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FTIR spectroscopic metabolome analysis of lyophilized and fresh *Saccharomyces cerevisiae* yeast cells†

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The yeast *Saccharomyces cerevisiae* is widely used as a biological eukaryotic model and also serves as a production organism in biotechnology. One of the methods used to avoid degradation of the yeast cell content is lyophilization. The use of lyophilized yeast cells has several advantages over fresh ones: samples can be easily transported and/or stored and variations of their metabolomic profiles do not occur during transport or storage. Fourier transform infrared (FTIR) spectroscopy is one of the most emerging approaches in modern biology that permits operation on very small quantities of whole cells without the need for extractions or purifications. This technique is very sensitive and not only allows the discrimination between different cell genotypes but also between different growth conditions. FTIR spectra provide interesting data on the metabolic status of the whole cell. Modern multivariate data processing was applied to analyse live fresh or lyophilized *S. cerevisiae* cells from different growth media. This study clearly demonstrates that yeast cells coming from an identical biological medium can be used indiscriminately for FTIR analysis whether they are analysed directly as live fresh cells or after lyophilization which is a freeze-drying process. Moreover, FTIR data obtained using lyophilized cells showed less variability.

1. Introduction

The yeast *Saccharomyces cerevisiae* is certainly the best studied eukaryotic organism.[1] Its genome was the first sequenced and many omic databases are available.[2,3] So, it is genetically, biochemically and physiologically a well characterized organism. Moreover, yeast growth can be controlled by adjusting the environmental conditions. It presents many advantages like fast doubling time, easy manipulation and inexpensive cultivation.

Due to these reasons *Saccharomyces cerevisiae* is widely used as a biological eukaryotic model for system biology studies,[4] as a production organism in biotechnology,[5] and it is particularly used in food microbiology.

One of the advantages of yeasts is that these organisms can tolerate freeze-drying for the purpose of easier storage or transport. Moreover, lyophilization was presented as one of the methods used to avoid degradation of the yeast proteome during cell harvesting[6] or for achieving parallel analysis.[7] Anyway, outcomes of freeze-drying yeast cells were studied in view of analysing single biochemical components such as proteins, ARN, *etc.*, but never the total components from whole cells.

Fourier transform infrared (FTIR) spectroscopy commonly involving transmission mode but also diffuse reflectance absorbance[8] or ATR[9,10] modes is one of the most emerging approaches in modern biology. It has been introduced in microbiology for species identification and strain characterization.[8,11–13] It is frequently used as a very sensitive and non-destructive method to allow discrimination of yeast,[14] to study their biochemical changes,[9,10] and to determine the level of contamination in yeast media.[10,15]

One of the strengths of FTIR studies is that they can provide data on the metabolic status of whole cells. To perform these analyses only very small quantities of whole cells are needed and extractions or purifications of the metabolome components[16] are not required. Thus, the advantages of FTIR spectroscopy contribute to choose this technique preferentially over conventional techniques used for metabolomic analysis such as chromatography coupled to mass spectrometry (MS) and nuclear magnetic resonance (NMR) methodologies.[17,18] However, a modern FTIR spectrometer and associated software platform for multivariate data analysis are rarely available in biological research laboratories. So, the use of lyophilized yeasts

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instead of fresh ones for FTIR analysis could be very interesting with the aim of making a material more convenient for transport or for preservation of a perishable material for a later assessment. Nevertheless, in previously published studies on yeasts using FTIR spectroscopy, the species were freshly prepared and analysed as soon as possible. To our knowledge, none of the FTIR spectroscopy studies using yeasts were carried out with freeze-dried ones.

Yeast can use almost thirty different compounds as the nitrogen source. Depending on the nitrogen-containing compound in the medium, nitrogen transport, anabolism and catabolism are subject to tight controls. There are two kinds of transcriptional controls acting on genes involved in transport and catabolism: specific ones affecting only a limited number of genes and nitrogen catabolite repression (NCR) acting on a wide variety of genes.[19,20] Thus, several nitrogenous compounds, gamma-aminobutyric acid (GABA) being one of them,[21] specifically induce transcription of the genes involved in their utilization. On the other hand, NCR is typically exerted on the many genes involved in the utilization of non-preferential nitrogen sources when a good nitrogen source (*e.g.*, ammonium, glutamine, and asparagine) is available in the medium.[19,22]

