Ctr. 51BM/2016: Summary

- **Project title: "Investigation on Function and Micro-Structure of the Cellulase Secretion System by High Resolution Imaging Techniques"**
- **Acronym: CESESYS**
- **Year of completion: 2017**
- **Project duration: 15 months**
- **Romanian partner: University Politehnica of Bucharest**
- **Project director: Dr. Stefan G. Stanciu**
- **Chinese partner: Guangxi Academy of Sciences in Nanning**
- **Chinese Team Leader: Prof. Guang Wu**

With their joint work in the frame of CESESYS, the Center for Microscopy-Microanalysis and Information Processing, part of University Politehnica of Bucharest (CMMPI-UPB) and the Guangxi Academy of Sciences in Nanning (GXAS) have aimed at establishing a set of research directions that will lead towards solving a set of technological problems in the domain of renewable energy production and exploitation based on materials derived from biological sources. More precisely, this collaboration tackled scientific subjects of interest for yielding novel types of biomass plantations with increased efficiency for biofuel production, which in the future are likely to play a key role in the domain of renewable energy. CMMPI-UPB and GXAS have designed and explored research avenues that combine the aplication of high resolution imaging techniques and cutting-edge biological engineering approaches that pave the way for resolving various aspects behind the secretion system of cellulases which are poorly understood at the time being: (i) Identification of a cell's characteristics that hint towards its abilities to secret celllulases; (ii) Identification of sub-cellular locations of cellulases, (iii) Identification of secretion pathways specific to cellulases and understanding the way in which these can determine the modality by which the cellulases are secreted

The importance of these subjects for increasing the efficiency of biomass plantations in the purpose of biofuels production is closely correlated to the fact the cellulose is the main polymer in biomass and cellulases can hydrolyze it to cellobiose, which can be converted to glucose by β-glucosidase. Cellulases are currently the third largest industrial enzyme worldwide, by dollar volume, because of their use in cotton processing, paper recycling, as detergent enzymes, in juice extraction, and as animal feed additives. Moreover, cellulases will become the largest volume industrial enzyme, if ethanol, butanol, or some other fermentation product of sugars, produced from biomass by enzymes, will become a major transportation fuel, as many studies predict.

The two partners have worked together to design novel workflows that combine latest hour technologies in high-resolution imaging (that operate at both micro and nanoscale) and methods of biological engineering (mainly microorganisms engineering and enzyme engineering), which in the future might lead towards resolving important aspects with critical

roles in designing novel biological species capable to yield high quantities of cellulases in short time intervals. In a first step of CESESYS the two teams have worked in parallel on two different (but complementary) research directions. The GXAS team has performed in their own laboratories a series of studies aimed at selecting a series of microorganisms with different roles in the secretion of cellulases, that represent viable solutions for performing studies that are relevant with respect to ellucidating some of the above mentioned aspects. As a result of these studies, GXAS has selected four biological species that further on in CESESYS stayed at the core of the experiments jointly run by GXAS and CMMPI-UPB: Escherichia coli., Clostridium beijerinckii, Saccharomyces cerevisiae and Enterobacter cloacae. The importance of these species for the project's theme is connected to their potential to generate enyzmes that contribute to cellulose's hydrolysis, a process that is necessarry for the synthesis of biofules based on cellulose. On a paralel activitiy track, in the first part of the project the CMMPI-UPB team has optimized a multimodal imaging platform (previously developed in-house) to facilitate the characterization of biological specimens supplied by GXAS. Various characteristics of the laser sources used for excitation have been evaluated, so that phototoxic effects and photodamage can be avoided. In the same time CMMPI has worked on designing efficient sample preparation protocols so that the same specimen instances supplied by GXAS can be investigated with various combinations of microscopy and nanoscopy tehcniques that rely on different contrast mechanisms. The imaging sessions have relied on a multimodal configuration which incorporates two interconnected modules that can investigate via multiple complementary imaging techniques correspponding regions of a sample of interest. By using one of the imaging modules of the system we have acquired nanoscale data sets via atomic force microscopy (AFM) and scattering-type Scanning Near-Field Optical Microscopy. AFM was used to characterize the above mentioned biological species from the point of view of morphology/topography, while s-SNOM was used as a tool to interrogate in a quantitative manner optical properties of biological samples that can be placed in corresppondence with their biochemical composition. By using a second imaging module we have acquired confocal laser scanning microscopy (CLSM) data sets, which have been mainly used for characterizing autofluorescent properties of the four selected biological species in the purpose of placing these properties in corresppondence with their efficicency to secret celullolitic enzymes with roles in celllulose degradation and biofuel production.

