A sense of discovery

Investment by Technology Innovation Agency's Bioprocessing Platform in FTIR technology, assisting Biotechnology Start-ups with scientific insights and discovery breakthroughs.

FOURIER TRANSFORM INFRARED | APPLICATION NOTE

FTIR spectroscopy – Background

Molecules are the units of matter where atoms are connected with covalent bonds. If these bonds are given some energy, *i.e.* infrared radiation, they will bend, rotate or stretch more vigorously and radiation of that frequency will be absorbed. Each bond in a molecule has its own frequency which it absorbs when infrared radiation is passed through a sample of an organic compound: some frequencies are absorbed, and some pass through without being absorbed. An infrared spectrum shows which frequencies were absorbed and which passed through.

FTIR stands for Fourier Transform InfraRed, the preferred method of infrared spectroscopy. Fourier Transform decodes the spectrometer data to produce a spectrum representing its molecular 'fingerprint' that analysts can use to identify or quantify the material. The usefulness of infrared spectroscopy results from different chemical structures (molecules) producing different spectral fingerprints.

FTIR can be a single purpose tool or a highly flexible research instrument. With the FTIR configured to use a specific sampling device – transmission or ATR (attenuated total reflectance) – the spectrometer can provide a wide range of information such as the identification of an unknown, quantification of additives or contaminants, kinetic information *etc*. In addition to traditional Chemistry applications, FTIR is extensively used in applied microbiology and biotechnology of various types of microorganisms, including fungi, bacteria, yeasts or algae.

Interpretation of FTIR spectra

Typically, an FTIR spectrum is recorded between 4000 and 400 cm^{-1} (corresponding to 2500 to 25000 nm) in transmittance mode even if the spectrum is collected by measuring reflectance. In transmittance mode the peaks appear to be "upside down" compared to absorbance. 100% transmittance intensity means that no light has been absorbed. When a statement is made that a compound absorbs certain frequency energy, the transmittance at the wavelength corresponding to this frequency will be lower than 100%.

The region of the spectrum which is mostly used to identify functional groups is between 1400 and 3500 cm^{-1} as most functional groups of organic molecules give characteristic absorptions in this region. There is a wealth of FTIR frequency tables which provide typical positions of peaks representative of the specific groups, *e.g.* ketones, esters, hydrocarbons *etc*. It must be emphasized that the actual absorption frequency is affected by the group's molecular environment, so the tables show wide bands rather than single frequencies. Similar information is often given in a chart form – see Figure 1 below.