Considering the sensitivity of *S. cerevisiae* cells to nitrogen compounds, we carried out FTIR analysis to study live fresh or lyophilized *S. cerevisiae* cells from three growth media with different sources of nitrogen. These analyses were managed with the aim of assessing if both fresh and lyophilized yeast samples could be used indifferently in FTIR based studies of cell metabolome. An unsupervised multivariate analysis method such as Principal Component Analysis (PCA) and a supervised classification method such as Partial Least Squares-Discriminant Analysis (PLS-DA) were applied to compare the spectral behaviour of the yeast *S. cerevisiae* cells in a given growth medium with two sampling methods.

2. Materials and methods

2.1. Yeast strain and growth conditions

The *S. cerevisiae* strain 23344c (Matα ura3), isogenic to the wild type Σ1278b, was used in this study. Cells were grown in the minimal medium (0.17% Difco yeast nitrogen base without amino acids) supplemented with uracil 0.2 mM and containing 2% glucose as a carbon source and 10 mM proline (MP) or 10 mM ammonium sulphate (MA) as a nitrogen source. When indicated, cells from MP were incubated with 0.1 mM GABA for 1 hour (MG).

2.2. Sample preparation and FT-IR measurements

The experimental procedure used in this study is illustrated in Fig. 1. Cells from 3 ml cultures with an optical density (OD) of 1.0 at 600 nm were collected by centrifugation and washed once with distilled water. Then, sample preparation was performed according to two different procedures. (i) Fresh cells (FR): the washed cellular pellets were immediately resuspended in 0.1 ml of distilled water. (ii) Lyophilized cells (LY): the washed cellular pellets were immediately kept at −80 °C and lyophilized. Before use, the dried cellular pellets were resuspended in 0.1 ml distilled water and left for 1 hour with occasional agitation. For comparison of the FTIR spectra from FR or LY cells, not only sample preparation is important but also how the deposition for FTIR measurement is managed. Therefore, the samples from both procedures were vortexed. The OD of each sample was again controlled with the aim of adjusting the density of the cell concentration. This is of particular importance so that the thickness of each deposit will be homogeneous for the FTIR measurements.[23] A 5 µl volume per well (five deposits per condition) was immediately transferred onto an IR-light-transparent 384-well silicon microtiter plate for FT-IR analysis and the sample was vortexed between each deposit. Samples were dried in vacuum as reported[24] to generate a suitable sample film for FTIR measurements.

FTIR measurements were performed in transmission mode using a Tensor-27 FTIR spectrometer equipped with a HTS-XT accessory for rapid automation of the analysis (Bruker Optics, Ettlingen, Germany). Spectra were recorded using the spectral window of 900 to 3190 cm^{-1} which is assumed to be the spectral region that is the most informative for biological samples. Moreover, the 900–3190 cm^{-1} spectral range has been chosen instead of the classical 900–3200 cm^{-1} because of hardware and software specificities. The spectral resolution used is 4 cm^{-1} because it seems to be a conventional resolution in yeast analyses. Background spectra were collected before each experiment and subtracted from each acquired spectrum. For each spectrum, 128 scans were co-added and averaged.

Experiments were carried out in triplicate, meaning 15 spectral data for each condition.

2.3. Data pre-processing and analysis

2.3.1. Signal to noise ratio calculation. In this study, the signal to noise ratio (SNR) was calculated as the standard

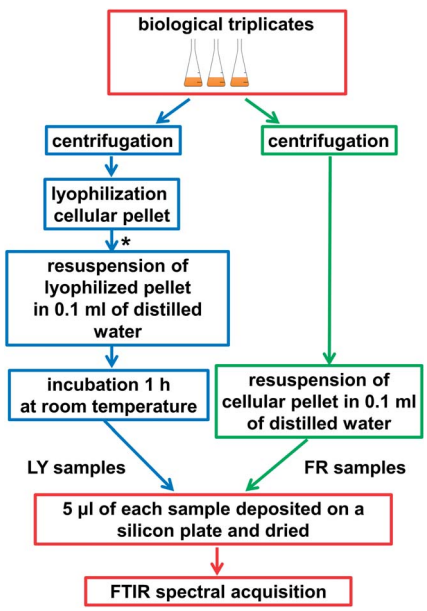

Fig. 1 Flow diagram of the experimental procedure used. The asterisk indicates the step where samples can be stored.

