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# microPAT: A protocol for direct in vitro NMR observation of lactic acid bacteria fermentations

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Lactic acid bacteria (LAB) are widely used in the food industry as starter cultures to manufacture fermented food, and more recently there has been an increasing interest in using LAB for biopreservation of food products. The study of the metabolism of these bacteria is therefore of great interest, and NMR is the only omics analytical technology that has the potential to measure their metabolome non-destructively. In vivo NMR can provide valuable information about the dynamic metabolism of microorganisms, for example for studying the adaptive responses of microorganism to different environmental and stress factors such as substrate, cohabitation, deleterious metabolites, as well as temperature and pH. This paper describes a protocol for *in vitro* NMR measurement of the fermentation of lactic acid bacteria. We call it microPAT (microbial process analytical technology) [1] as it monitors in real time the microbial processes of the fermentation. Some of the experimental issues that were encountered during the process of developing the protocol as well as the selected approach to overcome them are presented.

#### Introduction

MR can provide valuable information about the dynamic metabolism of microorganisms. Already in 1984 *in vivo* <sup>31</sup>P NMR was used to measure the intracellular inorganic phosphate (Pi) and relative levels of glycolytic intermediates of *L. lactis* [2]. *In vivo* <sup>13</sup>C-NMR has later been used extensively to investigate regulation of the carbohydrate metabolism in *L. lactis* [3-5]. One of the interesting applications of *in vivo* NMR has been to study the adaptive stress responses of microorganisms, when they are exposed to different environmental perturbations. A great advantage of NMR in this context is the ability to perform real-time non-destructive and non-invasive measurements of the rapid metabolic changes of microorganisms under the stress conditions [6, 7].

In order to study the metabolism of selected strains of LAB, an efficient analytical protocol was recently developed for real-time *in vitro* NMR measurements of LAB fermentations, which covers all the steps of the analysis, from sample preparation, over data acquisition and preprocessing, to the extraction of the kinetic metabolic profiles [8].

# The protocol for real-time in vitro NMR measurements of LAB fermentations

In the following, some of the experimental issues that were encountered during the development of the protocol will be accounted for along with the selected approach to overcome the problems. In addition, the experimental design that the protocol was applied to, and the strategy for extracting the metabolic profiles are presented.

#### Glycerol (cryo-protectant) removal

The bacterial cells that were used in the experiment where frozen at -80°C in glycerol solution until use. The glycerol gave rise to intense resonances in the NMR spectra which necessitated washing the cells twice with the chemically defined interaction medium (CDIM)

prior to sample preparation, to decrease the glycerol concentration. Figure 1 shows an example of the spectra from a sample without the cell wash. Glycerol signals are even more intense than glucose signals, and would definitely limit the receiver gain. Therefore, it was necessary to wash the cells before inoculation, and washing the cells twice with CDIM enabled sufficient reduction of glycerol.

## **Alignment**

Changes in pH or temperature may induce chemical shift changes for pH or temperature sensitive compounds. This is not a desirable source of variation when using multivariate data analysis techniques. Therefore, post-acquisition alignment techniques are necessary prior to multivariate analysis of the data. One of the methods that has proved to be very useful for this purpose is the *i*coshift algorithm [9, 10]. It allows for the simultaneous alignment of a series of spectra, either relative to a reference signal, or in the user-defined intervals.

For the in vitro time series NMR data from the fermentations, the strong buffer ensured that only minor changes of the pH occurred and as a result, no significant chemical shift changes were observed for most of the nutrients and metabolites. However, for compounds such as acetic acid and histidine which are highly pH sensitive, even the small changes in the pH resulted in large chemical shift changes during each fermentation, as well as between the fermentations of the different samples. Such chemical shift changes are of course larger in samples with the higher glucose concentrations as they tend to produce more acidic metabolites by fermentation. In the experimental design that the time series measurement protocol was applied to [8], icoshift was used to align each of the 16 timeseries data, first relative to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) that was added to the samples as an internal standard for chemical shift calibration, and then in defined intervals containing the selected metabolites. Intervals were defined for glucose,

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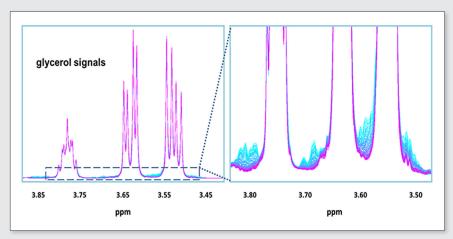


Figure 1. Strong signals from glycerol in the samples without washing the cells prior to inoculation. Glycerol signals compared to the glucose signals, that can be observed in the zoomed figure, are much stronger.

pyruvate, acetate, alpha-acetolactate, formate, glutamine, aspartate, adenosine, inosine, and adenine. The 16 batches of time-series data were then augmented row-wise (along time-dimension) and *i*coshift was used again to align the selected signals. Figure 2 shows the alignment process schematically.