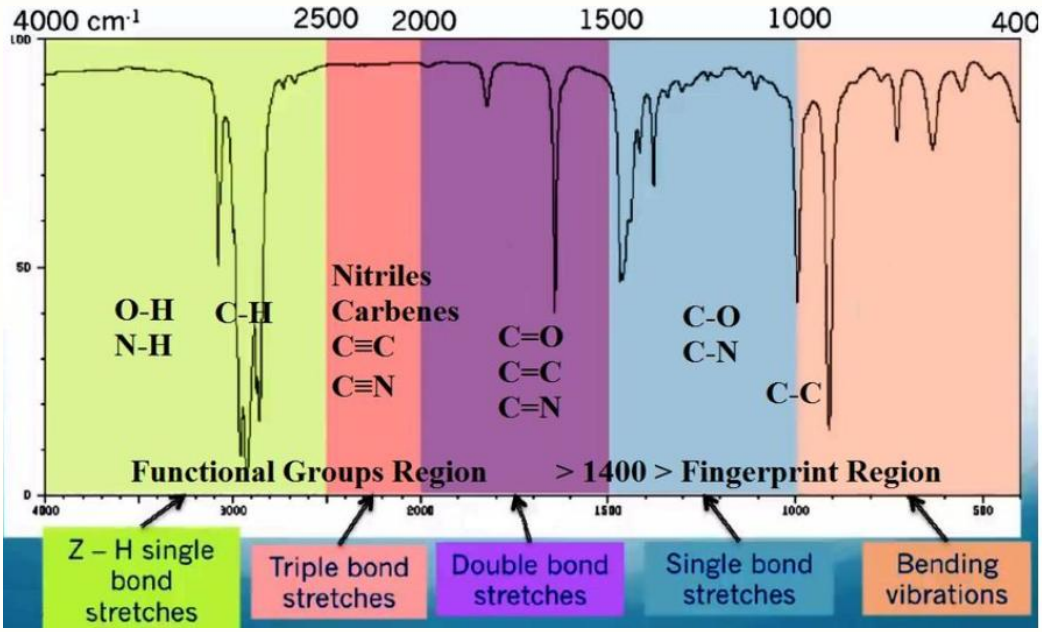

Figure 1. FTIR spectrum showing characteristic absorbance regions of organic functional groups.

The region between 400 – 1400 cm^{-1} of the spectrum is more complex and typically has a lot of peaks which are very close together and thus could be difficult to identify. These peaks are not from specific bonds but are a result of the structure of the molecule as a whole. This region is completely different for each molecular structure, even for those with the same functional group. This region is identified as the fingerprint region. Generally, the infrared bands for inorganic materials are broader, fewer in number and appear at lower wavenumbers than those observed for organic materials. If an inorganic compound forms covalent bonds within an ion, it can produce a characteristic infrared spectrum.

Interpretation of the FTIR spectrum must be done with a degree of caution. There are several rules which allow for judicious assigning of the molecular structure:

- Concentrate on the 1400 and 3500 cm^{-1} region first to identify the typical functional groups,
- Use a frequency table to identify possible functional groups for each frequency,
- Use the fingerprint region (below 1400 cm^{-1}) to confirm or elaborate on the structural elements,
- Do not try to assign every single peak in the spectrum!
- Cross check with the literature data from trusted publication on similar sample matrices wherever possible,
- Note both the negative and the positive evidence for the presence of a particular functional group in different parts of the spectrum,
- Accept that sometimes the band intensities may vary significantly for the same functional group in different molecular environments,
- If sample is in solution, some frequency bands can shift from their “normal” positions due to hydrogen bonding. This shift is generally within ~ 5 cm^{-1}. The solution pH can influence the spectrum even stronger, resulting in larger shifts from the expected position.
- Be cautious when focusing on small wavenumber changes: this can be meaningful in some instances while totally ignored in others.

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Overall, FTIR spectroscopy cannot provide exhaustive information on the molecule's structure, thus it is best used in conjunction with other analytical methods such as chromatography, nuclear magnetic resonance, mass spectroscopy and elemental analysis.

Use of FTIR at Sawubona Mycelium

Sawubona Mycelium started using FTIR to characterise isolated materials as far back as 2021, but it has only been able to reap the full advantage of this technique since April 2023, when TIA Bioprocessing Platform acquired its own FTIR instrument and provided Sawubona with unlimited access. Sawubona Mycelium's interest lies in developing sustainable biomolecules production from submerged fermentation of various basidiomycetes' fungal strains. The major components of interest are commercially lucrative biopolymers chitin, glucans and glycoproteins. In addition, a variety of biologically active small molecules, such as lipids, peptides and phenolic compounds present in fungi can be obtained from the fermentation products.

Chitin (poly-ß-1,4-N-acetyl-D-glucosamine) and its deacetylated form, chitosan (poly-ß-1,4-D-glucosamine), are natural biodegradable polymers with a broad range of applications in food, pharmaceutical and agricultural industries: chitosan belongs to the most versatile and promising functional biopolymers, with superior material properties and interesting biological activities. An increasing market demand for high-quality chitosan exceeds the current global production, which is based primarily on deacetylation of chitin from shells of crustaceans. Fungal lipids are another potentially commercially attractive product as, depending on the fungal strain, the composition of the fats extracted from mycelia can be similar to that of either plant or fish oils, in addition to containing unique bioactive terpenoids.

