IMPROVED METHOD FOR INTRACELLULAR pH DETERMINATION IN FILAMENTOUS FUNGI

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Abstract

Until now, it was difficult to accurately determine the intracellular pH of filamentous fungi growing in large bioreactors. In order not to change the pH_i values during the preparation of samples and ^{31}P-NMR measurements, a new technique was developed that quenched the metabolism immediately after taking the cells out of the growing environment. It has been done by immersing the mycelium in cold buffered methanol at -40 °C. The temperature of the samples was maintained low even during the accumulation of scans in the NMR magnet. Since frozen cells were surrounded by liquid buffered methanol, sufficient motion of molecules containing phosphorous was possible to enable the formation of high-resolution NMR spectra. The method has proven to be reliable enough for monitoring the changes of apparent intracellular pH that occur during hypoxia in a fermenter.

Introduction

For studying metabolic regulation of filamentous fungi during biotechnological processes information about changes in the intracellular pH (pH_i) is of extreme importance. There are several previous reports on pH values in fungal cells obtained by ^{31}P-NMR technique, where measurements were conducted in narrow 10 or 20 mm NMR tubes. The cells were kept aerated by the direct introduction of air into the NMR tube[1] or by continuous perfusion into the medium[2] that has been aerated outside the magnet. However, such systems just mimic the conditions in a larger fermenter; therefore, the physiological state and the intracellular pH may differ from that in the bioreactor. In order to detect physiological levels of many phosphate containing metabolites and ortho-phosphate, it is necessary to perform NMR experiments on dense suspensions of cells, generally 10-50% of wet weight/volume.[3] Under such conditions it is often difficult to maintain cells in a well oxygenated physiological state, which is of utmost importance for pH_i determination. Namely, it is well known that intracellular pH is very prone to

changes, induced by altered oxygen tension or nutrient conditions.[2] Under oxygen limitation ATP synthesis is reduced and consequently ATP depletion slows the activities of proton pumps involved in cellular pH regulation, which seem to be major factor responsible for intracellular acidification.[4]

Due to the unwanted effects that may occur in the cells during prolonged preparation and examination of samples, it was practically impossible to observe pH_i changes continuously during growth in a fermenter. In order to overcome this problem, a method that rapidly quenches metabolism immediately after cells are removed from the growth medium was introduced. It was done by submerging the mycelium in buffered methanol, cooled to –40 °C. The method was originally described for yeast cells,[5] and later was applied for filamentous fungi, including *Aspergillus niger*.[6]

Results

A sample of approximately 250 ml of suspension was poured into the pre-cooled Dewar flask directly from the fermenter, so that the temperature of the fermentation broth instantly dropped from 30 °C to about 10-15 °C. In less than 30 seconds the mycelium was collected by suction filtration and immersed in buffered methanol at -40 °C. To find out how rapidly metabolic changes occur in chilled mycelium the position of ortho-phosohate peak in NMR spectrum of the mycelium, which was poured directly into the cold methanol was compared to peak position in spectra of the cells that were kept in a Dewar flask at reduced temperature (15 °C) for different time periods. No differences in position of inorganic phosphate peaks were observed between the samples where the metabolism was quenched instantly and the samples kept in the Dewar flask for 30 and 60 seconds. Minor shift of the peak was recorded in the cells that stayed untreated at 15 °C for 90 seconds, while by prolonged incubation the differences increased.

Before further tests, it was important to know whether the cells remained intact after immersion in the cold methanol. Methanol could dissolve lipid components in the membrane and the cells would become permeable. By liquid chromatography supernatants of buffered methanol with mycelium were checked for the presence of

intracellular metabolites. Aliquots (1 ml) were taken at 30 minutes intervals. Although the samples were concentrated about 100 fold by evaporation, only carbohydrates characteristic for the growth medium, glycerol and imidazole were recorded by liquid chromatography. However, the amount of substances slowly increased with time (Table 1), but no unknown peaks of possible intracellular metabolites could be detected, even after extended incubation of mycelium in the cold methanol.