It is important to perform the alignment in a supervised manner, and the alignment results should be investigated before subsequent data analysis in order to ensure that the algorithm has not aligned resonances from different compounds in overlapping signals, or signals that sit very close to each other. For instance, in the case of the doublet of lactate at 1.32 ppm, it was observed that the doublet of threonine overlapped with the lactate doublet. Lactate and threonine signals, with and without alignment are shown in Figure 3. As the concentration of lactate at the first time points is zero, the algorithm erroneously aligns threonine at these points with the lactate doublet. Due to this problem and also the fact that lactate signal did not change its chemical shift during fermentation, icoshift was not used for this signal in the final processed data.

#### Line broadening

As the number of cells increases during the LAB fermentation, the samples become thicker and can become inhomogeneous because of cell coagulation. The inhomogeneity in the sample will

result in line broadening of the signals in the NMR spectra towards the end of the fermentations. Therefore, for a time-series measurement of LAB fermentation, signals will get broader during the fermentation, as long as the cells are propagating. Lineshape changes by time will definitely lead to errors in quantitative multivariate analysis of the metabolic changes of such data, by methods like principal component analysis (PCA) [11], partial least squares (PLS) regression [12], and multivariate curve resolution-alternating least squares (MCR-ALS) [13, 14] that have bilinearity of the data as their main principle. It has previously been shown by designed artificial 'metabolic' experiments that Reference Deconvolution can be helpful in improving the multivariate analysis results of NMR data, by enhancing the line-shapes and bilinearity of the data [15]. Based on this, Reference Deconvolution was used on the in vitro NMR data, in order to enhance the quality of the spectra and to solve the line broadening problem.

#### Recovery of the lactate doublet

In the recorded time-series data of LAB fermentations, lactate signals were quite broad, and for instance the signal of the methyl group in lactate at 1.32 ppm, showed that a very broad signal was building up over the time course of fermentation instead of the expected doublet form. Figure 4a shows the lactate doublet for *L. rhamnosus* 

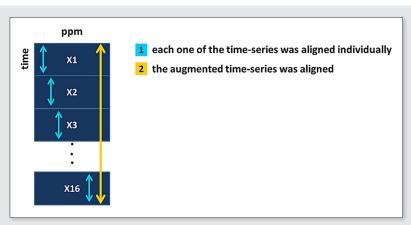


Figure 2. The schematic illustration of the alignment procedure of the data.

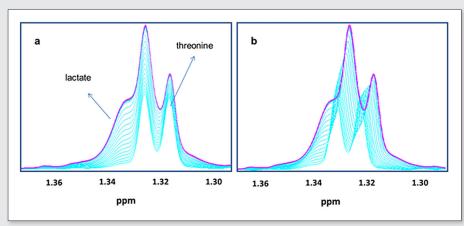


Figure 3. Lactate and threonine signals: a) without alignment, b) after alignment. In (b), the signals are misaligned.

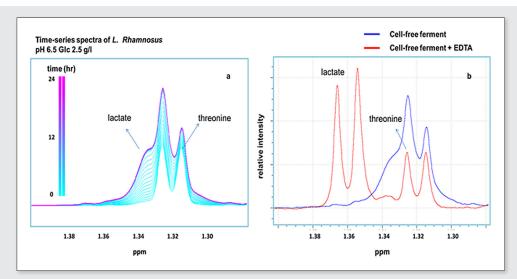


Figure 4. Recovery of lactate doublet by addition of EDTA. a) time-series spectra of one of the *L. rhamnosus* samples, colored by time, b) spectra of the cell-free ferment of the sample with and without added EDTA.

sample at pH 6.5 and the glucose concentration of 0.25 g/l. In order to verify that the broadening of lactate signals occurred due to the formation of Na/Ca lactate, cell free supernatant of the sample at the end point of fermentation was prepared using 0.2  $\mu m$  filters, and ethylenediaminetetraacetic acid (EDTA) was added to the solution. EDTA can form chelates with Na $^{+}$ /Ca $^{2+}$  ions, and therefore release lactate. Figure 4b shows the spectra of the cell free supernatant with and without the added EDTA. After adding EDTA, the sharp doublet form of lactate is recovered which confirms that the lactate line broadening is due to the formation of Na/Ca lactate. The chemical shift is higher after addition of EDTA, due to the slight change in the pH of the sample. This test was merely performed in order to explain the observed line broadening. For the purpose of the data analysis, the original samples and spectra were used.

#### Experimental design and sample preparation

The experimental design, as shown in Figure 5 consisted of two strains of LAB, *Lactobacillus rhamnosus* DSM 20021 and *Lactobacillus plantarum subsp. plantarum* DSM 20174, two levels of glucose concentration (2.5 and 0.25 g/l), two pH values (pH 6.5 and 5.5), and duplicate fermentation batches. Samples for the *in vitro* 

NMR measurements were prepared according to the protocol that is shown in Figure 5.