The aim of Sawubona Mycelium is to co-produce high-value metabolites from mycelia biomass (BM) and/or filtrate through metabolic pathways by varying the fungal strains as well as the carbon and nitrogen sources in the fermentation media. Sawubona Mycelium has identified around a dozen of strains of both well-known medicinal fungi and locally grown non-toxic mushroom species to carry out the experimental work on selecting the most efficient producers of chitin, biologically active lipids and other biomolecules.

The traditional approach for monitoring and developing the production of different constituents in fungal cells is based on the extraction or separation of the produced metabolites followed by further qualitative and quantitative analysis using different analytical procedures. Such approach requires significant amount of biomass for processing and analysis, since different metabolites need to be extracted and analysed in different and often expensive and time-consuming ways. When combined with the need to change the media for submerged fermentations as well as the cultivation conditions, the overall scope of the research becomes excessive, highlighting the need to employ a fast-screening methodology. This is where FTIR technology can step in and help to identify the presence of the compounds of interest even at the early stage of production. By virtue of being able to show the presence of all functional groups, the FTIR spectra of the unprocessed fermentation products invariably have multiple overlapping bands. Fortunately, the major classes of compounds of interest such as glucans, proteins, lipids and chitin do not have very "busy" spectra themselves and are known to have separate characteristic absorption peaks which allows for simultaneous "detection" of glucans, proteins etc in biomass and supernatants.

Over the past years using both literature sources and own research, Sawubona Mycelium has compiled an in-house FTIR frequency table[1] which helps with identification of various polymers (Table 1).

Table 1. FTIR frequency table for fungal biopolymer characterisation.

Frequency, cm-1	Assignment	Characteristic of a functional group
3400-3500	stretching OH	OH of glucose rings, water
3200-3400	stretching N-H	NH in amines and amides
2950-2970	stretching CH	CH_3 groups
2910-2940	stretching CH	CH_2 groups
1720-1750	stretching C=O	carboxylic acids, esters, uronic acids, fats
1640-1660	Amide I: C=O	chitin, protein
1620-1640	stretching -C-0	carboxylic acids, carboxymethyl-, uronic acids,
1630, broad	bending OH	Water
1610-1625	CONH	chitin, protein
1545-1560	Amide II: NH	Chitin
1530-1540	Amide II: NH	Protein
1420-1430	bending -C-0	carboxylic acids/salts
1370-1380	Deforming C-H	Chitin
1320-1330	deforming C-C	carboxylic acid
1320-1325	NH2	chitosan
1310-1320	Amide III: C-N	Chitin
1300-1310	deforming OH	Sugars
1160-1165	-C-O-C-, -C-C-	beta-Glucan
1150-1155	-C-O-C-, -C-C-	alpha-Glucan
1070-1080	-C-O-, -C-C-	beta-Glucan
1040-1050	-C-O-, -C-C-	carboxymethyl-
1035-1045	-C-O-, -C-C-	beta-Glucan
1020-1025	-C-O-	alpha-Glucan
~930	-C-C-	glucose ring, alpha-Glucan, -1,3 and 1,4-1,6, carboxyl
880-895	deforming C-1-H	beta-Glucan, -1,3; chitin/chitosan
870-880	C-1-H and C-O-C	mannogalactan
845-855	deforming C-1-H	alpha-Glucan, -1,3
820-830	C-1-H and C-O-C	alpha-Glucan
800-805	C-1-H and C-O-C	mannogalactan
780-790		beta-Glucan
760-770		alpha-Glucan
530-540		alpha-Glucan

With the use of the above table, it became quite easy to identify the presence of beta- and alpha-glucans, chitin, uronic acids and lipids in the complex biomass or supernatant matrix. A telling example of how useful FTIR screening of biomass is, comes from the time when Sawubona Mycelium did not have access to the instrument and had to rely on sending isolated biopolymers for external analysis. In 2022, many months were spent trying to isolate chitin from the fermented *Schizophyllum Commune* (SC) biomass with next to no results. When the FTIR instrument became available in 2023, the scans of the dried SC mycelia samples showed none to barely notable absorbance in the 1540-1560 cm^{-1} spectral range, demonstrating that this particular organism has very low chitin content and should not be used as a chitin source.

