3D reconstruction of Artificially Structured Microbial Consortia (ASMC) by image analysis

F. Castillo-Olvera[1], C. A. González-Ramírez[1*], J. S. Andrews[2], G. H. Markx[3], G. A. Vázquez-Rodríguez[1], E. M. Otazo-Sánchez[1], F. Prieto-García[1]. ([*]e-mail: ccr_gonzalez@yahoo.com)

[1] Área Académica de Química, Universidad Autónoma del Estado de Hidalgo. Carretera Pachuca-Tulancingo km 4.5, C.P. 42076, Mineral de la Reforma, Hidalgo, México.

[2] Department of Civil and Structural Engineering, The University of Sheffield, Sheffield, S1 3JD, UK.

[3] Department of Chemical Engineering, Heriot Watt University, Edinburgh, EH14 4AS, Scotland, UK.

Abstract A biofilm is formed when microorganisms are attached to a surface and, within the whole biofilm; microorganisms are only a small volume fraction, which is complemented by the extracellular matrix of the structure. Inside the structure, fluids flow carrying, in and out, dissolved nutrients and intermediate metabolic products, which can be consumed by other bacterial species in the biofilm. In order to study these phenomena, Artificially Structured Microbial Consortia (ASMC) were constructed using *Acinetobacter* sp. C6 and *Pseudomonas putida* R1, this strain is a Green Fluorescent Protein (GFP) producer that was monitored. ASMC were constructed for degrading benzyl alcohol, which is initially transformed into benzoic acid, by *Acinetobacter* sp. C6, and then it is consumed until full aerobic degradation by *Pseudomonas putida* R1. The progress in time of ASMC was followed using confocal laser scanning microscopy (CLSM) obtaining cross-section micrographies for image analysis. 3D reconstruction of these images was achieved using information from the plane and confocal cross-sections of the ASMC. This image analysis technique takes into account the edge of each cross-section, which is defined by the GFP producer species. Pixels intensity was estimated using data from bitmap files that were obtained after processing the confocal images; this information was useful for studying biofilm morphology and carrying out 3D image reconstruction.

Keywords *Acinetobacter* sp. C6; biofilm; image analysis; *Pseudomonas putida* R1; 3D reconstruction.

INTRODUCTION

In nature, microorganisms frequently grow as attached members of complex communities (Costerton *et al*., 1995). Although these communities are diverse, it is possible to take advantage of microbial ecology for bioremediation purposes, an example of multi-component biofilms is a community including the following three components, algae, bacterial and exopolimeric substances (Lawrence *et al*., 1998; Yang *et al*., 2000). Microscopy has long been considered an essential investigative tool in the study of complex micro-biological environments, such as those encountered in modern non-invasive biofilm research, which may be conducted *in situ* at cellular level (Kyana *et al*., 2005). CLSM it is found to be one of the most versatile and effective methods, due to the possibility of quantifying spatiotemporal parameters in biofilms (Beyenal *et al.*, 2004). When combining CLSM with fluorescent strains, it is possible to obtain a quantitative evaluation of three-dimensional biofilm structures (Kyana *et al.*, 2005). The

key, for identifying additional applications of combined microscopy techniques, consists in developing research that involves specific tools, which are initially isolated, such as: gene transfer, ASMC micro-fabrication, fluorescence detection, spectroscopy, CLSM, image processing, analysis and projection, as well as 3D reconstruction; in order to integrate these tools for new applications (Lawrence *et al.,* 1998).

OBJECTIVE

To analyse the three-dimensional morphology from CLSM cross-section images of a micro-colony, constructed into an ASMC, by reconstructing the 3D structure.

