For data analysis, the so-called severity factor, Ro, defined as in Eq. (3) was considered, where "t" represents the digestion time in minutes, "T" is the treatment temperature in °C, and "T_{ref}" is 100 °C, a reference temperature [12,22].

$$\text{Severity factor } \text{Ro} = \text{t} \cdot exp\left(\frac{\text{T} - \text{T}_{\text{ref}}}{14.75}\right) \quad (3)$$

2.2. Confocal Laser Scanning Microscopy (CLSM)

Variations of the lignin content between treated and untreated samples were assessed by CLSM, discriminating the leaves and stems portions of the LCW. Under the conditions used to observe the samples, lignin exhibits autofluorescence and can be easily distinguished by this technique from other components without contrast reagents [36–38]. The images were obtained using an Olympus FV300/BX61 confocal microscope (Olympus, Japan). The samples were first dried, fixed on glass slides, and covered with a thin glass lid. They were first observed under an optical microscope (UPLFL 10X). After establishing the proper focus, multiple stacks of 1024p x 1024p pictures were taken with laser excitation at 405nm and fluorescence emission detection at 550nm, characteristic of lignin, in scanning mode [36]. Magnification was at 10X as well as an objective lens (UPLFL 10X). Images were analyzed using the ImageJ open-source software (https://imagej.nih.gov/). The stack was averaged over the Z-axis and converted to a 0-255 grayscale (8-bit). Mean intensity was calculated from these images averaging all pixels of the best-focused image, as suggested by Hernández-Hernández et al. (2016, 2014) [38,39].

2.3. Scanning electron microscopy (SEM)

Changes in surface morphology of untreated and delignified samples were examined under scanning electron microscope Carl Zeiss NTS-Supra 40 (Carl Zeiss AG, Oberkochen, Germany). Before analysis, the samples were fixed on the sample plates using carbon tape as a non-conducting adhesive. Then, the samples were subjected to gold sputtering to increase their conductivity before taking the micrographs.

3. Experimental design

The acid-oxidative hydrolysis of the LCW is a multivariable process in which the variables interact with one another. Therefore, experimental design techniques present a more balanced alternative to the *ceteris paribus* (i.e., one variable at a time) approach to obtain information and improve the conditions for the delignification stage with good holocellulose yield [40–42]. The Statistical Design of Experiments (DoE) combined with the Response Surface Methodology (RSM) analysis covers only a fraction of the experimental space. The DoE-RSM method allows drawing effective conclusions while estimating the interactions between the different variables. Methods for experimental design can be broadly divided into two categories:

i) One includes designs used to explore a potentially large number of input variables to discover those that are statistically significant and estimate their magnitude. Plackett-Burman (PBSD) and Factorial Fractional designs are examples of these methods [42].

ii) The other category includes methods for optimizing a process given a reduced number of variables selected previously. A typical scenario is Central Composite (CC) or Box Behnken Design (BBD) and a RSM analysis on linear or quadratic models. Moreover, RSM analysis allows fine-tuning of any process variables, an issue to be considered when an industrial scale is aimed [42]. The objectives of the