A new tool to monitor biofilm growth in industrial process waters

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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 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.
Keywords: Biofilm monitoring; biofilm control; particle videomicroscopy; enzymatic control.

1. Introduction

A bacterial biofilm is an organized community of microorganisms attached to a solid surface submerged 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 characterised by various factors such as surface material, nutrient availability, hydrodynamics, source of microbiological contamination, 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 towards closing water circuits drives paper mills to work at process conditions that favour the growth of microorganisms, since the re-use 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 procces 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 optimisation of any anti-microbial or antifouling program depends on the information obtained from the monitoring of biofilm formation and growth[10]. Ideally, this information should be acquired on-line, in-situ, non-destructively 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 analysed and (2) non-destructive techniques for on-line biofouling monitoring. Among the destructive techniques, the most frequently used are: 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 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 non-destructive 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 turbidity[12] and optical fibre sensors [27-28]), methods based on the measurement of changes in pressure drop and/or heat or electrical transfer resistance due to biofilm accumulation[29] and electrochemical techniques (impedance[30] and capacitance[31]). 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 fibre sensors are based on the measurement of the light intensity received by a detector not involving therefore taking any actual image.
In this study a new on-line 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 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 to 1% is converted into a wet web, which have a solids level of about 15 to 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 comercial in-situ imaging system developed by Lasentec from Mettler Toledo AutoChem (USA), 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 on-line 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 saphire 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 saphire window by a new one with two scratches to favour 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 inmediately adjacent to the external window surface. The light reflected by the particles attached to the window is carried to the detector through an optic fibre cable. The detector generates a black and white image of a 850 x 654 μm^2 surface with an approximate resolution of 3-5 μm[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 each 4 h from all the chord lengths obtained during the analysis of the 10 images collected during 5 min.

2.2. Experimental set-up

The experimental set-up, which is based on the one developed by Pedersen[36] 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 similar way as it occurs in a paper mill. The biofilm is developed mainly by aerobic and facultative anaerobic bacteria being possible the existence of anaerobic bacteria in the centres 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 centre 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 set-up 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 assure turbulent flow. Since the hydrodynamic conditions affect notably

the biofilm build-up and it is very difficult to reproduce the hydrodynamic conditions of the mill at laboratory scale, the effect of the enzymes on biofilm build-up was studied at constant and turbulent hydrodynamic conditions, which allows comparing 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, establishing the effect of each treatment on the biofilm build-up 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).

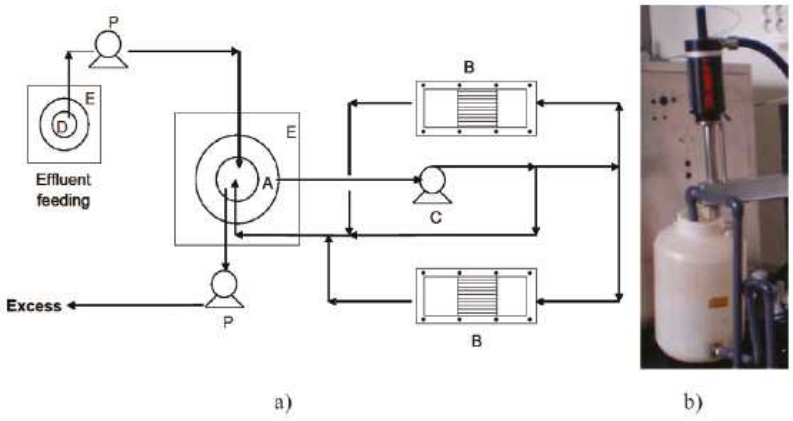

Figure 1. a) Continous flow system: Stirred tank (A), flow chamber with coupons (B), centrifugal pump (C), feeding tank (D), Isothermic bath (E) and peristaltic pump (P). b) Probe location in the system.