METHODS

ASMC were constructed following a dielectrophoretic technique using interdigitated castellated microelectrodes that were prepared by photolithography (Andrews *et al*., 2004). 3D reconstruction was performed using CLSM images of an ASMC made of two species: *Pseudomonas putida* R1, which is a GFP producer strain that consumes benzoic acid, and *Acinetobacter sp*. C6, which is benzoic acid producer and benzyl alcohol consumer (Tolker-Nielsen *et al*., 2000; Xu *et al*., 2003). This consortium was selected for developing experiments with different structural arrangements of the ASMC in order to follow benzyl alcohol degradation and benzoic acid production/degradation as an example of potential wastewater treatment applications, by using this sort of multi-species arrangements. ASMC were monitored by CLSM obtaining focal and confocal (transversal and longitudinal cross-sections) images of the microbial consortium structure (Andrews, 2004). Digital images are processed and converted into bitmap files for an accurate collection of information from pixels intensity and axis coordinates of each green pixel within the image, these data are useful when plotting these numbers to reconstruct the original image of focal and cross-sectional views of the micro-colony. The whole process is accomplished using a purpose-made Visual Basic® program (Gonzalez-Ramirez, 2006). This program was designed for calculating each pixel intensity and colour composition. A graphical reconstruction of the real image is performed using data obtained with this program. Collected data are stored in specific text files according to the Red-Green-Blue (RGB) format, in this case only the green colour was of interest. Further image reconstruction was achieved by plotting numerical data obtained from the bitmap file analysis. In order to carry on with a three dimensional reconstruction, z-axis limits (contour of image depth) were measured by reconstructing both, transversal and longitudinal cross-sections of the confocal image. Additionally, green colour intensity, from pixels of the image, was considered for evolving an interpolated and full view across the z-axis (Beyenal *et al.*, 2004).

RESULTS

Images obtained by CLSM of ASMC constructed using interdigitated castellated microarrays were processed taking cross-sections from the confocal image and also using the focal view of a micro-colony within the biostructure.

A micro-colony was chosen from the whole ASMC and cross sections of the image were cut off the image in order to carry out the image analysis process that has been above described.

Information about green intensity and x-y-z coordinates of each pixel was collected from each section of the image. Once this numerical information was stored in specific files, it was performed the image analysis and reconstruction algorithm, in order to obtain a 3D image from real data generated using CLSM. Valuable information from the image

was generated since green color intensity has a direct relationship with metabolic cell activity.

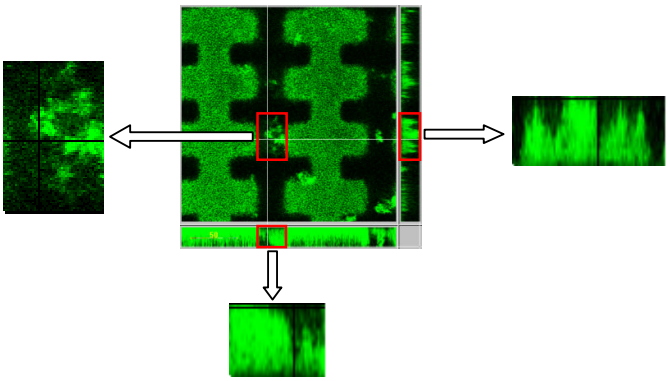

Figure 1. Confocal image, including cross sections, of the micro-colony that was analyzed and 3D reconstructed (kindly provided by Andrews, J.S., 2004).

3D image reconstruction was achieved using pixels information from the confocal image. Focal and confocal sections of the image were used considering a *x-y-z* axis distribution, in order to place these data in corresponding three dimensional planes. Image analysis process was carried out selecting green pixels from the image and measuring green intensity in a 0-1 scale, meaning 0 a completely black pixel and 1 an absolutely white pixel, with intermediate values being related to pixels color intensity. Axis coordinates were obtained from the image analysis software and related to a real micrometric scale of the image, in order to generate a real scale reconstruction.

It is also important to mention that green fluorescent *Pseudomonas putida* R1 was imbedded within a polymeric matrix and in a consortium formed with *Acinetobacter* sp. C6, which has no fluorescence to be detected. Therefore, some of the green pixels in the 3D reconstruction may appear not attached to the biofilm, however, it is because both *Acinetobacter* sp. C6 and the polymeric matrix were not visible in the image but green pixels were present and visible inside them.

Figure 2 shows results of the 3D reconstruction using numerical data obtained from image analysis of focal and confocal sections of the image. These data are stored in numerical format and are useful for quantitative assessment of the biofilm structure, estimation of morphological parameters and quantification of internal metabolites concentration profiles, in order to evaluate mathematical models about environmental biofilms.