deviation of absorbance differences between two concomitant wavenumbers in the 1625–1675 cm^{-1} spectral range (maximum signal) divided by the standard deviation of absorbance differences between two concomitant wavenumbers in the 1800–1900 cm^{-1} (minimum signal) spectral range. This can be expressed as follows:

$$\mathrm{SNR} = \frac{\mathrm{std}\left(\int_{n=1625}^{1675}(X_{n+1} - X_n)\right)}{\mathrm{std}\left(\int_{n=1800}^{1900}(X_{n+1} - X_n)\right)} \quad (1)$$

where X is a vector containing IR absorbance values for wavenumbers $900 \leq n \leq 3190$ cm^{-1}.

All acquired spectra presenting a SNR ≤ 25 based on this calculation were systematically excluded from this study. Such a calculation takes into account the influence of atmospheric water vapor and carbon dioxide spectral interferences for a more realistic assessment of the spectral signal quality.

2.3.2. Extended Multiplicative Scatter Correction (EMSC). Several sources and factors contribute to the measured IR spectra. Not all contributions are equally relevant; some of them even could mask the information one is looking for. As a HTS-XT Tensor-27 spectrometer adequately corrects water vapor interferences, the most important and undesirable spectral contribution in this study is scattering effects of IR light. Scattering effects, such as Mie scattering,[25] give rise to a distorted background that renders detailed interpretation of biochemical changes in the samples difficult. Using the EMSC method, the variance due to non-resonant Mie scattering effects is corrected and the spectra are normalized to have comparable intensity.[24]

2.3.3. Principal Component Analysis (PCA). Prior to PCA, all the spectra (900–3190 cm^{-1}) were converted into a second derivative using a 7-point Savitzky–Golay algorithm.

To investigate the possible differences and/or similarities due to the various treatments of the yeast cells, Principal Component Analysis (PCA) was used. PCA is an unsupervised multivariate analysis method[26] that decomposes the spectra in a dataset into "Principal Components" (PCs) or "loading vectors", which are based on the variance in the dataset. These PCs contain, in descending order, less and less of the variance of the dataset. Thus, generally, only the first few PC's are required to describe most of the information contained in the whole spectral dataset. The "quantity" of each PC in a given spectra is named the "score" (S), and the unique score derived from each spectrum can be used to classify and/or group spectral data.[26] Indeed, each spectrum of a dataset is characterized by the sum of all calculated PCs, found in all spectra, weighted by their respective scores:

$$\mathrm{Sp} = \mathrm{PC}_1 \times S_1 + \mathrm{PC}_2 \times S_2 + \mathrm{PC}_3 \times S_3 + \ldots + \mathrm{PC}_n \times S_n \quad (2)$$

where Sp is a given spectrum of a spectral dataset, PC corresponds to the loading vectors, S to PC scores, and n to the number of calculated PCs.

2.3.4. Partial Least Squares-Discriminant Analysis (PLS-DA). Partial least-squares discriminant analysis (PLS-DA) is a supervised classification method based on partial least squares (PLS) regression.[27,28] This analysis permits the relation of spectral data from a group of reference samples (training set) to a user defined class of samples. If the spectral data are collected in a matrix X and the class is defined as Y, a PLS-DA model can be expressed as

$$\mathrm{pY} = \mathrm{X}b + e \quad (3)$$

where pY is the probability that X belongs to the sample class Y, b is characterized as the vector of regression coefficients, and e is the model error.

Thus, a PLS-DA model can be used to assess the belonging of a new spectral dataset X′ (validation set) to the user defined sample class Y. Here, sample class Y corresponds to the sampling method of yeast samples (FR or LY) and/or to the medium in which they were grown. X and X′ correspond to the spectral data acquired on fresh or lyophilized yeast samples grown in various media.

The PLS-DA analyses were performed using training datasets composed of 3 independent experiments. For each calculated model, cross-validation was performed by randomly splitting the training set into a calibration set (70% of spectra from the training set), and a test set (30% of spectra from the training set). The test set was used for internal validation of the developed model. External validation was then processed using a "prediction dataset" composed of X independent spectral experiments, not used for model calibration.