The following section gives various examples of how examining and comparing FTIR spectra of various fungal mycelia can draw light on the presence of compounds of interest.

Application of FTIR spectroscopy for fungal BM characterisation at Sawubona Mycelia

Example 1. Assessing potential chitin content of biomass samples

The spectrum of fungal chitin is shown in the Figure 2 below. The dominant spectral bands are situated at 1100-1000, 1650 and 1375 cm^{-1}, corresponding to -C-O- and C-C- bending, -N-H- amide stretching and -C-H deforming bonds respectively. Despite their high intensity, they cannot be used for detecting chitin presence in fungal biomass because of their overlap with

[1] Developed by Sawubona Mycelium team during fungal mycelium R&D processing, available and accessible by all TIA Bioprocessing Tenants

the major bands due to glucans, 1100-900, 1630 and 1370 cm^{-1}, which are the major constituents of biomass. However, the medium intensity bands at 3276 (-N-H amide stretching), 2923 (CH_3 stretching) and 1553 cm^{-1} (-N-H amide bending) can distinguish chitin from glucans. The 2923 band is not unique to the acetyl groups of chitin and can also come from other cell wall constituents, namely lipids, thus the 1550 cm^{-1} peak becomes the main distinguishing feature. Proteins have a number of amide bonds in their structure as well, but they tend to absorb at slightly lower wavenumbers, 1540-1530 cm^{-1}.

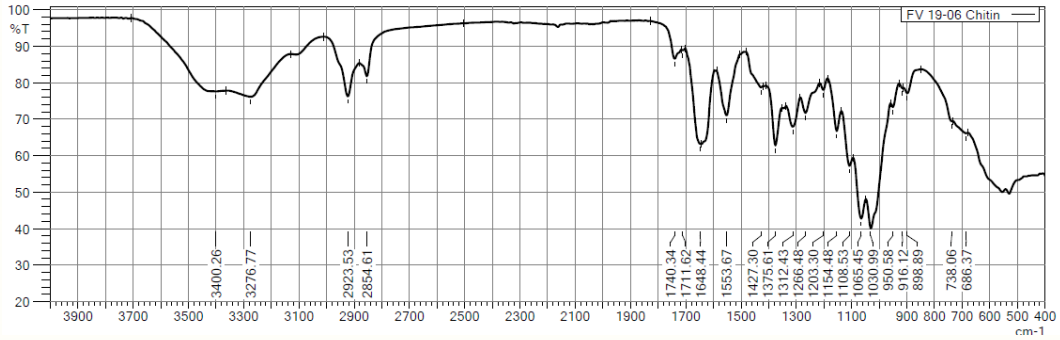

Figure 2. Spectrum of fungal chitin isolated from *Flammulina Velutipes* mycelium.

Figures 3a and 3b show FTIR spectra of several SC biomass samples: the peak at ~1550 cm^{-1} is barely noticeable for the samples from Feb and Nov 2022 as well as Jan and May 2023 mycelia. The biomass sample spectra from Jun and Jul 2023 display more prominent bands at 1550 cm^{-1}, suggesting chitin presence at a low level. (The fermentation media composition was the same for all batches, hence the reason behind the variation is not clear.)