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 37ºC in a medium prepared to enhance the production of polysaccharides. The medium contained glucose (20 g/L), yeast extract (0.5 g/L), $(NH_4)_2SO_4$ (0.6 g/L), KH_2PO_4 (3.18 g/L), K_2HPO_4 (5.2 g/L), $MgSO_4$ x $7H_2O$ (0.3 g/L), $CaCl_2$ (0.05 g/L), $ZnSO_4$ x $7H_2O$ (0.2 mg/L), $CuSO_4$ x $5H_2O$ (0.2 mg/L), $MnSO_4$ x H_2O (0.2 mg/L), $FeSO_4$ x $7H_2O$ (0.6 mg/L) and $CoCl_2$ (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 reaching a mature biofilm, which in this case took 10 days. Temperature was kept at 30ºC by using thermostatic baths (Figure 1a) and pH was controlled with addition of NaOH 0.1% solution. Every 24 h 500 mL of medium were replaced by sterile culture medium in order to add 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 ºC and at a pH of 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 folowing two hours 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, Krogshoejvej 36, DK-2880 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 optimisation 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 untill dryness and then it was weighted. The result was expressed in mg/cm^2. The other coupon of the pair extracted was employed to determine the colony forming units per square centimetre of coupon surface (CFU/cm^2). This coupon was removed with sterile forceps and flushed with 9 mL 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 serie of dilutions was performed and it was plated on plate count agar. Plates were incubated at 30 ºC 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 activies). The experiments were conducted in series. Before each trial the system was cleaned and desinfected 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 are 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 above. 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 lengths 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, $\overline{C}$ (µm), is calculated from each chord length distribution with equation (1).

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

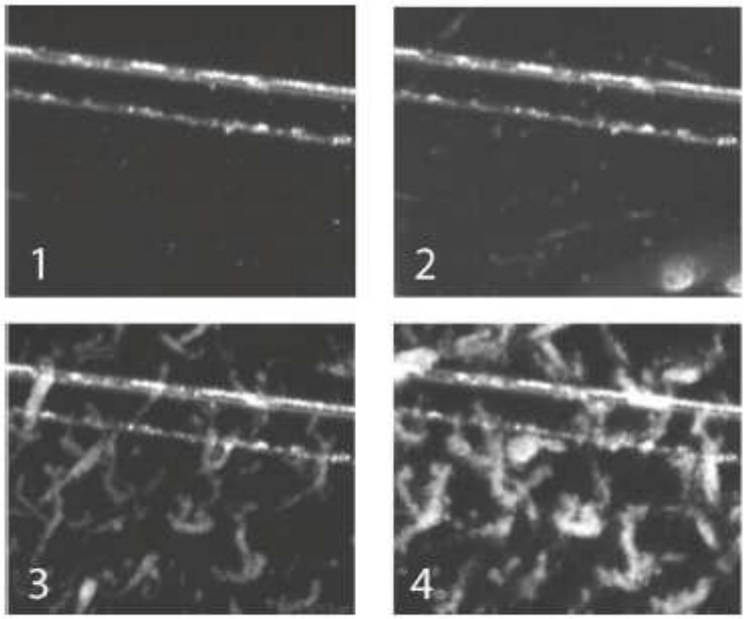

Figure 2. Images obtained with PVM device during single strain culture experiments.

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) µm, [6-8) µm ,.., [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 $\overline{C}$ obtained at the beginning of the trial corresponds to the scratches on the window. Therefore, to obtain the values of $\overline{C}$ associated to the formation of biofilm, it is necessary to subtract the initial $\overline{C}$ value from the $\overline{C}$ values obtained during the trial. The variable representing the biofilm grown is the increment of the $\overline{C}$ value during the trial.

The appearance of a base line 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 images analysis corroborates that biofilm formation in culture medium can be measured using the PVM as on-line monitoring and that the evolution of the square weighted mean chord lentgh represents the biofilm growth sucessfully.