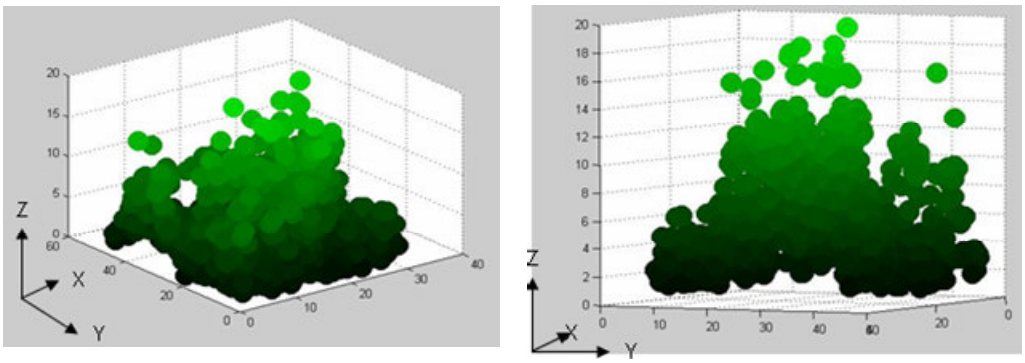

Figure 2. 3D-reconstructed images of a micro-colony constructed within an Artificially Structured Microbial Consortium (ASMC).

CONCLUSIONS

A three dimensional reconstruction of a micro-colony constructed within an ASMC was achieved by using CLSM and considering numerical information from pixel coordinates (*x*-*y*-*z* axis) and green color intensity. This information is also useful for measuring contour limits with respect to the *z*-axis from cross-sections of a confocal image. It was also possible to obtain benzoic acid concentration profiles, in a 3D structure, by relating green color pixel intensity with benzoic acid concentration and metabolic activity of the GFP producer strain. After analysing this information it is possible to relate biofilm behaviour prediction using mathematical models with 3D real data from experiments on ASMC. By using this information 3D mathematical models will find an accurate source of information through the image analysis process that has been proposed in this work. This will lead to a reliable source of data for mathematical modelling validation purposes.

ACKNOWLEDGEMENTS

University of Manchester, Autonomous University of Hidalgo State (UAEH) and National Science and Technology Council (CONACyT) of Mexico, for the support and funding provided.

REFERENCES

Andrews, J.S., 2004. Confocal image of a biofilm structured. *Private communication*.

Andrews, J.S., Mason, V.P., Thompson, I.P., Stephens, G.M. and Markx, G.H., 2006. Construction of artificially structured microbial consortia (ASMC) using dielectrophoresis: Examining bacterial interactions via metabolic interactions within environmental biofilms, *Journal of Microbiological Methods*, 64, 96-106.

Beyenal, H., Donovan, C., Lewandowski Z., Harkin, G., 2004. Three-dimensional biofilm structure quantification, *Journal of Microbiological Methods*, 59, 395-413.

Costerton J.W., Lewandowski Z., Caldwell D.E., Kurber D.R. and Lappin-Scott H.M., 1995. Microbial biofilms, *Annual Reviews Microbiology*, 49, 711-745.

Gonzalez-Ramirez, C.A., 2006. Analysis of Transport in Environmental Biofilms. A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences. School of Chemical Engineering and Analytical Science.

Kyana, M., Guanb L., Lissc, S., 2005. Refining competition in the self-organising tree map for unsupervised biofilm image segmentation, *Neural Networks*, 18, 850-860.

Lawrence, J.R., Neu, T.R., Swerhone, G.D.W., 1998. Application of multiple parameter imaging for the quantification of algal, bacterial and exopolymer components of microbial biofilms, *Journal of Microbiological Methods* 32, 253-261.

Tolker-Nielsen T., Brinch V.C., Ragas P.C., Andersen J.B., Jacobsen C.S. and Molin S., 2000. Development and dynamics of *Pseudomonas sp*. Biofilms, *Journal of Bacteriology*, 182-22, 6482-6489.

Xu T., Petridou S., Lee E.H., Roth E.A., Vyavahare N.R., Hickman J.J. and Boland T., 2003. Construction of high-density bacterial colony arrays and patterns by the ink-jet method, *Wiley interscience*.

Yang, X., Beyenal, H., Harbin, G., Lewandowski, Z., 2000. Quantifying biofilm structure using image analysis, *Journal of Microbiological Methods*, 39, 109-119.