, an open stirred tank, two flow chambers for housing the coupons, a centrifugal pump for water recirculation, and two peristaltic pumps for water refreshment (Figure 1a). Tanks are open to allow the dissolution of the oxygen from the air into the water in a way similar to how it occurs in a paper mill. The biofilm is developed mainly by aerobic and facultative anaerobic bacteria, the existence of anaerobic bacteria being possible in the centers of the colonies where the oxygen level is very low. Costerton et al. observed that although the water channels appear to transport oxygen into the biofilm, the diffusion limitations and oxygen utilization produce very low oxygen levels at the center of cellular microcolonies.³⁷ These direct observations of living biofilms may explain the existence, and even the physiological activity, of anaerobes within mixed biofilms in aerobic environments. The

use of closed tanks in the experimental setup could increase the presence of anaerobic, even pathogen, bacteria. Each flow chamber contains 10 PVC coupons with spaces of 5 mm between them. The total volume of the circuit is 10 L. The centrifugal pump is set up to move this volume of liquid with a flow of 0.29 L/s. Fresh sterile culture medium or fresh process waters are pumped from the feeding tank to the stirred tank discontinuously (in the case of culture medium) or continuously (in the case of process waters). The total volume of process water or culture medium is kept constant through the removal of exceeding wastewaters or culture medium from the stirred tank.³⁸ According to data coming from different mills, a hydraulic retention time of 12 h was selected for the system when it worked with process waters (the flow through the peristaltic pumps was 0.83 L/h). The flow through the coupon chambers was 0.10 L/s to ensure turbulent flow. Since the hydrodynamic conditions affect notably the biofilm buildup and it is very difficult to reproduce the hydrodynamic conditions of the mill at laboratory scale, the effect of the enzymes on biofilm buildup was studied at constant and turbulent hydrodynamic conditions, which allows comparison of the results obtained in the four cases (the case of the control sample, without treatment, and the three cases in which an enzymatic control agent is used) and, therefore, establishment of the effect of each treatment on the biofilm buildup by comparing it with the control sample. The PVM probe was inserted from the top of the stirred tank near the inflow of the tank, immersed into the process water or culture medium (Figure 1b).

2.3. Experimental Procedure. The first set of experiments, involved the monitoring of the growth of biofilm caused by the bacteria *E. cloacae* E-22114, isolated from a paper mill that produces 100% recycled paper using a mixed recovered paper as raw material. This strain was cultured at 37 °C in a medium prepared to enhance the production of polysaccharides. The medium contained glucose (20 g/L), yeast extract (0.5 g/L), (NH₄)₂SO₄ (0.6 g/L), KH₂PO₄ (3.18 g/L), K₂HPO₄ (5.2 g/L), MgSO₄·7H₂O (0.3 g/L), CaCl₂ (0.05 g/L), ZnSO₄·7H₂O (0.2 mg/L), CuSO₄·5H₂O (0.2 mg/L), MnSO₄·H₂O (0.2 mg/L), FeSO₄·7H₂O (0.6 mg/L), and CoCl₂ (0.2 mg/L). The formation and evolution of biofilm during the experiment was monitored with the PVM probe, and pH and temperature sensors were used to maintain the optimal conditions for bacterial growth until a mature biofilm was reached, which in this case took 10 days. Temperature was kept at 30 °C by using thermostatic baths (Figure 1a), and pH was controlled with addition of NaOH 0.1% solution. Every 24 h 500 mL of medium was replaced by sterile culture medium in order to add a new carbon source. This was carried out by turning on the two peristaltic pumps for 36 min each 24 h at a flow rate of 0.83 L/h.

The second set of experiments was intended to study the effects of biofilm control products on the formation of biofilm caused by the natural flora present in the process water of the paper mill. The 10 L system was filled with process waters from the forming wire of the paper machine, and the experiment was carried out at 30 °C and at pH 6.8–7.0 during 96 h (the natural flora requires a shorter time than the single culture to form a mature biofilm). Every 24 h process waters were collected from the mill and within the following 2 h used to fill the feeding tank and restock the fresh process water consumed.

The effects of different enzymatic products (all commercially available from Novozymes A/S, Bagsvaerd, Denmark) on the formation of biofilm were determined. The enzyme dosage was 1000 ppm (expressed as activity per liter of water) for all tests.