2.4. Statistical evaluation

All results were considered to be significant when the calculated p value was equal to or less than 0.05 for both analysis of variance (ANOVA) and Mann–Whitney tests.

2.5. Software

All spectral data and statistical analysis were processed using Matlab 2012a (Mathworks, Natick, MA) based software. Matlab routines were written in-house using the SAISIR package.[29]

3. Results and discussion

In most cases, reports dealing with studies of yeasts with FTIR spectroscopy contribute to the identification, differentiation and typing.[11,14,30] Moreover, FTIR spectroscopy is considered to be a very sensitive technique so that spectra can reveal small variations due to culture or treatment parameters.[23] Therefore, it is particularly useful to analyse the metabolomic alterations caused by internal or external stress.[31,32]

The aim of this study is to demonstrate that yeast cells coming from an identical biological medium can be used indiscriminately for FTIR analysis whether they are analysed directly as live fresh cells (FR) or after lyophilization (LY). The major relevance of such a study lies in the possibility of a postponed analysis without altering the biochemical constituents of the sample. This could be very useful in case of inevitable transport for FTIR analysis to a distant laboratory. Moreover, yeast cells are used in some industrial fields like the

food-processing industry where control analyses are required, FTIR spectroscopy being commonly used. In case of a second assessment, a freeze-dried sample stored could be used to avoid degradation.

For this purpose, we will first show that the growth media used could be well differentiated. Then we will study by different statistical methods the potential for using either FR or LY cells for FTIR spectroscopy analysis.

3.1. PCA processing of cells from different growth media

S. cerevisiae cells from three different growth conditions were used for this study, *i.e.* (i) minimal medium containing ammonium (MA), (ii) minimal medium containing proline (MP) and (iii) minimal medium containing proline and incubated for 1 hour with GABA (MG). MA and MP are two minimal media containing two different nitrogen compounds, *i.e.* ammonium, a preferred nitrogen source where NCR is active, and proline, a non-preferred nitrogen source where NCR is relieved. Consequently significant changes in the metabolomes of cells from these media are expected.[20] Moreover, considering MP and MG media now, when we compared cells from proline medium treated or not with GABA, only five genes were activated in response to GABA addition.[33] So, slight differences between the FTIR spectral signatures of these cells are expected in agreement with the growing media. As shown in Fig. 2, spectra from the two sampling conditions and the three media exhibit imperceptible differences. In the depicted region the main spectral contributions are derived from proteins, showing the very intense and broad amide I band around 1650 cm^{-1} and the amide II band around 1540 cm^{-1}. The region between 900 and 1200 cm^{-1} is dominated by the intense absorption of carbohydrates with a band located at 1040 cm^{-1} attributed to glycogen.[9,34] We observed that the different nitrogen sources used to grow yeast cells produced slight metabolic changes affecting these regions. This could be due to the fact that the three media contain glucose as the carbon source and that the genes differentially expressed in these media are limited.[20,33] Next, we considered for each medium the difference between the average of FTIR spectra from FR cells and LY cells as shown in Fig. 3. As expected, it reveals that for each medium there are differences between spectra from LY and FR cells. The use of statistical analysis will be required to highlight if the subtle alterations observed are or not statistically significant. Therefore, PCA was processed separately on LY and FR cells in the whole spectral range after pre-processing and the second derivative of the spectral data with the aim of demonstrating that FTIR signatures depend on the medium but not on the sampling method.

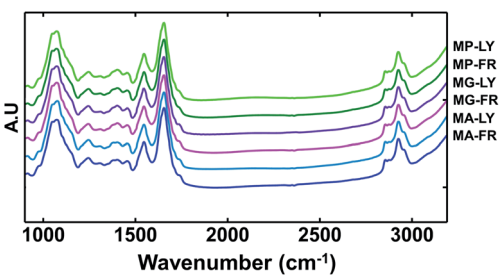

Fig. 2 Average spectra after pre-processing treatment for both sampling conditions (FR or LY) in each growth medium (MA, MP and MG) in the 900–3190 cm^{-1} spectral range.

