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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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Running title

FTIR to follow-up microbial growth during SSF

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 1651cm^{-1} and 1593cm^{-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 1651cm^{-1} to 1593cm^{-1} , 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.

Keywords: Attenuated Total Reflectance · Fourier Transformed Infrared · microbial pretreatment · *Phanerochaete chrysosporium* · quantitative analysis - Solid state fermentation

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 Transformed 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 sample 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 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

Phanerochaete chrysosporium MUCL 19343 was used as a white-rot fungus for the 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 NaNO₃, 0.5 g KCl, 0.5 g MgSO₄ · 7 H₂O, 0.5 g FeSO₄ · 7 H₂O, 1.0 g KH₂PO₄ and 20.0 g Glucose per liter distilled water and sterilized at 121 °C for 20 minutes.

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 minutes. For the inoculation, approximately 4 cm² of PDA agar with the *P. chrysosporium* culture was minced into

small pieces, suspended and homogenized in 3 ml sterilised 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 above 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 one minute to embrittle the sample and was placed in the mixer mill (Retsch MM 2000). The milling was carried out for two minutes 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 μ l 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 μ m 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 Na₂CO₃) 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 the 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 allow enough mycelium production but no sporulation because they 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.

2.3.4 FTIR macrospectroscopy

The Macro-unit part of the FTIR spectrometer (Bruker, LUMOS Macro-Unit) was applied to acquire spectra from the samples. Before each FTIR measurement, an infrared background was collected from the clean ATR diamond crystal. For analysis, the sample was positioned in the FTIR macro-unit. Spectrum analysis was performed by the OPUS spectroscopy software (version 7.5). The spectrum was collected from 600 cm^{-1} to 4000 cm^{-1} . For each FTIR spectrum, 32 scans were averaged. Sample preparation took a few minutes work but needed in total three to four hours, due to the lyophilisation step. A single FTIR analysis required not more than 5 to 10 minutes, including the introduction of the sample on the measuring unit and performing multiple scans.

2.4 Modelling microbial growth as determined by FTIR

The growth was modelled by the logistic growth kinetics, i.e.,

$$\frac{dX(t)}{dt} = \mu \cdot \left(1 - \frac{X(t)}{X_{\max}} \right) \cdot X(t)$$

where X is the amount of microbial biomass, $X_{\max}$ is the maximum amount of biomass and μ is the specific growth rate. For fitting the *lsqnonlin* function in Matlab (MathWorks, MA, USA) was applied.

3 Results and discussion

Samples were analyzed with the glucosamine assay and Fourier Transformed Infrared measurement in attenuated total reflectance mode (FTIR-ATR). Fermentations and sample preparations were executed as described in Section 2.2.

3.1 Glucosamine assay

Measurement of the fungal growth can be based on determining the glucosamine content which is the main constituent of chitin in the cell wall. A calibration curve for D(+)-glucosamine hydrochloride (Sigma, Belgium) solubilized in water in the range of 5 to 100 $\mu\text{g}\cdot\text{ml}^{-1}$ with correlation coefficient of 0.995 was obtained. The glucosamine content of mold samples was calculated to be 53.36 mg glucosamine $\cdot(\text{g mold})^{-1}$.

Samples of shake flask experiments and bioreactor experiments were in the lower range of the calibration curve (maximum 23 $\mu\text{g}\cdot\text{ml}^{-1}$). Moreover, when the colorimetric assay was executed with samples of shake flask experiments and bioreactor experiments, it was visible that the samples which contained wood gave a purple color after the 65°C incubation in the water bath in contrast to the pink color of the calibration points and the mold sample free of wood. Glucosamine measurements were obtained after subtracting the absorbance value of the wood blank, i.e., sample of wood before inoculation, from the absorbance values of the samples. The glucosamine content of the samples was converted to the amount of mold biomass in the sample by using the determined conversion factor of 53.36 mg glucosamine $\cdot(\text{g mold})^{-1}$. Results are shown in Table 1.

The mold concentrations vary between 8.5 and 79.1 mg mold per g dry weight of sample. From triplicates, the reproducibility was evaluated and was found to be 6% for the low concentrations, i.e., in the samples obtained at the start of the experiments, and 0.6% for the higher concentrations.