The optimization of the dosage of enzyme was not the objective of this research; hence, a high dosage was employed to enhance the effects of the enzymes on biofilm formation and to allow carrying out an appropriate screening of the enzymatic treatments. The flow chamber was opened every 24 h to remove two coupons. One of the coupons was used to quantify biofilm formation, based on its dry weight. This coupon was placed in a stove at 105 °C until dryness, and then it was weighed. The result was expressed in mg/cm². The other coupon of the pair extracted was employed to determine the colony forming units per square centimeter of coupon surface (CFU/cm²). This coupon was removed with sterile forceps and flushed with 9 mL of sterile saline solution to remove slightly adhered cells and excess aqueous medium. Complete biofilms were swabbed from one side of each coupon with sterile cotton swabs and transferred to a vial containing a known volume of sterile saline solution. This suspension was vortexed carefully during 20 s to disperse cells. Then, with each sample obtained, a series of dilutions was performed and it was plated on plate count agar. Plates were incubated at 30 °C for 48 h.

The images obtained with the PVM probe were processed, displayed, and stored in sequences by the PVM control software. Images were taken during the 4 days of the experimentation, after which, they were analyzed.

2.4. Enzymatic Treatments. Three enzymatic preparations, commercially available, having different lytic activities according to Novozymes, were studied in the 10 L system: Pectinex Smash (various pectinolytic activities), Viscozyme L (pectinolytic, cellulolytic, hemicellulolytic, and polygalacturonolytic activities), and Novozyme 863 (polygalacturonolytic, pectolytic, and hemicellulolytic activities). The experiments were conducted in series. Before each trial the system was cleaned and disinfected with a solution of ethanol with a concentration of 70%.

3. RESULTS AND DISCUSSION

3.1. Single-Strain Culture Experiments. The first set of experiments was conceived to assess whether the PVM technique was suitable to monitor the formation of biofilm and to select the best statistic of the chord-length distribution to represent the biofilm formation process, given that single-species culture is easier to control and more predictable than natural flora. With this purpose, an experiment using as pure culture *E. cloacae* E-22114 was carried out. The strain was grown in the medium referred to in preceding text. In this experiment a series of images were taken with the PVM probe to monitor the formation of biofilm on the surface of the probe window in real time. Figure 2 shows some of the images obtained. The chord length distributions obtained from the analysis of the sequence of images were studied and different statistics were calculated (e.g., the total number of counts, the mean chord length, etc.) to identify which of them presented an evolution appropriately reflecting, in a simple way, the growth of biofilm. Figure 3 shows that the evolution of the square-weighted mean chord length can be used as an indirect measurement of the biofilm growth. This was expected, to some extent, since the increase of this variable is a function of the increase in the percentage of the window area covered by deposits, and it is more sensitive to changes in the large chord lengths than other statistics, which reduces the noise due to bubbles. The square-weighted mean chord length, \bar{C} (μm), is calculated from each chord length

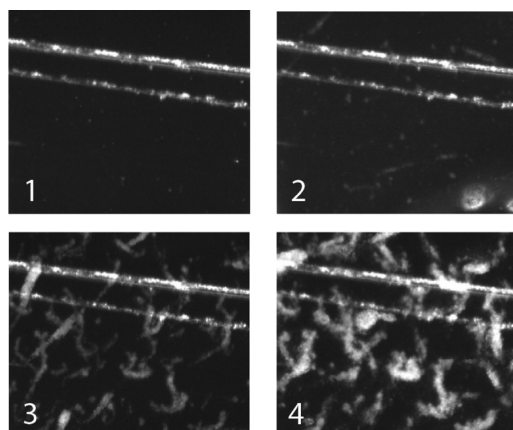


Figure 2. Images obtained with PVM device during single-strain culture experiments.

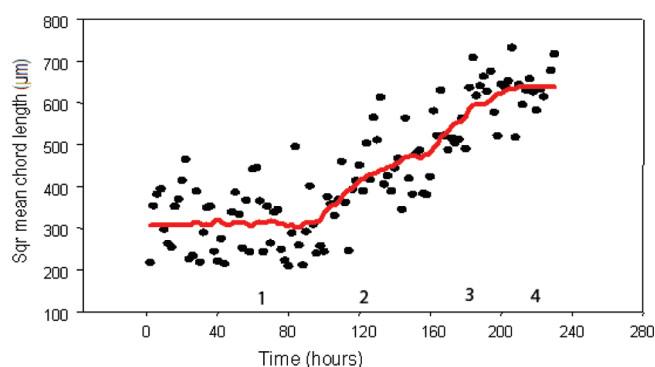


Figure 3. Evolution of the mean of the square-weighted chord length distribution during single-strain culture experiments.

