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FTIR as an easy and fast analytical approach to follow up microbial growth during fungal pretreatment of poplar wood with *Phanerochaete chrysosporium*

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ABSTRACT

Since the determination of the fermentation kinetics is one of the main challenges in solid state fermentation, the quantitative measurement of biomass growth during microbial pretreatment by FTIR spectroscopy in Attenuated Total Reflectance mode was evaluated.

Peaks at wave numbers of 1651 cm^{-1} and 1593 cm^{-1} showed to be affected during pretreatment of poplar wood particles by *Phanerochaete chrysosporium* MUCL 19343. Samples with different microbial biomass fractions were obtained from two different experiments, i.e., shake flask and fixed-bed reactor experiments. The glucosamine concentration was compared to the normalized absorbance ratio of the 1651 cm^{-1} to 1593 cm^{-1} peak, measured by FTIR-ATR, and resulted in a linear relationship. The application of a normalized absorbance ratio in function of time provided a graph that was similar to the microbial growth curve.

Application of FTIR in ATR mode to follow-up kinetics during solid state fermentation seems to be a fast and easy alternative to laborious measurement techniques, such as glucosamine determination.

1. Introduction

Solid state fermentation (SSF) has been increasingly researched for biological detoxification of agro-industrial residues, bioconversion of biomass, biotransformation of crop-residues for nutritional enrichment, and the production of value-added products, such as, antibiotics, alkaloids, plant growth factors, enzymes, organic acids, biopesticides, biosurfactants, biofuel, aroma components, etc. The application of SSF in this work is for the pretreatment of poplar wood used as lignocellulose source for an improved microbial production of chemicals. The mold *Phanerochaete chrysosporium* is applied to degrade the lignin compound of the wood and to make the wood substrate more accessible for the enzymatic degradation of the polysaccharides in the subsequent saccharification process.

During solid state fermentation (SSF), microorganisms are growing on a solid substrate or support in the absence or near absence of free water. This highly heterogeneous system consists therefore of three phases, i.e., solid phase and liquid phase, as mentioned before, and a gas phase being the surrounding air that will provide the oxygen. The heterogeneous nature of solid state fermentation makes the follow-up of the microbial growth very difficult and is an obstacle in research and optimization of the processes.

Microbial biomass estimation is performed by indirect techniques by measuring cell components such as ergosterol, glucosamine, proteins or DNA. These methods are typically very laborious and time-consuming. Alternatively, on-line measurements of the oxygen uptake rate (OUR) by a gas analyzer can be used. This OUR value is connected to the microbial growth but needs calibration with one of the techniques mentioned above.

In this paper, the use of Fourier Transform Infrared spectroscopy in Attenuated Total Reflectance mode (FTIR-ATR) as a fast and easy quantitative technique for biomass estimation during SSF is proposed. ATR is based on a total internal reflection and has the advantage that it overcomes the weaknesses of the FTIR methodology with KBr windows, such as sample preparation and lower reproducibility. FTIR-ATR is suitable for the analysis of liquid and solid samples with minimal (e.g. lyophilisation) or no sample preparation. Moreover, the depth of the IR light penetration is not dependent on the sample thickness. Accordingly, this technique is suitable for the study of heterogeneous samples that contain highly infrared absorbing components such as polysaccharides, lignin and sugars (Baker et al., 2014).

In the area of microbiology, several publications have been presented where FTIR spectroscopy has been used as an alternative to the labor-intensive routine analysis for *identification of fungi* due to its

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powerful advantages such as rapidity, direct analysis of the sample, automation and minimal sample preparation. Wenning et al. (2002) successfully developed a procedure for the identification of food-borne yeasts using FT-IR micro- and macrospectroscopy in ATR mode with the generation of spectral libraries. Fischer et al. (2006) has managed to identify airborne filamentous fungi and yeast with FTIR in transmission mode. Salman et al. (2010) used FTIR spectroscopy in ATR mode to identify fungal phytopathogens. Schäwe et al. (2011) made it possible to observe the relative abundances of the species in a binary culture with FTIR in transmission mode.

Whereas all cited references describe on the use of FTIR for microbial identification purposes, the quantitative analysis of the fermenting microorganism itself and their growth in complex heterogeneous samples from an actual solid state fermentation mixture by FTIR-ATR has not been reported yet.

2. Material and methods

2.1. Bacterial strain

The white-rot fungus *Phanerochaete chrysosporium* MUCL 19343 was used for solid state fermentation experiments. The inoculum was taken from Petri plates in which the *P. chrysosporium* was grown on Potato dextrose agar (PDA) for 5 days at 27 °C and stored at 4 °C until use.

2.2. Experimental set-up

The medium was prepared by solubilizing 3.0 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g $FeSO_4 \cdot 7\ H_2O$, 1.0 g KH_2PO_4 and 20.0 g Glucose per liter distilled water and sterilized at 121 °C for 20 min.

Poplar wood pellets (Saw mill Caluwaerts Willy, Holsbeek, BE) were sieved through a 5 mm, 3.15 mm and 2 mm screen. Pellets between 2 mm and 5 mm were used as substrate for solid state fermentation. The humidity of the wood was measured by drying the wood sample until the weight was constant. The resulting humidity was 7%.