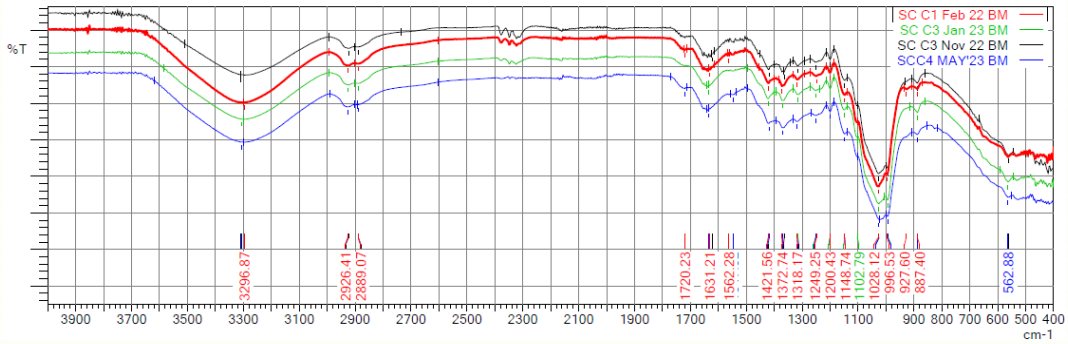

Figure 3a. Spectra of SC BM with non-detectable chitin content.

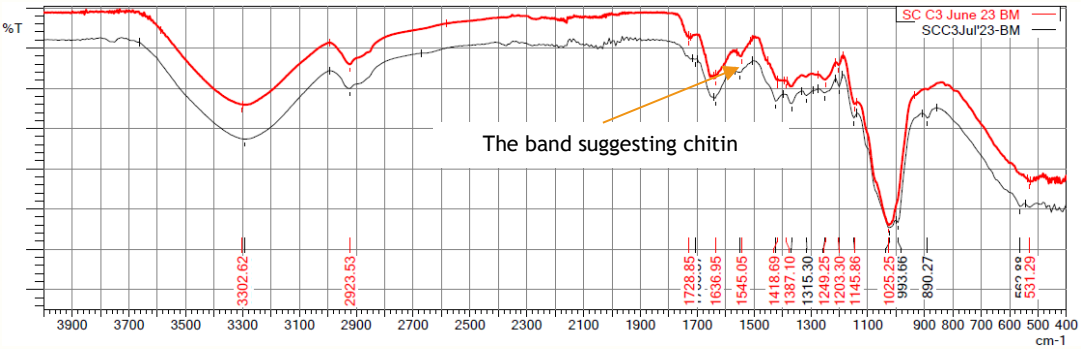

Figure 3b. Spectra of SC BM with very low chitin content.

In contrast to SC, several other fungal organisms fermented by Sawubona Mycelium displayed considerably higher chitin content, based on the intensity of the peak at ~1550 cm^{-1} as well as other characteristic spectral bands, including the ones at 2923 and ~3280 cm^{-1} – see Figure 4.

The ease of isolation of chitin and subsequently its deacetylated derivative, chitosan, from the mycelia of these organisms has been successfully demonstrated experimentally.