distribution with

$$\bar{C} = \frac{\sum_{i=1}^{90} n_i M_i^3}{\sum_{i=1}^{90} n_i M_i^2} \quad (1)$$

The measurement interval of the PVM covers from 5 to 2000 μm . This gap is divided into 90 intervals to obtain the histogram. (e.g., [5–6], [6–8], ..., [1900–2000] μm), “ i ” represents the interval number (1, 2, ..., 90), n_i is the number of counts detected with dimensions between the boundaries of the interval i , and M_i is the midpoint of the interval i (μm).

The value of \bar{C} obtained at the beginning of the trial corresponds to the scratches on the window. Therefore, to obtain the values of \bar{C} associated with the formation of biofilm, it is necessary to subtract the initial \bar{C} value from the \bar{C} values obtained during the trial. The variable representing the biofilm grown is the increment of the \bar{C} value during the trial.

The appearance of a baseline region corresponding to the induction period (slow initial microbial attachment) is observed in Figure 3, followed by an exponential growth, which is detected between day 5 and day 8. Finally, the square mean chord length remained constant between day 8 and day 10, which indicates that the biofilm maturation was reached. The evaluation of image analysis corroborates that biofilm formation in culture medium

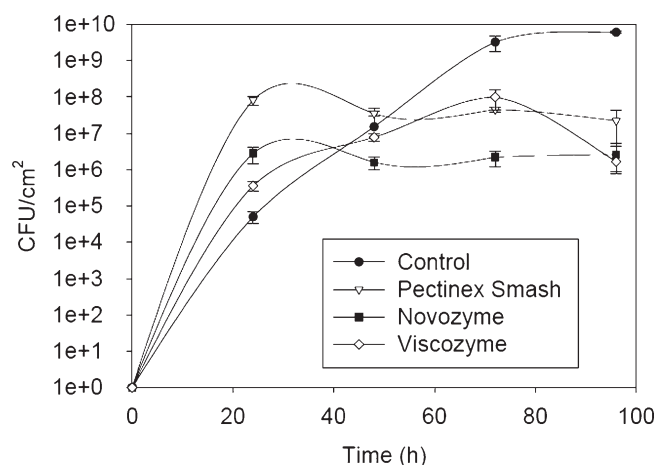


Figure 4. Effects of the selected enzymatic treatments on biofilm buildup in the 10 L system in natural flora experiments (where, for example, $1\text{e}+10$ represents 1×10^{10}). Viable counts on coupons.

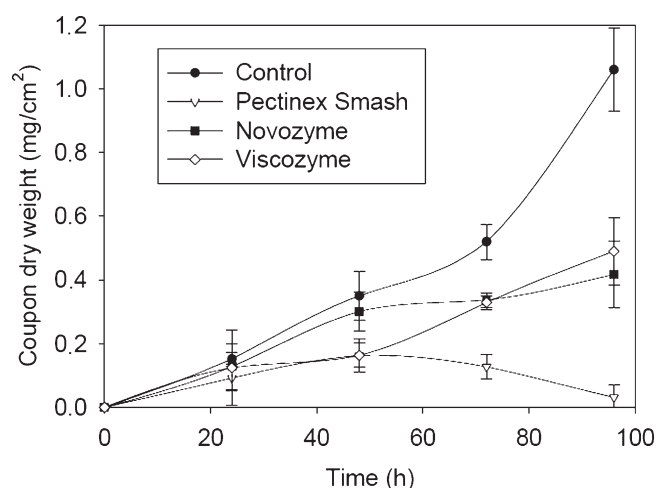


Figure 5. Effects of the selected enzymatic treatments on biofilm buildup in the 10 L system in natural flora experiments. Dry weight of biomass in the coupons.

can be measured using the PVM as online monitoring and that the evolution of the square-weighted mean chord length represents the biofilm growth successfully.

3.2. Natural Flora Experiments. The PVM methodology was used to study the effects of three enzymatic treatments on biofilm formation with natural flora present in the paper mill process water. Figures 4–6 show the results of biofilm buildup on coupons as CFU/cm² and as coupon dry weight (mg/cm²) and on the PVM window as square-weighted mean chord length (μm). Bacterial population was measured by the plate count of aerobic bacteria in the waters; the results of the counts are expressed as the number of colony forming units per milliliter (CFU/mL). During the experiments, the total bacterial count in the culture medium did not vary significantly as it was always between 10^7 and 10^8 CFU/mL.