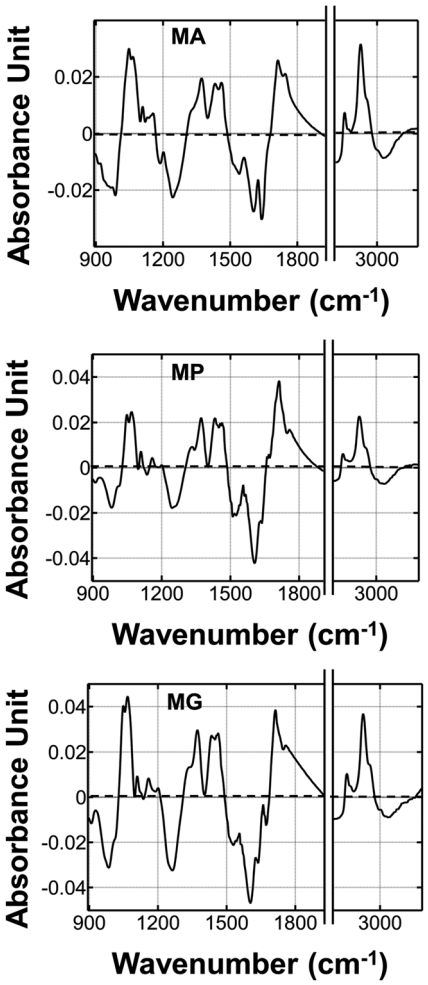

Fig. 3 Spectral variations between fresh (FR) and lyophilized (LY) *S. cerevisiae* cells grown in three different minimal media containing ammonium (MA), proline (MP) or GABA (MG) as the nitrogen source. For each medium, average FTIR spectra from FR cells (dotted line) are deducted from average FTIR spectra from LY cells (continuous line).

The corresponding score plots for the two best separating principal components are shown for FR and LY cells in Fig. 4a and b, respectively. In these figures, scatter plots represent a normal distribution fit on bivariate data represented here by the two more discriminative PCs. PC loadings used in this study are available in the ESI (Fig. 1S†). PC1 and PC2 together constitute 99.4% of the total variance of spectral data for FR yeasts while PC1 and PC3 constitute 84.4% for the LY cells. These results show that FTIR spectra from *S. cerevisiae* cells growing in one of the three different media (MA, MP or MG) are easily clustered in both FR and LY cell samples. However, MA and MP are well separated while data points of both MP and MG are close for FR cells if not overlapped for LY yeasts. These results indicate that

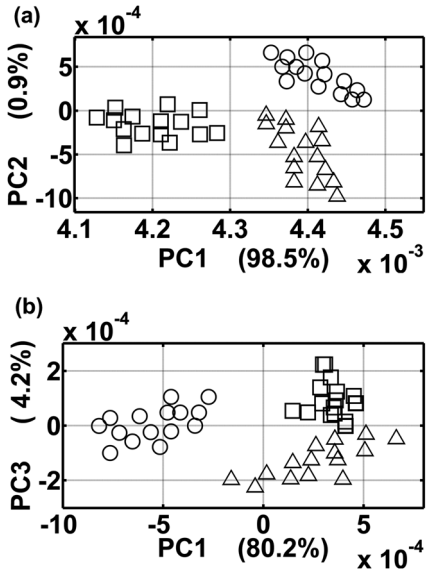

Fig. 4 Scatter plots of the PC scores from pre-processed spectral data acquired on *S. cerevisiae* cells grown in three different minimal media containing ammonium (MA), proline (MP) or GABA (MG) as the nitrogen source. All three parallel measurements were included in the PCA. (a) Fresh cells (FR) from MA (○), MP (△) and MG (□). (b) Lyophilized cells (LY) from MA (○), MP (△) and MG (□).

it was easier to separate the MP and MA media than the MP and MG media in agreement with gene expression studies.[20,33] After all, FTIR analyses allow efficient separation of cells from the three media for both sampling methods.

Distribution of the samples of each medium is depicted by scatter plots and shows that lyophilized ones are more homogeneous than fresh ones. This suggests that the lyophilization of yeast samples could be considered as a fixation treatment, limiting undesirable biochemical alterations induced by degradation of the cell content that occurred when fresh samples were slowly desiccated prior to FTIR analysis.[35] Therefore, the freeze-drying procedure could increase the robustness of discrimination between growth media.