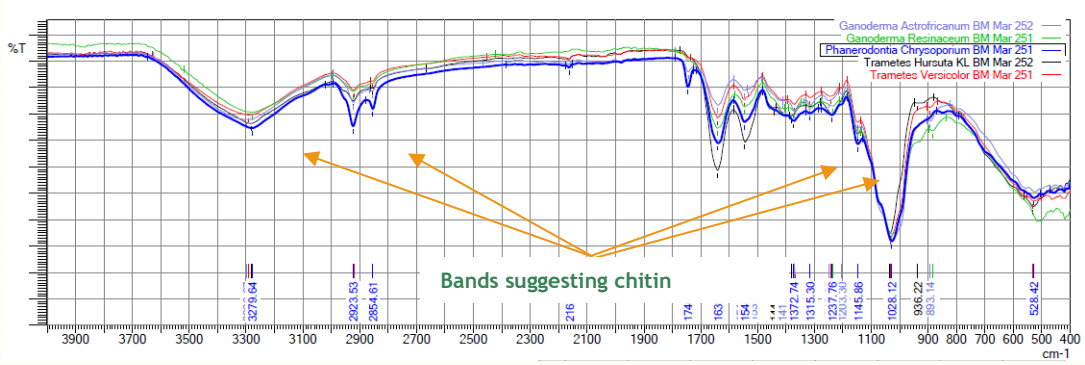

Figure 4. Spectra of the biomass from 5 fungal strains, *Ganoderma Resinaceum, Ganoderma Austroafricanum, Trametes Versicolor, Trametes Hirsuta* and *Phanerodontia Chrysosporium*, showing appreciable chitin presence.

<u>Example 2. Evaluating the effect of fermentation conditions on the composition of fungal mycelium</u>

The influence of cultivation conditions on the fermentation outcome is not a new phenomenon. Understanding the degree of this effect prior to processing the fermentation products is vital for downstream work. Sawubona Mycelium has been experimenting with a variety of fermentation conditions such as oxygen feed/degree of aeration, glucose and nitrogen source in order to increase the production of target compounds.

Figures 5 and 6 show the spectra of *Pleurotus Sajor-Caju* (PSC) and *Dacryopinax Spathularia* (DS) respectively, fermented under different conditions.

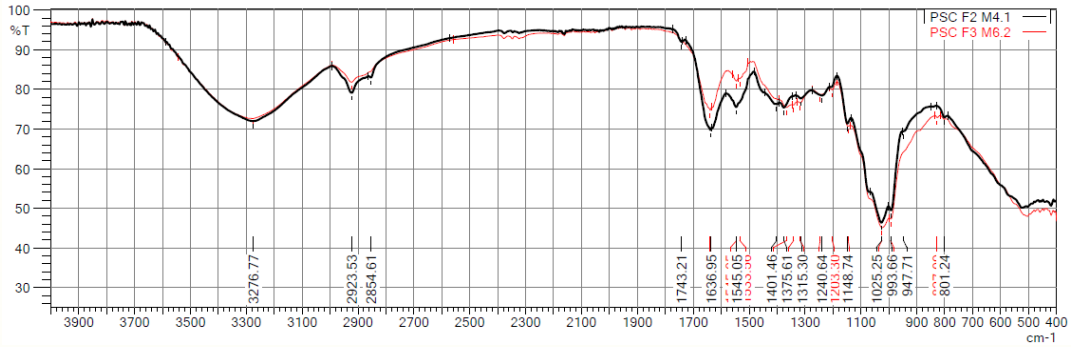

Figure 5. Spectra of *Pleurotus Sajor-Caju* BM samples fermented using Media 4.1 vs 6.2.

The spectra of PSC BM fermented with Media 4.1 shows considerably more pronounced peak at ~1550 cm^{-1}, characteristic of the chitin content. The IR results are consistent with the actual amount of chitosan (the product of chitin deacetylation) isolated from the 2 biomasses: the yield of acid-soluble chitosan from the 4.1 media material was 11% on the dry BM basis compared to only 1.4% when media 6.2 material was used for extraction.

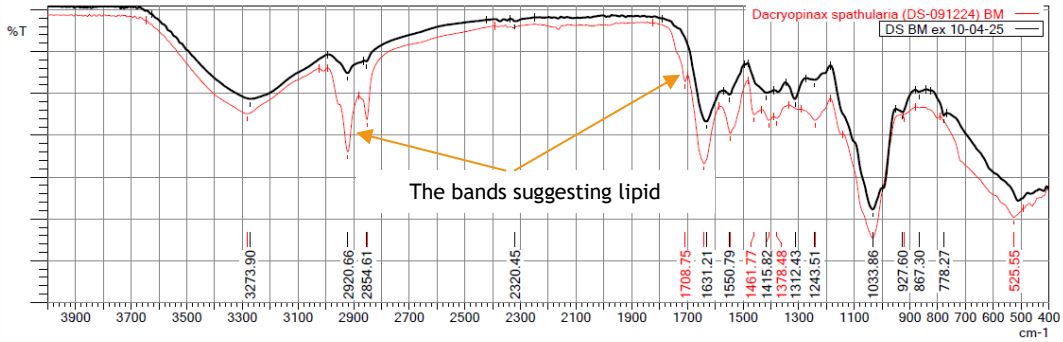

Figure 6. Spectra of 2 *Dacryopinax Spathularia* BM samples fermented under different conditions.