3.2 Analysis of samples with FTIR-ATR

For FTIR analysis of highly heterogeneous surfaces it is a general technique to use macrospectroscopy, because the absorbance signal will be averaged. However, in our research, it was the purpose to measure the mold concentration in the solid state fermentation samples, i.e., the amount of mold per mass of sample, and not the amount of mold on the sample surface. In our research the analysis of non-milled samples did not provide spectra that allowed quantitative measurement of the mold. For this, two reasons can be proposed. The first is that hard and big particles such as our wood samples did not allow a good contact surface at the applied pressures in the macro-unit. The second reason is that the measuring depth in the unit does not allow to measure the amount of mold relative to the amount of wood. This is related to the principle of the technology, which only allows determination of amounts relative to another compound. Based on these results we used the homogenized milled samples for analysis. Quantitative determination by FTIR requires a calibration curve of the sample absorbency at a characteristic wavenumber that is specific for the system under investigation. Therefore, the first step is to find the probe band that changes in intensity with the amount of mold. Using the created spectrum library to identify the presence of mold in the sample did not provide any result. This was obviously due to the heterogeneous nature and complexity of the samples. It is common practice to eliminate the instrumental artefacts in the spectra by normalizing to a constant absorption band. As a reference band 1021 cm^{-1} was chosen which is assigned to the C-O stretching vibration of carbohydrates, such as the polysaccharides cellulose and chitin (Hon and Shiraishi 2000).

Visual inspection of the mold spectrum showed that the most prominent peak was at 1651 cm^{-1} . This was compared to the spectra of the samples from the shake flask and bioreactor experiments, which are presented in Figure 1 and Figure 2, respectively. The most significant changes that can be observed here are in the $1500\text{-}1700\text{ cm}^{-1}$ range. The blank sample, which consists of only poplar wood, has a characteristic pattern in this area, with peaks at 1593 and 1732 cm^{-1} and a plateau at 1651 cm^{-1} . The same pattern can be seen by careful inspection of the spectra in the work of Zhou et al. (2011). For the different samples of the fungal pretreatment process, two main peaks seemed to be affected, i.e., at 1651 cm^{-1} and at 1593 cm^{-1} . Both peaks increased during fermentation. The absorption bands in the region of $1700\text{-}1500\text{ cm}^{-1}$ correspond to the protein bonds, the $1700\text{-}1600\text{ cm}^{-1}$ range especially belongs to the stretching of the C=O bond (Amide I region), while the $1600\text{-}1500\text{ cm}^{-1}$ range to the C=N and C–N–H stretching (Amide II region) (Wharfe et al. 2010). Since water is absorbing in the Amide I region, it's important that samples are lyophilized before FTIR measurement.

Figure 1
and 2

This absorbance increase at 1651 cm^{-1} most likely appeared because of the presence of proteins which were produced by *P. chrysosporium* (Santos and Corso 2014). For the peak at 1593 cm^{-1} , a slight increase is visible. This peak lies in the Amide II region and can also be caused by the proteins. Additionally, the 1593 cm^{-1} peak has been assigned as one of the most characteristic bands for lignin in the fingerprint region (Zhou et al. 2011). However, as one of the main goals of fungal pretreatment is the delignification of the samples, a decrease of the lignin content is expected. Possibly there is a balance between an increase of protein and a decrease of lignin, resulting in the slight increase in the 1651 cm^{-1} peak. Based on these results the absorbance at 1593 cm^{-1} and the absorbance ratio at 1651 cm^{-1} and 1593 cm^{-1} are determined and summarized in Table 2.

Table 2

The absorbance values are relatively low due to the dilution by the wood obtained by

the milling step in the sample preparation. However, this can make it possible to correctly quantify higher concentrations of mold.

3.3 Evaluation of quantitative FTIR measurement

To evaluate the different FTIR data for their ability to present the microbial growth kinetics during solid-state fermentations, regression lines against the established glucosamine measurement were used. Hereto, the resulting glucosamine amount of each sample is presented on the x-axis and the corresponding FTIR-ATR absorbance value on the y-axis. Figure 3 presents the data and linear regression lines for the absorbance at 1651 cm^{-1} and the absorbance ratio of 1651 and 1593 cm^{-1} peaks. The correlation between the absorbance at 1651 cm^{-1} and the amount of glucosamine shows a low linearity with a correlation coefficient of 0.711. Only the ratio of the absorbance at 1651 cm^{-1} and 1593 cm^{-1} corresponds to the glucosamine determination by a linear trend with a correlation coefficient of 0.927. This is a good correlation, considering the lower accuracy of the glucosamine determination on wood samples and the heterogeneity and complexity of the sample with varying concentrations such as increasing chitin amount, decreasing lignin content and increasing protein concentration. It seems that when the absolute absorbance of peaks is used, values of this absorbance are sensitive to the normalization procedure which relies on the assumption that the C-O reference peak is a valid reference vibration for all samples. The results show that calculation of the ratio of the 1651 cm^{-1} versus 1593 cm^{-1} peak eliminates the possible errors associated with the normalization procedure. It is hence important to apply such a procedure in complex mixtures such as the one investigated here.