3.2. PCA processing of cells from different sampling methods

Now we have to demonstrate that lyophilization does not affect the global biochemical information given by the FTIR fingerprint allowing us to perform FTIR analysis equally with LY or FR cells. The differentiation of yeast cells from different growth media seems to be relatively easy using FTIR spectroscopy. But now considering one specific growth medium, we examined if it is possible to use either live yeasts or freeze-dried ones for the FTIR analysis.

For this purpose, PCA was processed on the spectral data of FR or LY cells from MA on one hand, or MP on the other hand. ANOVA was then applied to PC scores to determine the statistical significance of PC average scores depending on the sampling methods (FR or LY) of cells from the two media. The ANOVA plot not only provides the mean score of a given PC score but also presents the advantage of displaying intuitively the statistical significance of mean score differences between each tested condition.[24] For each medium, the ANOVA plot of the top three more discriminative PCs is presented for a spectral data comparison between FR and LY cells (Fig. 5). In both cases, it was impossible to differentiate significantly spectra of FR cells

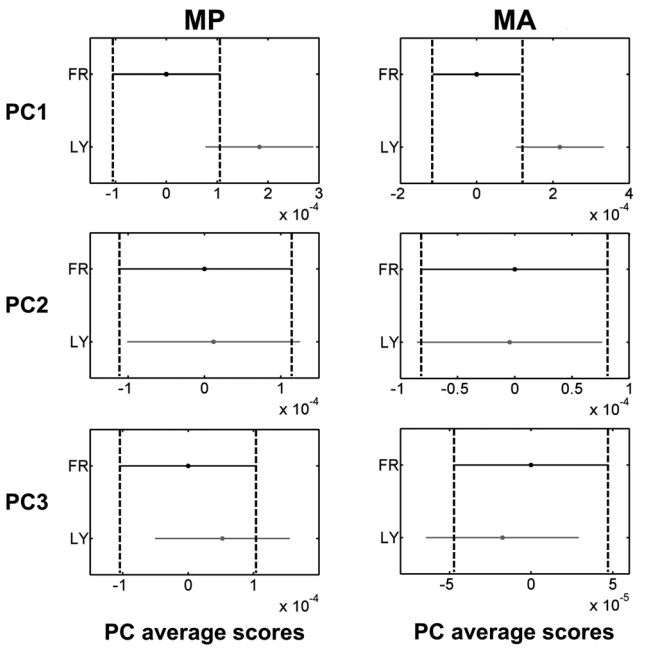

Fig. 5 PCA-based discrimination of yeast cells from proline medium (MP) or ammonium medium (MA) depending on the sampling method: fresh (FR) or lyophilized (LY) cells. Mean scores of the first three PCs (PC1; PC2; PC3) for pre-processed spectral data from three experiments are presented. Horizontal bars shown in the graphs are computed so that mean PC scores being compared are significantly different if their intervals are disjoint (p value <0.05), and are not significantly different if their intervals overlap (p value >0.05).

Table 1 PLS-DA classification results for a discrimination of the media without considering the FR or LY sampling method

	Training set			Prediction set		
Medium	Samples correctly classified	% Variance	False negative	Samples correctly classified	% Variance	False negative
MA-(FR + LY)	19	95	1	9	90	1
MP-(FR + LY)	18	90	2	8	80	2
MG-(FR + LY)	19	95	1	9	90	1

Table 2 PLS-DA classification results for a discrimination of the media considering the FR or LY sampling method

	Training set			Prediction set		
Medium and cell sampling method	Samples correctly classified	% Variance	False negative	Samples correctly classified	% Variance	False negative
MA-FR	7	70	3	1	20	4
MA-LY	9	90	1	2	40	3
MP-FR	8	80	2	1	20	4
MP-LY	9	90	1	1	20	4
MG-FR	8	80	2	2	40	3
MG-LY	9	90	1	2	40	3

or LY cells (p-value >0.05). PC loadings used in this study are available as ESI (Fig. 2S†).