# Extracting the metabolic profiles – multivariate curve resolution (MCR) and second derivative approach

In order to model the metabolic profiles, as shown in Figure 6, MCR-ALS or second derivatives of the signals were used. In the subsequent data analysis, we tried to model as many signals simultaneously as possible, because it is both statistically preferred, and will also reduce the analysis time. Because of the strong buffer system, the position of most of the signals remained stable during the fermentation, and MCR-ALS was used on the augmented data to model their profiles during fermentation. However, for some of the pH sensitive compounds changes in chemical shifts during fermentation were observed. For the singlets from acetate and pyruvate a perfect alignment was not achieved, and instead the minimum of the second derivative of the signal was used to model the profile. Figure 6 shows the pyruvate signal in L. rhamnosus sample at pH 6.5 and glucose concentration of 2.5 g/l, and its second derivative that was used for the subsequent modelling of the pyruvate concentration profile.

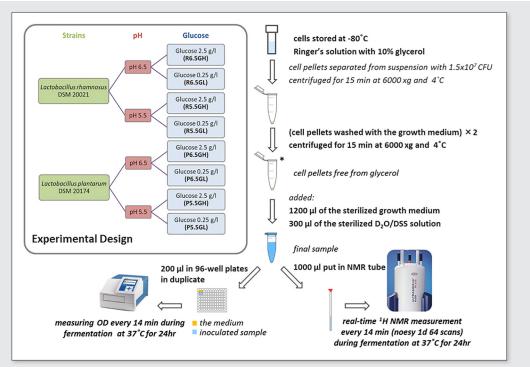


Figure 5. a) The Experimental Design for the in vitro NMR measurement of LAB fermentation, and b) the developed protocol for in vitro NMR studies of bacterial fermentation.

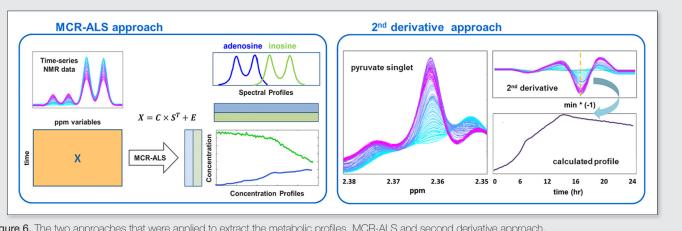


Figure 6. The two approaches that were applied to extract the metabolic profiles, MCR-ALS and second derivative approach.

The chemical shift of inosine differed for the two pH values, and at pH 5.5, the inosine doublet partly overlapped with the adenosine doublet. Because of this overlap, a suitable alignment could not be achieved between pH 6.5 and 5.5 samples. However, as the signal position was fairly stable in individual time-series and also between the samples with the same pH value, adenosine and inosine signals were modelled by two component MCR-ALS models for pH 6.5 and 5.5 separately.

# Outreach

NMR spectroscopy is a unique method for in vivo/vitro investigation of bacterial metabolism. An efficient in vitro NMR protocol was developed for real-time investigation of bacterial metabolism. The protocol has been applied to an experimental design and allowed for detailed real-time kinetic analysis of 11 major metabolites that are involved in the glycolysis, pyruvate catabolism, amino acid catabolism and cell energy metabolism, which led to new microbiological knowledge about the strains [8].

One of the key points in the protocol was the application of reference deconvolution for enhancing the quality of the time-series NMR spectra. As the first application of Reference Deconvolution to metabolomic NMR data, it proved to be a necessary and elegant solution to the problem of the inhomogeneity of the samples that is encountered in in vitro NMR measurements of cells. Moreover, a strong buffering system was required, as pH changes during fermentation and having pH as one of the design factors demands to keep it fairly stable during the fermentation.

The only limiting factor for the application of NMR for in vitro investigation of bacterial fermentation is the inherent low sensitivity of NMR techniques compared to analytical techniques such as mass spectrometry. Due to numerous analytical limitations, even by the application of the most sensitive analytical techniques, we are still far from capturing the whole image of the metabolome. On the other hand, by using techniques such as *in vitro* NMR that have lower sensitivity, we will be limited even further and will be able to cover only the most abundant metabolites. Despite this, the obvious advantages of *in vitro* NMR techniques cannot be overestimated and the use of this technique, especially in parallel with other techniques such as chromatography and mass spectrometry can provide a very strong tool to obtain new insight into the metabolism of the organisms of interest. Using hyperpolarization techniques, such as dynamic nuclear polarization (DNP) that can increase the sensitivity of NMR by several orders of magnitude [16] can also broaden the application of NMR for studying bacterial metabolism.

The new *in vitro* NMR protocol can be used in biopreservation studies for improved strain selection, investigations of the effects of different design factors on the excretion of antifungal metabolites, investigations of potential synergies (cohabitation), investigations of the effect of different substrates. As a part of the microPAT concept, *in vitro* NMR analysis should be compared to inhibition assays against pathogenic bacteria or molds at the end of the fermentations. To this end a new software called PCluster was developed for quantifying mold growth and inhibition [17]. The combination of PCluster and *in vitro* NMR measurements will allow for the identification of antimicrobial metabolites, for quantifying how different internal and external factors affect inhibition, and for optimizing different parameters to stimulate the excretion of antifungal metabolites.

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