2.2.1. Solid-state fermentation in shake flasks

Parallel SSF experiments in shake flasks were carried out with 6 g wood. After the moisture content of the wood was brought up to 70% by the addition of 12.7 ml distilled water, the shake flasks were autoclaved at 121 °C for 60 min. For the inoculation, approximately 4 cm^2 of PDA agar with the *P. chrysosporium* culture was minced into small pieces, suspended and homogenized in 3 ml sterilized medium. One tube with dispersed inoculum was poured into each of the shake flasks. The shake flasks were incubated in a shaking incubator (Sanyo Orbital Shaker) at 200 rpm and 27 °C. Samples of approximately 0.5 g were taken after soft homogenization of the reaction mixture with a glass rod immediately after inoculation and weekly from every shake flask and prepared by lyophilisation and ball milling before glucosamine assay and FTIR analysis.

2.2.2. Solid-state fermentation in a fixed bed reactor

The reactor consists of a double-walled cylinder which is heated by flowing water from a temperature controlled water bath through the jacket. It contains a perforated plate to carry the substrate, medium and microorganism. Air is introduced at the bottom of the reactor and exits at the top with a constant flow rate that is controlled by a mass flow controller (El-flow, Bronkhorst). Before entering the reactor the air is passing through a gas washing bottle to bring its humidity to 100% and through a heating device, i.e., a spiral placed in the water bath. Loading and inoculation of the reactor is obtained by first preparing an inoculated shake flask in the same way as described in Section 2.2.2 and introducing its content into the sterilized reactor. A humidity sensor sterilized by ethanol is placed in the reactor. The same sensor is also applied to obtain the reactor temperature. After the installation of the reactor, control of mass flow and reactor temperature via the BlueSens software (BlueSens, Germany) was started. Values of reactor temperature, humidity and flow rate were fixed at 27 °C, 100% humidity and 0.05 l/min, respectively, and monitored. Glucosamine and FTIR analysis were performed on samples taken at the start and after 1.35 weeks of fermentation. No intermediate sampling was performed because mixing the reactor bed is necessary for homogeneous sampling and it was experienced before that mixing is causing a relapse in the microbial growth. When drying of the upper part of the solid bed was visible, 10 ml of sterile water was added.

2.3. Analysis

2.3.1. Sample preparation

Before the glucosamine assay and the FTIR analysis, the samples were lyophilized (CHRIST ALPHA 1-2 LDplus) and grinded with a ball mill. Hereto, the lyophilized sample was measured into a ball mill together with two stainless steel grinding balls of 15 mm diameter. The ball mill was immersed in liquid nitrogen for 1 min to embrittle the sample and was placed in the mixer mill (Retsch MM 2000). The milling was carried out for 2 min at a frequency of 70 Hz.

2.3.2. Glucosamine determination

The glucosamine assay was carried out according to a modified method of Said et al. (2010), i.e., instead of 0.5 g, 45 mg of sample was applied to enable the total degradation of the chitin, which is the primary component of the fungal cell wall. The samples were measured into tubes and hydrolysed with 400 µl 60% v/v sulfuric acid for 24 h at room temperature. Then the sample was diluted with 8.6 ml water to reduce the sulfuric acid concentration to 0.5 M. The tubes were autoclaved at 121 °C for 1 h. Afterwards the hydrolysates were neutralized with 10 M NaOH and 0.5 M NaOH, brought to 9 ml with water and filtered through a 0.2 µm polyether sulfone membrane. The resulting solutions were measured with the glucosamine colorimetric assay where 1 ml of the solution was mixed softly with 1 ml acetyl acetone reagent (2% v/v of acetyl acetone in 0.5 M Na_2CO_3) and incubated in a boiling water bath for 20 min. After cooling of the solutions 6 ml ethanol was added followed by 1 ml Ehrlich reagent (2.67% w/v of *p*-dimethylaminobenzaldehyde in a 1:1 mixture of ethanol and concentrated HCl). The solutions were carefully mixed and incubated at 65 °C in a thermostat (Laude C12) for 10 min and cooled to room temperature. The absorbance of the solutions was measured at 530 nm against a blank wood sample. A calibration curve was prepared with D-(+)-Glucosamine hydrochloride in water. Glucosamine content of the mold without substrate was also determined. *P. chrysosporium* of five days old was scraped from the PDA agar plate. The five days of incubation allowed enough mycelium production without sporulation because spores are only formed after 7 to 10 days. The prepared sample was diluted 10 times and 20 times before the colorimetric assay in order to reach the optimal glucosamine concentration which suits to the calibration range.

2.3.3. FTIR microspectroscopy

The FTIR microscopy measurements were performed with the Bruker LUMOS FTIR microscope. The FTIR spectra were recorded in the region of 4000–600 cm^{-1} with 4 cm^{-1} spectral resolution in ATR mode. For each FTIR spectrum, 64 scans were averaged. Each sample was measured 4 to 7 times in distinct randomly selected areas of the sample. The background was measured according to the aperture which means a new background was taken according to the measurement position.

From the measured spectra a spectrum library was created. The measured samples were potato dextrose agar (PDA, Oxoid, UK), *P. chrysosporium* scraped from PDA, xylan (from corncob, Roth, DE), lignin (extracted from poplar wood), poplar wood and cellulose.