In the case of the investigated E.coli species, we placed a special focus of attention on identifying shape modifications that can be placed in correspondence with an increased efficiency for producing cellulolitic enyzmes. Our studies on Clostridium beijerinckii were aimed at understanding on how this microorganism would behave if cultured together with a related specie, Clostridium termitidis, on mixed substrates of cellulose and glucose, taking into consideration that the metabolites of one of the two species could have an impact over the efficiency of the other one. The studies of GXAS and CMMIP-UPB on the instances of S. Cerevisiae addressed two asepcts. The first one conisted in designing synthesis and investigation protocols that would allow an accurate understanding on when a S. Cerevisiae microorganism chooses to secret the enzymes necesssary for hidrolysis into the extracellular environment and when it chooses to display these on the cell surface. The second direction of research was aimed at establishing a set of investigation protocols that could allow resolving

the mechanisms by which the surfacee displayed enzymes can contribute to the digestions of cellulose in fermented sugars, a process which is not well uderstood at the time being. Our studies focused on Enterobacter cloacae were targeted on characterizing specific aspects of this microorganism that allow it to play a duble role: degrading the cellulose and simultaneously producing an electric current. At the time being the mechanisms behind this ability are not well understood, but once more light will be shed over these processes specific to Enterobacter cloacae, new hybrid strategies for generating renewable energy will become possible (biofuels generated via cellulase based hidrlysis and electricity produced during cellulose degradation).

Biofuels based on cellulosic biomass represent a promising alternative to fossil fuels, and currenntly many countries around the globe place intense efforts on designing and implementing more efficient synthesis strategies. Among these efforts lie also efforts focued on implementing at an industrial scale stages for preprocessing the biomases used for biofuels generation, so the the enzymatic hydrolysis of cellulose takes place at an accelerated pace, in the absence of secondary products that pose threats over human health or the environment. An important segment of these techniques of biomass preprocessing refer to the biological treatment using microorganisms (such as the bacteria studied in the frame of CESESYS). Currently, many research directions related to this subject are followed but despite this there are still many fundamental aspects relevant for the efficient generation of cellulases from microrganisms that are poorly understood. The investigations started during this project established the foundations of new research avenues that in the future will yield new perspectives for the in depth understanding of the cellulases' secretion system, which is of critical importance in the biofuel industry.

The work and knowledge transfer that took place in the CESESYS project led to a new research project/grant that will be implemented between 2017 and 2020 by GXAS and CMMPI-UPB. This project entitled "Exploiting the application of single molecule imaging technology in enzyme engineering Research, 2017-2020" was funded by the Guangxi Sci. Res. And Tech. Development Plan (total budget of 1,200,000 RMB), will combine the competences available at GXAS in the domains of biomass and enzyme engineering, and those available at CMMPI-UPB in the domain of optical imaging at high and ultra-high resolutions, to develop new methods and protocols by which enzymes can be thoroughly characterized at a single molecule level. Understanding in high detail how cellulases are secreted, and how microorganisms can be modified to produce a higher enzyme yield, will be of critical importance for consolidating their utilization in catalysis processes employed for biofuel generation at industrial scales.