Furthermore, in most of the studies dedicated to the differentiation of microorganisms, only specific regions of the spectra are used because they are characteristic of these species such as the spectral window 900–1200 cm^{-1} corresponding mainly to the polysaccharides.[14,36] We investigated if using one specific spectral window the differentiation between spectra of FR and LY cells from one medium could be achieved. Once again, it was impossible to statistically discriminate FR from LY cells (results not shown).

Then, we can assume that PCA is an inefficient statistical method to distinguish FTIR spectra obtained from the two sampling methods for one specific medium.

3.3. Discriminant partial least squares regression (PLS-DA) for analysing fresh and lyophilized cells

As previously presented, FR and LY yeast cells were analysed with FTIR spectroscopy with the aim of showing that either of them could be indifferently used for these spectroscopic analyses. Therefore we will investigate if the discriminant partial least squares analysis (PLS-DA) could differentiate FR and LY cells coming from one growth medium. PLS-DA is a supervised analysis based on PLS regression but where the response variable indicates the class of the samples. Moreover this method of analysis could be considered more subtle[37] than PCA to establish a differentiation between the two sampling methods FR and LY. PCA contributes to differentiate samples but above all permits us to have an image of the distribution of the various media as a function of the sampling methods without any *a priori* treatment. By contrast, because of its supervised nature, PLS-DA does not permit evaluation of the distribution of spectral data but is a more effective approach for data classification. The PLS-DA approach was applied on a spectral dataset composed of spectra recorded from both FR and LY cells incubated in MA, MP or MG media.

First, we have developed a PLS-DA model to discriminate the growth conditions (MA, MP, MG) without considering the method of preparation of samples (FR or LY). Results for the training set and prediction set (external validation) for the calculated model are shown in Table 1. These results demonstrate that analysing both FR and LY cells from a particular growth medium such as MA, MP and MG had no consequence on the discrimination between media: the media were all well identified. In the same way the good percentage of variance for the prediction rate (between 80 and 90%) leads one to suppose that this method's accuracy is satisfying. These results are in agreement with those obtained above where the media were well separated using PCA (Fig. 5).

Then, we developed a PLS-DA model in order to discriminate not only the growth media (MA, MP, and MG) but also the cell sampling method, *i.e.* FR or LY. Thus, six groups of samples (MA-FR, MA-LY, MP-FR, MP-LY, MG-FR and MG-LY) have to be considered for this classification. Results of this PLS-DA classification are shown in Table 2. Considering results from the training set, spectra from LY cells were accurately classified for each medium. This was not the case for FR cells. Then results from the prediction set (external validation) depict that FTIR spectra could not be discriminated according to the sampling procedure even for LY cells and whatever the medium considered. Therefore, results in Table 2 show that the PLS-DA could not permit us to claim that a spectrum has been acquired from LY yeast cells rather than FR ones. However, even if the PLS-DA method mixes up FTIR spectra from the two sampling conditions, it permits differentiation of the FTIR spectra according to the media of origin. So, the differences between the media are easily detectable due to their impact on the metabolism of yeast cells. On the other hand, the differences between both sampling conditions FR and LY seem to be so slight that FTIR spectroscopy could not detect features of a specific condition.

4. Conclusion

In this work we demonstrate that either FR or LY yeast cells can be equally used for discriminating and classifying yeast samples based on IR spectral features related to metabolomic changes induced by the type of minimal growth media used. The use of LY cells has several advantages over FR ones: samples can be easily transported and stored and variations of their metabolomic profiles do not occur during transport or storage. Moreover, FTIR data obtained using LY cells showed less variability. Therefore it seems that the lyophilization procedure mimics other cellular fixation methods such as treatment of mammalian cells with formaldehyde.

FTIR spectroscopy together with chemometric analysis methods could not allow differentiating FR and LY yeast cells. Chemometric analysis such as PCA or PLS-DA permits

differentiation of the growing media of the yeast cells while it never permit us to highlight significant differences between the FTIR spectra from LY to FR yeast cells. This spectroscopic technique is well known for its sensibility and currently used to exhibit subtle features. In this case, FTIR spectroscopy did not permit distinguishing of FTIR spectra of one sampling method from the other allowing us to consider that LY yeast cells could be used for FTIR analysis instead of or even in conjunction with FR cells. This supports our proposal that the yeast cell lyophilization procedure is not only adequate but also advisable to transport and preserve samples before being subject to FTIR measurements.

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