The 2 spectra of *Dacryopinax Spathularia* on the Figure 6 differ considerably in two IR regions: 2900-2800 as well as 1700-1500 cm^{-1}. The difference in the intensities of the characteristic chitin amide peak at ~1550 cm^{-1} is clearly seen, however much higher intensity of the doublet around 2900-2800 cm^{-1} is attributable not only to a higher chitin content, but also to the presence of a number of lipids which are known to contain a lot of methyl and methylene groups. The low intensity bands in the 1740-1710 cm^{-1} range confirm the presence of functional groups associated with lipids. Ethanolic extraction of the two biomasses confirmed a large difference in both the quality and the quantity of lipids present in these materials.

A large difference in the biomass spectra can also be seen for *Ganoderma Austroafricanum* fermented using media 4.1 and 10.2 (Figure 7).

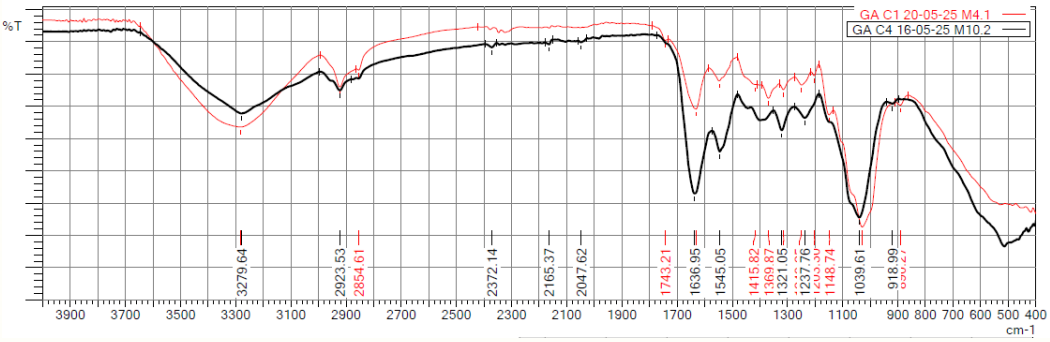

Figure 7. Spectra of *Ganoderma Austroafricanum* BM samples fermented using Media 4.1 vs 10.2.

The spectra look significantly different despite originating from the same organism. The spectrum of the material made with media 4.1 shows the presence of lipids due to the bands in the 2900-2800 cm^{-1} range and at ~ 1740 cm^{-1}, while these peaks are barely present in the biomass made with media 10.2. On the other hand, the latter material has a more pronounced chitin amide peak at ~1550 cm^{-1}. Closer examination shows other differences such as additional peaks in the spectrum of the media 10.2 biomass, suggesting substantial presence of a carboxylic acid/salt (~1630 and 1400 cm^{-1}), later turned out to be Calcium oxalate. The other notable difference is the shape of the main "glucan" peak in the 1100-900 cm^{-1} range: the difference in the peak profile is suggestive that different ratios of alpha and beta-glucans are found in the biomasses grown in medias 4.1 and 10.2.

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Conclusion

Access to the FTIR instrument at TIA Bioprocessing platform has allowed Sawubona Mycelium not only to characterise a number of important biomolecules isolated from fungal mycelia, but to be able to screen the biomass and predict its potential in producing compounds of interest, such as glucans, chitin/chitosan and lipids.

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