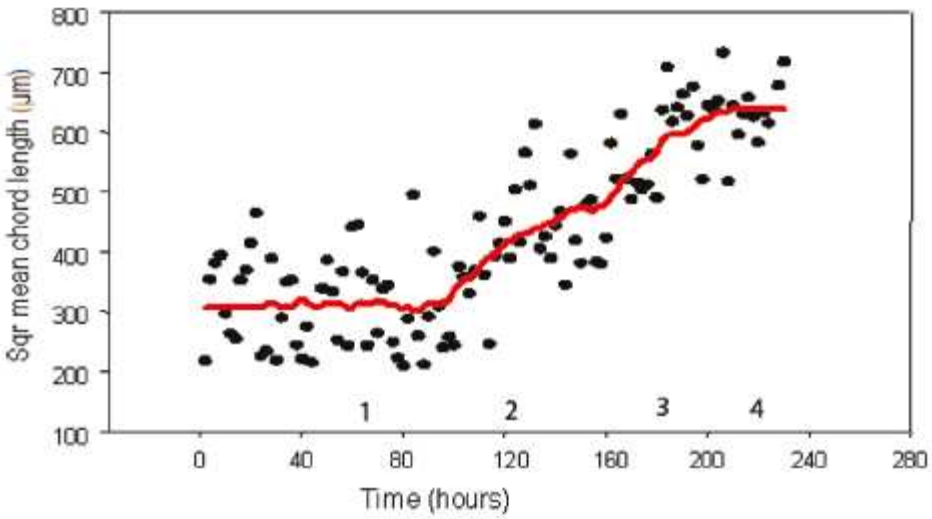

Figure 3. Evolution of the mean of the square weighted chord length distribution during single strain culture experiments.

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, 5 and 6 show the results of biofilm build-up on coupons as CFU/cm^2 and as coupon dry weight (mg/cm^2) and on the PVM window as square weighted mean chord length (µm). Bacterial population was measured by plate count of aerobic bacteria in the waters; the results of the counts are expressed as number of colony forming units per (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.

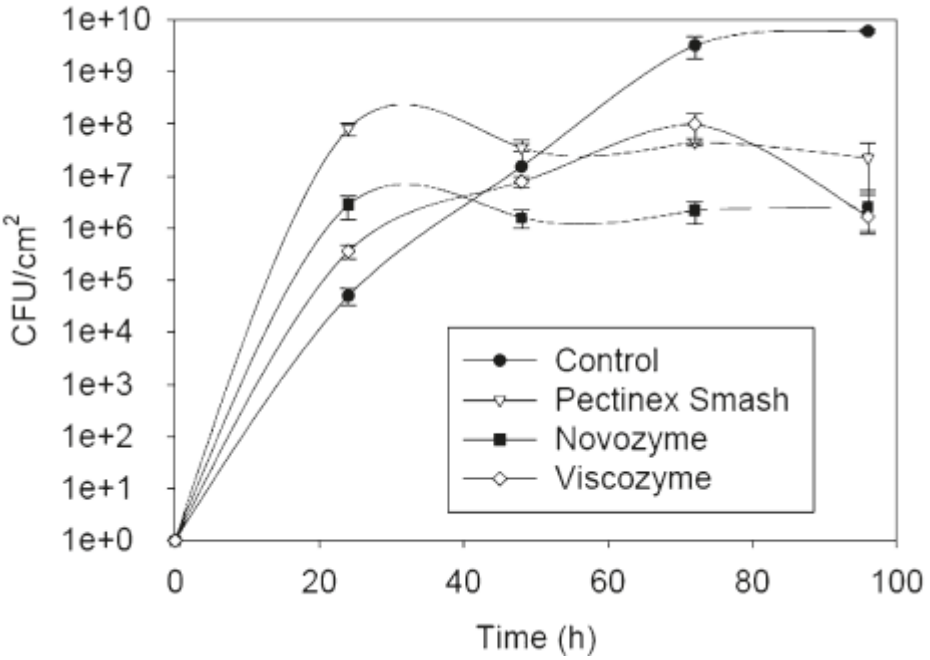

Figure 4. Effects of the selected enzymatic treatments on biofilm build up in the 10 L system in natural flora experiments. Viable counts on coupons.