The final values of dry weight and the CFU/cm² (96 h) on coupons extracted from the treated samples were significantly lower than the values of these parameters obtained from the coupons extracted from the control sample (Figures 4 and 5). This suggests that the treatments tested inhibit the formation of

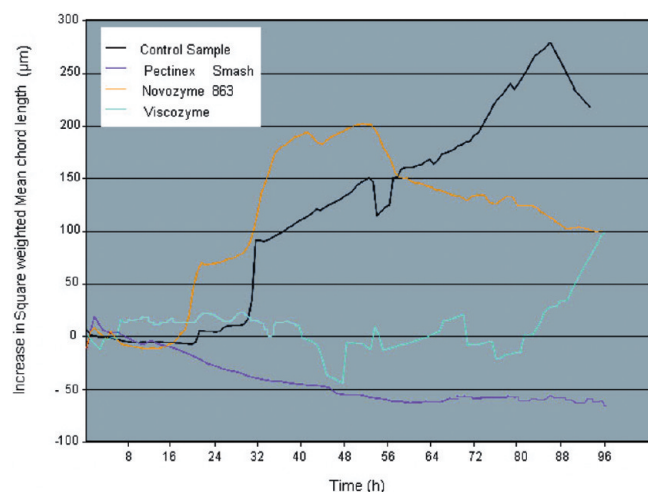


Figure 6. Effect of the treatments on the evolution of the square weighted mean chord length in natural flora experiments.

biofilm. Taking this into account, it is somehow surprising that the enzymatic treatment of the process water increased the number of CFU/cm² after 24 h with respect to the control sample (Figure 4). However, the dry weight of the biomass on the coupons extracted at 24 h was very similar in the four cases (Figure 5). This indicates that the treatment did not increase the mass of biofilm, but it increased the adherence of the cells to the coupons during the first stages, reducing the amount of micro-organisms removed during the extraction and washing out of the coupons with 9 mL of sterile saline solution. This effect is in accordance with the observations made by other authors that have proved that the presence of salts, disinfectants, or some other compounds, or other bacteria can increase the adhesion of bacteria and even the biofilm maturation.^{39,40}

At 48 h, the differences among the values of CFU/cm² decreased (Figure 4), but the dry weights of the biomass on the coupons, when no treatment or Novozyme treatment was used, were significantly higher than in the other two cases (Figure 5). Differences among the treatments increased with time because the amount of dry biomass on coupons extracted from the control sample increased continuously, while it remained constant in the case of using Novozyme or even decreased when Pectinex was used (Figure 5). This is in concordance with the increase of CFU/m² observed in the control sample and the constant results for CFU/cm² between time 48 and 96 h when Pectinex or Novozyme were used. Therefore, these two treatments do not prevent the bacteria attachment, but they inhibit the production of polysaccharides. These polysaccharides protect bacteria and attach other micro-organisms to them forming the biofilm. As a result, although these treatments are not biocide and do not attack the bacteria, they reduce the formation of the biofilm successfully, both by limiting the number of colonizing bacteria on the coupon surface and by hampering the formation of a biofilm on it, specially in the case of using Pectinex; but their effect was not evident within a short time.

It could seem that the treatment with Viscozyme could be more effective at shorter times than Novozyme (Figure 5); however, after 72 h the amount of biomass on the coupons increased, and at the end (96 h) it was higher than the biomass on the coupons extracted from the samples treated with the other

enzymes. During the first 48 h, the treatment affected polysaccharides production or stability and the adherence of bacteria was even lower than in the presence of other enzymes as shown by the lower value of CFU/cm² at 24 h (Figure 4); but after that time the effect was not strong enough to avoid the growth of biofilm, and the biomass weight increased with a similar trend to that of the control sample, although reaching lower coupon weight values.

To compare the results obtained through the PVM with the results obtained from the conventional techniques used at the industrial level (dry weight and coupon colonization), it must be taken into account that the first method is online and on-site and biofilm is not manipulated during the experiment, while conventional techniques require a manipulation that may cause the loss of part of biofilm, especially when it is weakly adhered to the surface, which is supposed to occur in the presence of enzymatic treatments.