Figure 3

The correlation between the FTIR and the glucosamine measurement results in the following equation for the amount of mold in the sample:

$$mg\ mold / g\ sample = 251.8 * A(1651\ cm^{-1}) \cdot (A(1593\ cm^{-1}))^{-1} - 164.15$$

Figure 4

In Figure 4, the absorbance ratio has been given as a function of time. The curve was modelled to determine the kinetics as measured by FTIR. In most cases, fungal growth kinetics in solid state fermentation are following the logistic growth (Mitchell et al. 2004). As shown in Figure 4, the simulated curve is in agreement with the absorbance ratio.

4 Conclusion

The FTIR measurement in ATR mode of solid state fermentation samples showed to provide an intuitive and accurate quantitative method for determination of the growth kinetics that can be easily used by occasional users of the equipment. Future studies will be performed to improve this method and to validate it for other solid state fermentations.

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Table 1: Experimental results of mass mold to solids ratio based on glucosamine determination in the SSF samples and 5.3 m/m% glucosamine/mold.

Table 2: Experimental results of the absorbance measurements of the 1651 and 1593 cm^{-1} peaks after normalization to the cellulose peak.

Table 1: Experimental results of mass mold to solids ratio based on glucosamine determination in the SSF samples and 5.3 m/m% glucosamine/mold.

Sample	Time (weeks)	mg glucosamine·(g DW) ⁻¹	mg mold biomass·(g DW) ⁻¹
Shake flask	0	0.82	15.5
experiments	1	3.24	61.1
	2	3.28	61.9
	3	4.19	79.1
Bioreactor	0	0.45	8.5
experiment	1.35	3.88	73.2

Table 2: Experimental results of the absorbance measurements of the 1651 and 1593 cm^{-1} peaks after normalization to the cellulose peak.

Sample	Time (weeks)	$A(1651\text{cm}^{-1})$	$A(1593\text{cm}^{-1})$	$A(1651\text{cm}^{-1}) \cdot (A(1593\text{cm}^{-1}))^{-1}$
Shake flask	0	0.171	0.245	0.699
experiments	1	0.210	0.247	0.852
	2	0.241	0.265	0.908
	3	0.288	0.306	0.942
Bioreactor	0	0.135	0.205	0.723
experiment	1.35	0.202	0.254	0.976

Figure 1: FTIR-ATR spectra and evolution during *P. chrysosporium* pretreatment in shake flasks.

Figure 2: FTIR-ATR spectra of starting sample and final sample during *P. chrysosporium* pretreatment in SSF reactor.

Figure 3: The use of regression lines for evaluation of FTIR-measurements with amount of mold based on glucosamine results. (left) Absorbency at 1651 cm^{-1} relative to glucosamine content, (right) absorbance ratio of peaks at 1651 cm^{-1} and 1593 cm^{-1} with respect to glucosamine content.

Figure 4: Evolution of (o) absorbance ratio of peaks at 1651 to 1593 cm^{-1} as measured by FTIR-ATR from samples of *P. chrysosporium* pretreatment and (—) fitting curve of logistic model.

Figure 1: FTIR-ATR spectra of mold and evolution during *P. chrysosporium* pretreatment in shake flaskss.

Figure 2: FTIR-ATR spectra of starting sample and final sample during *P. chrysosporium* pretreatment in SSF reactor.

Figure 3: The use of regression lines for evaluation of FTIR-measurements with amount of mold based on glucosamine results. (a) absorbency at 1651 cm⁻¹ relative to glucosamine content, (b) absorbance ratio of peaks at 1651 cm⁻¹ and 1593 cm⁻¹ with respect to glucosamine content.

Figure 4: Evolution of (o) absorbance ratio of peaks at 1651 to 1593 cm^{-1} as measured by FTIR-ATR from samples of *P. chrysosporium* pretreatment and (—) fitting curve of logistic model.

Highlights

- Microbial growth during solid state fermentation can be easily measured by FTIR-ATR
- Ratio $1651\text{ cm}^{-1} / 1593\text{ cm}^{-1}$ shows a good correlation with the glucosamine measurement
- FTIR-ATR is an accurate quantitative method for follow-up of the growth kinetics

Figure 1

Figure 2

Figure 3

Figure 4