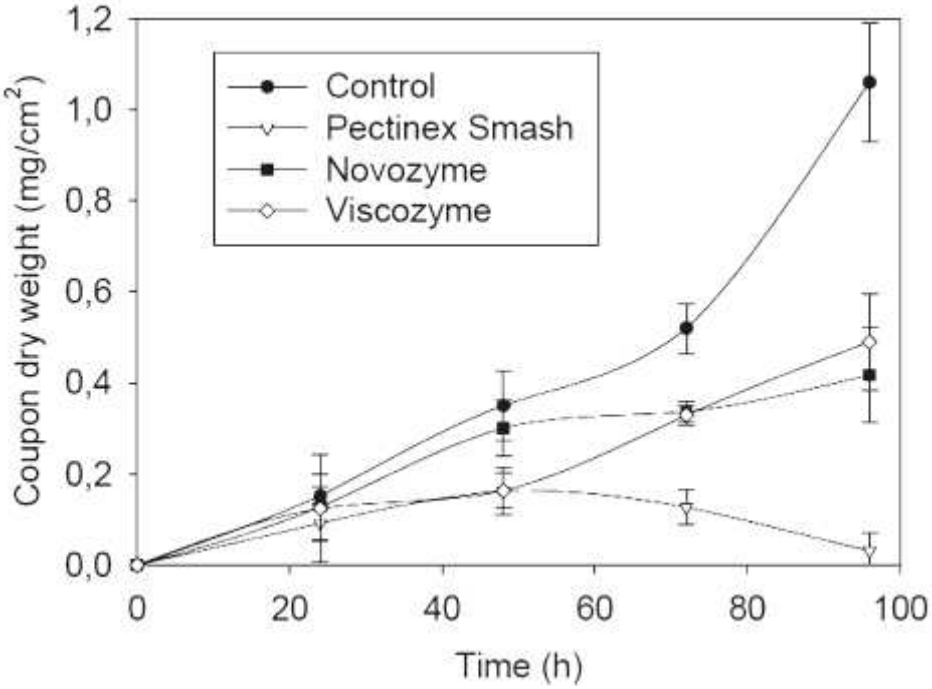

Figure 5. Effects of the selected enzymatic treatments on biofilm build up in the 10 L system in natural flora experiments. Dry weight of biomass in the coupons.

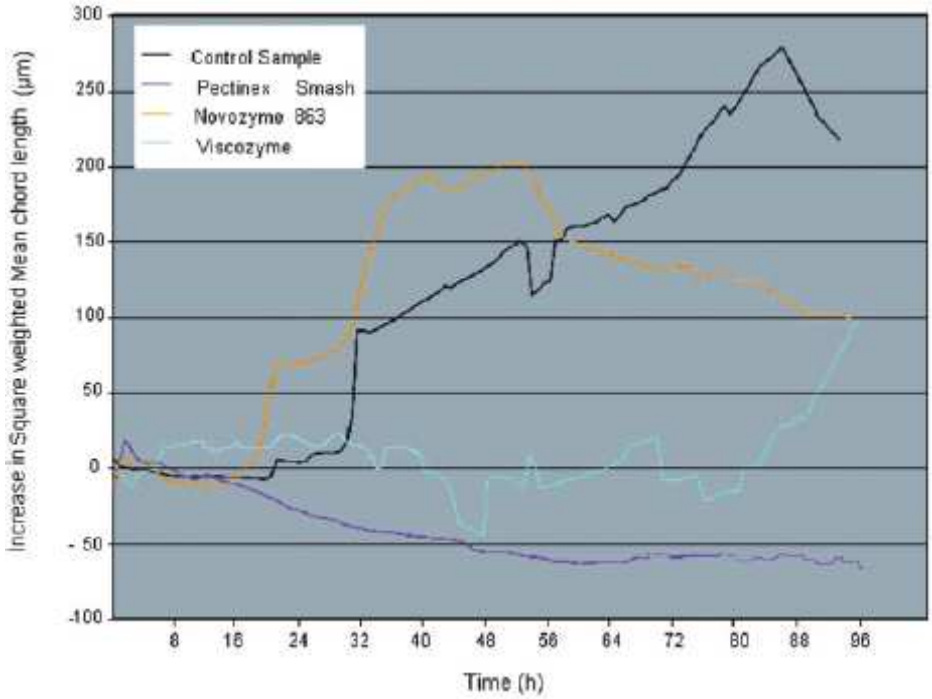

Figure 6. Effect of the treatments on the evolution of the square weighted mean chord length in natural flora experiments.