Figure 6 shows the evolution of square-weighted mean chord length during the trials carried out with process waters. In the case of the control sample, the square-weighted mean chord length started increasing at around hour 20 to reach a maximum at around hour 80. Images in Figure 7 show the building up of biofilm when no treatment is used. At least two colonies can be observed in the images taken at 36 h, and their number increased later. First, the colonies appear in the scratches, where the adherence is better because of the rougher texture. Later on (see image taken at 70 h), the size of these colonies increases and more colonies are also detected in other parts of the window. This is reflected by the increase in the square-weighted mean chord length (Figure 6), and it is in accordance with the increase in the CFU/cm² and in the weight of dry biomass on the coupons (Figures 4 and 5).

When the Pectinex Smash treatment was used, the square-weighted mean chord length decreased slightly from the beginning of the trial. Images show that this decrease was due to the presence of some bubbles at the beginning of the trial that disappeared slowly in the course of the experiment (Figure 7). Bubbles are distinguished from the rest of the particles because of their very high brightness and particular shape, which is clear when the images are watched on the screen, at full scale (Figure 8). The results in this case of the images obtained by the PVM show that the treatment with Pectinex Smash is the most effective to prevent the formation of biofilm. In the case of Pectinex Smash the results of coupons analysis demonstrated that the colonization was the lowest from the three treatments and that inhibition of biofilm formation was effective. The same is concluded from the analysis of the images taken by the PVM, with some interference caused by the existing bubbles in the process waters at the beginning of the trial.

When using Novozyme 863, the square-weighted mean chord length remained constant during the first 16 h of the trial, but, after that, its value increased up to a maximum value at around hour 50 and, finally, it decreased. The PVM images obtained in this case (Figure 7) show a bacterial colonization located in the scratch of the window which remained during the experimentation and generates polysaccharides that are poorly attached to the surface of the window as they are detached around hour 50. Figure 4 shows a maximum value of CFU/cm² at 24 h and a slight decrease later, when this treatment was used. The differences between this result and results given by the PVM images are due to the window surface nature, which is polished sapphire, and, therefore, the attachment of bacteria can be slower than the