The final values of dry weight and the CFU/cm^2 (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 biofilm. Taking this into account, it is somehow surprising that the enzymatic treatment of the process water increased the

number of CFU/cm^2 after 24 h with respect to the control sample (figure 4). However, the dry weight of 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 microorganisms 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/cm^2 decreased (figure 4), but the dry weights of 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/m^2 observed in the control sample and the constant results for CFU/cm^2 between time 48 h 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 microorganisms 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 presence of other enzymes as shown by the lower value of CFU/cm^2 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 on-line 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 surface, which is supposed to occur in 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/ cm^2 and in the weight of dry biomass on the coupons (Figures 4 and 5).

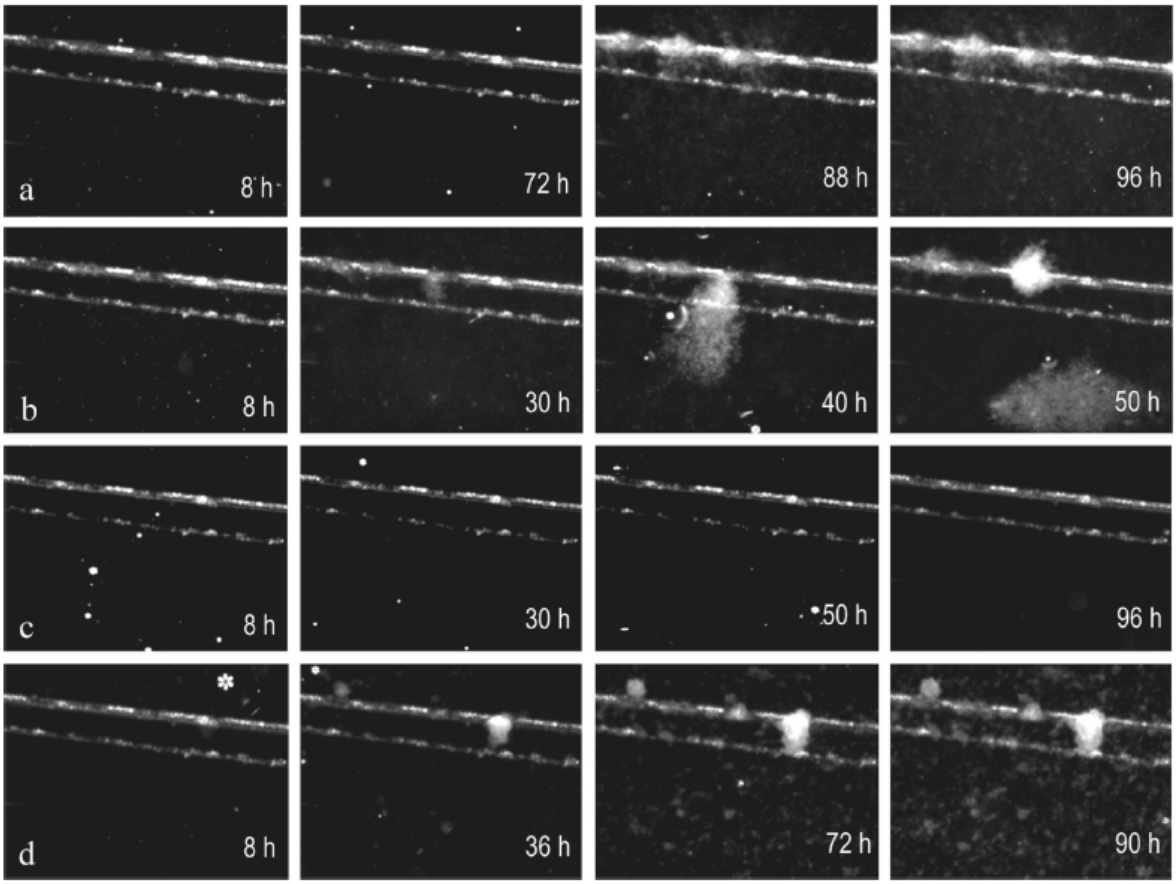

Figure 7. Images obtained with the PVM during the trials with the natural flora of papermaking process waters: a) Viscozyme®, b) Novozyme 863®, c) Pectinex Smash®, d) Control sample.

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/ cm^2 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 attachment of the bacteria to the PVC coupons. Therefore, the maximum value of the square mean chord

length was reached at hour 50 instead 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.

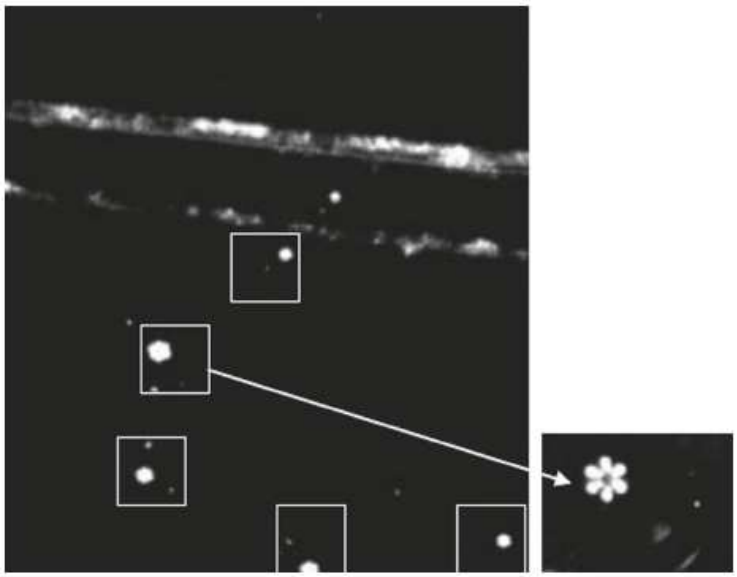

Figure 8. Identification of bubbles. The shape is the result of the reflexion of the six lamps of the probe in the bubble's surface.

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/ cm^2 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 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 reults obtained by the two tecniques: (1) the lower adherence of bacteria to the shapphire 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 sucessfully the formation of biofilm and they should be considered as an effective alternative to the biocides for the control of biofilm formation.

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REFERENCES

(1) Verhoef, R.; Schols, H.A.; Blanco, A.; Siika-aho, M.; Ratto, M.; Buchert, J.; Lenon, G.; Voragen, A.G.J. Sugar composition and FT-IR analysis of exopolysaccharides produced by microbial isolates from paper mill biofilm deposits. *Biot. Bioeng.* **2005**, 91(1), 91-105.

(2) Ratto, M.; Verhoef, R.; Suihko; M.L.; Blanco, A.; Schols, H.A.; Voragen, A.G.J.; Wilting, R.; Buchert, J. Colanic acid is an exopolysaccharide common to many enterobacteria isolated from paper-machine slimes. *J. Ind. Microbiol. Biot.* **2006**, 33(5), 359-367.

(3) Characklis, W.; Marshall, K. Biofilms and microbial fouling. *Adv. Appl. Microbio*. **1990**, 29, 93-138.

(4) Videla, H. Prevention and control of biocorrosion. *Int. Biodeterior. Biodegrad.* **2002**, 49, 259-270.

(5) Lahtinen, T.; Kosonen, M.; Tiirola, M.; Vuento, M.; Oker-Blom, C. Diversity of bacteria contaminating paper machines. *J. Ind. Microbiol. Biotech.* **2006**, 33(9), 734-740.