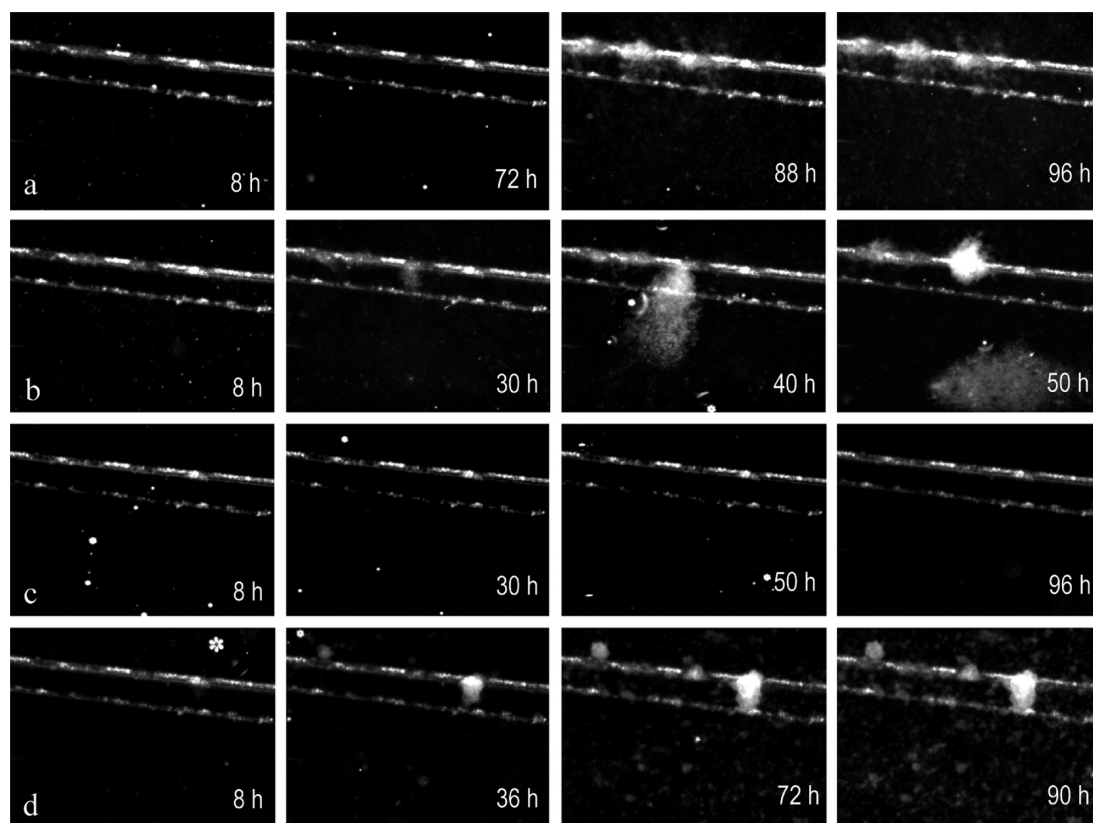


Figure 7. Images obtained with the PVM during the trials with the natural flora of papermaking process waters: (a) Viscozyme, (b) Novozyme 863, (c) Pectinex Smash, and (d) control sample.

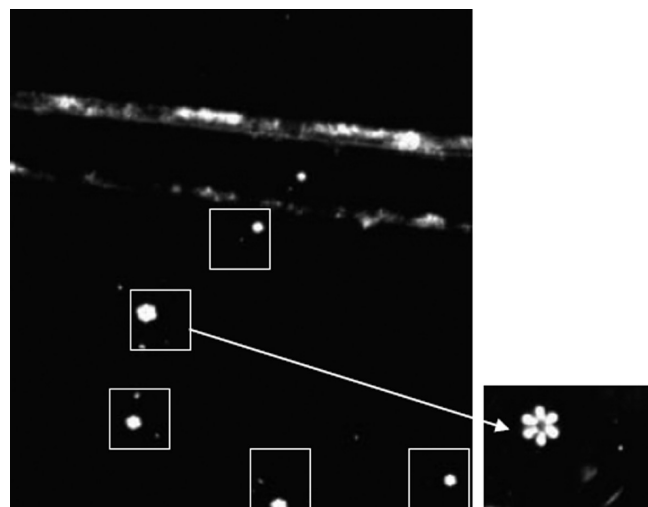


Figure 8. Identification of bubbles. The shape is the result of the reflection of the six lamps of the probe in the bubble's surface.

attachment of the bacteria to the PVC coupons. Therefore, the maximum value of the square mean chord length was reached at hour 50 instead of at hour 24. Furthermore, as shown in Figure 7, part of the material was separated from the colony at hour 50, which explains the observed decrease in the square mean chord length. This was not observed on the coupons because these polysaccharides were separated from the coupons extracted at 48, 72, and 96 h during some of the stages of the coupons analysis

procedure, which requires opening the coupons chamber, extracting the coupon, and manipulating it, stages that can lead to a loss of biomass. This is what is intended to be avoided by the use of PVM to monitor the biofilm growth.

When the treatment with Viscozyme was used, the increase in the value of the square-weighted mean chord length was delayed until around hour 80; then it increased until the end of the experiment. The values of the CFU/cm² obtained after 48 h were lower than those from the control sample, but a small decrease was observed at hour 96, which is probably due to the loss of cells during the manipulation of the coupons. The dry weight of the coupons shows a tendency similar to the control experiment, but with lower values than the control sample and lower slope of the curve. The slope increase was not observed until the coupon corresponding to hour 72 was extracted, but it is observed at hour 80 in the images taken by the PVM (Figure 7) and in the evolution of the square mean chord length (Figure 6). This difference is due to the different nature of the surfaces and is not a difference in the tendency, but only a difference in the attachment time.

4. CONCLUSIONS

The developed methodology based on taking images of biofilm formed on the PVM probe can be a powerful tool to (1) monitor biofilm formation and growth, (2) determine the effects of antimicrobial products on biofilm formation, and (3) select the most efficient enzymatic treatment and assess the effects of control systems on biofouling. The results obtained with the PVM probe in the absence of enzymatic treatment

matched those obtained with a conventional technique based on coupon biomass weight measurements. The conclusions extracted from the images and image analysis are in accordance with the conclusions extracted from the coupons analysis. There are only two sources of differences between the results obtained by the following two techniques: (1) the lower adherence of bacteria to the sapphire window, which can lead to a delay of the first stages of the biofilm formation, and (2) the loss of organic material of the biofilm during the manipulation of coupons.

The tried enzymatic treatments, especially Pectinex Smash, reduced successfully the formation of biofilm, and they should be considered an effective alternative to the biocides for the control of biofilm formation.

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