(6) Kolari, M.; Nuutinen, J.; Rainey, F.A.; Salkinoja-Salonen, M.S. Colored moderately thermophilic bacteria in paper-machine biofilms. *J. Ind. Microbiol. Biotech.* **2003**, 30(4), 225-238.

(7) Blanco, M.A.; Negro, C.; Gaspar, I.; Tijero, J. Slime problems in the paper and board industry. *Appl. Microbiol. Biot.* **1996**, 46(3), 203-208.

(8) Blanco, A. Microbiology in papermaking. *Recent Res Dev Appl Microbio Biot*. **2003**,1- 87.

(9) Blanco, A.; Negro, C.; Monte, C.; Fuente, E.; Tijero, J. The challenges of sustainable papermaking. *Environ. Sci. Tech.* **2004**, 38(21), 414A-420A.

(10) Ludensky, M. Control and monitoring of biofilms in industrial applications. *International. Biodeter. Biodegrad.* **2003**, 51, 255-263.

(11) Momba, M.; Kfir, R.; Venter, S.; Cloete, T. An overview of biofilm formation in distribution system and its impact on the deterioration of water quality. *Water S.A.* **2000**, 26, 59-66.

(12) Klahre, J.; Flemming, H. Monitoring of biofouling in papermill process waters. *Water Res*. **2000**, 34(14), 3657-3665.

(13) Patching, J.W.; Fleming, H.C. Industrial biofilms: formation, problems and control. In: Lens, P.; Moran, A.P.; Mahony, T.; Stoodley P.; O'Flaherty, V. Editors. *Biofilms in Medicine, Industrial Environmental Biotechnology.* IWA Publishing, UK. **2003**, 568–572.

(14) Flemming, H.C.; Tamachkiarowa, A.; Klahre, J.; Schmitt, J. Monitoring of fouling and biofouling in technical systems. *Water Sci. Technol.* **1998**, 38(8), 291-298.

(15) Simoes, M.; Simoes, L.C.; Vieira, M.J. A review of current and emergent biofilm control strategies. *LWT - Food Sci. Technol*. **2010,** 43(4), 573-583.

(16) Shi, X.; Zhu, X. Biofilm formation and food safety in food industries. *Trends food Sci. Technol.* **2009**, 20(9), 407-413.

(17) Meyer, B. Approaches to prevention, removal and killing of biofilms. *Int. Biodeter. Biodegrad.* **2003**, 51(4), 249-253.

(18) Torres, C.E.; Gibello, A.; Nande, M.; Martin, M.; Blanco, A. Fluorescent in situ hybridization and flow cytometry as tools to evaluate the treatments for the control of slime-forming enterobacteria in paper mills. *Appl. Microbiol. Biot.* **2008**, 78, 889-897.

(19) Costerton, J.; Lewandowski, Z.; Caldwell, D.; Korber, D.; Lappin-Scott, H. *Microbial biofilms Annu. Rev. Microb.* **1995**, 49, 711-745.

(20) Ludensky, M. An automated system for biocide test-ring on biofilms. *J. Ind. Microbiol. Biot.* **1998**, 20, 109-115.

(21) Wirtanen, G.; Salo, S.; Helander, I.; Mattila-Shaldom, T. Microbiological methods for testing disinfectant efficiency on Pseudomonas biofilm. *Colloid Surface B.* **2001,** 20, 37-50.

(22) Chavant, P.; Gaillard-Martinie, B.; Talon, R.; Hebraud, M.; Bernardi, T. A new device for rapid evaluation of biofilm formation potential by bacteria. *J. Microbiol. Method.* **2007**, 68, 605-612.

(23) Hadjiev, D.; Dimitrov, D.; Martinov, M.; Sire O. Enhancement of the biofilm formation on polymeric supports by surface conditioning. *Enzyme Microb. Tech.* **2007**, 40, 840-848.

(24) Christensen, B.; Sternberg, C.; Andersen, J.; Palmer, R.; Nielsen, A.; Givskov, M.; Molin, S. Molecular tools for study of biofilm physiology. *Method. Enzymol*. **1999**, 310, 20-42.

(25) Jass, J.; Costerton, J.; Lappin-Scott, H. Assessment of a chemostat-coupled modified Robbins device to study biofilms. *J. Ind. Microbiol. Biot*. **1995**, 15, 238-289.

(26) Morris, C.; Monier, J.; Jacques, M. Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. *Appl. Environ. Microbiol.* **1997**, 63, 1570-1576.

(27) Tamachkiarw, A.; Flemming, H. On line monitoring formation in a brewery water pipeline system with a fibre optical device. *Water Sci. Technol*. **2003**, 47, 19-24.

(28) Chandy, R.; Scully, P.; Eldridge, P.; Kadim, H.; Grapin, M.; Guy, M.; D´Ambrosio, M.; Colin, F. An optical fiber sensor for biofilm measurement using intensity modulation and image analysis. *IEEE J. Sel. Top. Quant*. **2000**, 6(5), 764-762.

(29) Janknecht, P.; Melo, L. On line biofilm monitoring. *Rev. Environ. Sci. Biotech.* **2003**, 2, 269-283.

(30) Ramaraja, P.; Ren, Z.; Mench, M.; Reagan, J. Impact of initial biofilm growth on the anode impedance of microbial fuel cells. *Biotech. Bioeng*. **2008**, 101, 101-108.

(31) Mauricio, R.; Dias, C.J.; Santana, F. Monitoring biofilm thickness using a non-destructive, on-line, electrical capacitance technique. *Env. Monit. Assess*. **2006**, 119, 599-607.

(32) Kougoulos, E.; Jones, A.G.; Jennings, K.H.; Wood-Kaczmar, M.W. Use of focused beam reflectance measurement (FBRM) and process video imaging (PVM) in a modified mixed suspension mixed product removal (MSMPR) cooling crystallizer. *J. Cryst. Growth*. **2005**, 273, 529-534.

(33) Negro, C.; Fuente, E.; Sanchez, L.M.; Blanco, A.; Tijero, J. Evaluation of an Alternative Flocculation System for Manufacture of Fiber-Cement Composites. *Ind. Eng. Chem. Res*. **2006**, 45, 6672-6678.

(34) Negro, C.; Fuente, E.; Sanchez, L.M.; Blanco, A.; Tijero, J. Polyacrylamide induced flocculation of a cement suspension. *Chem. Eng. Sci*. **2006**, 61, 2522-2532.

(35) Hamann, O.S.; Montague, T.W.; Reed, B.W.; Hokanson, J.V. Imaging apparatus of multiphase fluid medium - has image detector with CCD array which outputs image to electronics package, for suitable conversion. Pattent number: WO9845682-A. **1998.**

(36) Pedersen, K. Method for Studying Microbial Biofilms in Flowing Water Systems. *Appl. Environ. Microbiol.* **1982**, 43(1), 6-13.

(37) Costerton, J.W.; Lewandowski, Z.; Debeer, D.; Caldwell, D.; Korber, D.; James, G. Biofilms, the Customized Microniche. *J. Bacteriol*. **1994**, 176(8), 2137-2142.

(38) Torres, E.; Blanco, A.; Fuente, E.; Negro, C. Slime forming microorganisms monitoring in the paper industry. 4th International meeting on biotechnology, towards a sustainable bioeconomy, *Biotec 2008*, Granada, Spain, September 17-19, **2008**, 169.

(39) Jensen, A.; Larsen, M.; Ingmer, H.; Vogel, B.; Gram, L. Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of Listeria monocytogenes strains. *J. Food Protection*. **2007**, 70, 592-599,

(40) Carpentier, B.; Chassaing, D. Interactions in biofilms between Listeria monocytogenes and resident microorganisms from food industry premises. *Int. J. Food Microbiol.* **2004**. 97, 111-122.