Time (minutes)	30	60	90	120	150	180
Saccharose (mg/g w.w.biomass)	0.4	0.56	0.93	1.3	1.46	1.53
Glucose (mg/g w.w.biomass)	5.9	8.5	13.3	14.8	17.2	16.3
Fructose (mg/g w.w.biomass)	4.2	6.3	10.1	11.5	13.4	12.8
Glycerol (mg/g w.w.biomass)	0	0.09	0.32	0.36	0.42	

Table 1: 10 g of wet weight of mycelia was placed into 100 ml of buffered methanol and incubated at –40 ^{o}C. At 30 minutes intervals, aliquots of 1 ml of supernatant were taken, concentrated by evaporation and tested by HPLC. Only the components found in the medium plus about 2.7 mg/ml of imidazole (buffer) were detected. Data presented in table are the averages of results obtained from three independent measurements.

At –40 ^{o}C the cells were frozen and actually solidified. To mimic such conditions, a calibration curve for the chemical shift of ortho-phosphate measured at –40 ^{o}C caused by different pH values, was observed with a frozen buffer surrounded by liquid methanol. Under such conditions the inorganic phosphate peak shifted by about 0.5 ppm, in the pH range between 7.4 to 6.2 (Figure 1). The proton activities designated as pH values of water buffer solutions used for the calibration curve were determined at 22 ^{o}C and were reproducible. Under liquid conditions, when 40 ml of calibrating buffer were simply

mixed with 60 ml of methanol and then cooled to –40 °C, the ortho-phosphate peak shifted for more than 1 ppm in the same pH range (Figure 1).

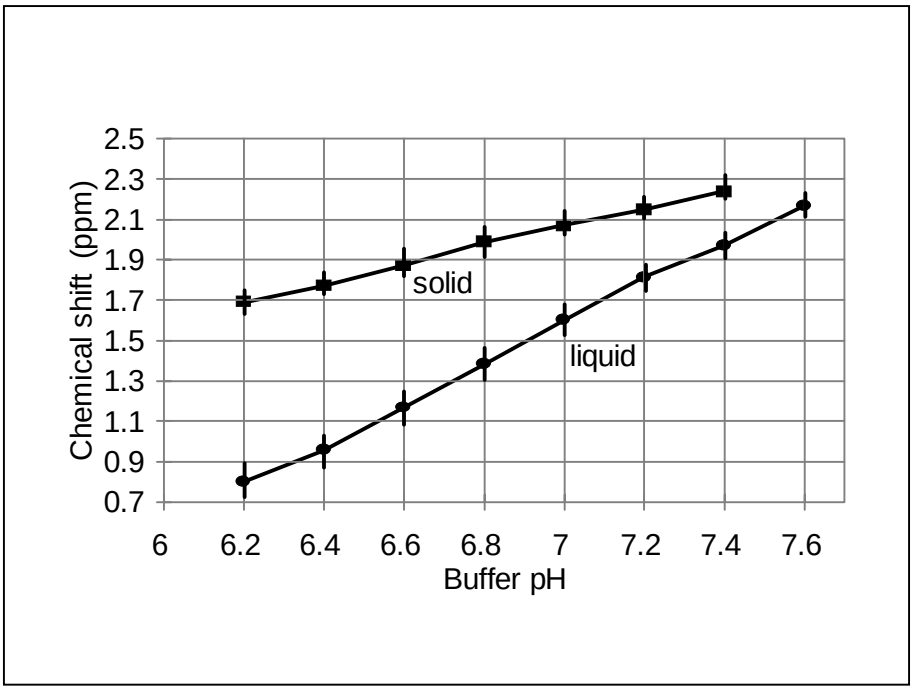

Figure 1: Chemical shift of ortho-phosphate peak recorded at –40 °C with respect to different proton activities of buffers determined at 22 °C. Different shifts were recorded when the buffer containing phosphate was frozen, pulverised and than mixed with cold buffered methanol (■); or simply mixed with cold methanol to the final concentration of 60% v/v (•).

A remarkable effect of a slight change in temperature on chemical shift was observed. By raising the temperature from -40.0 °C to -37.0 °C a peak of ortho-phosphate moved from 2.020 to 1.964 ppm. Therefore, the scans were started at least 5 minutes after the NMR tube with the mycelium was placed into the magnet, in order to allow the temperature to stabilise at –40 ± 0.1 °C.

To find out whether the mycelium could be stored under the liquid nitrogen, spectra of freshly prepared samples were compared to those of the same mycelium kept under liquid nitrogen for three months. Again, the differences in position of ortho-phosphate

peak between both samples were only within the accuracy of a chemical shift (data not shown).

There were practically no differences in the resolution of spectra recorded at –40 and at +25 °C (Figure 2).

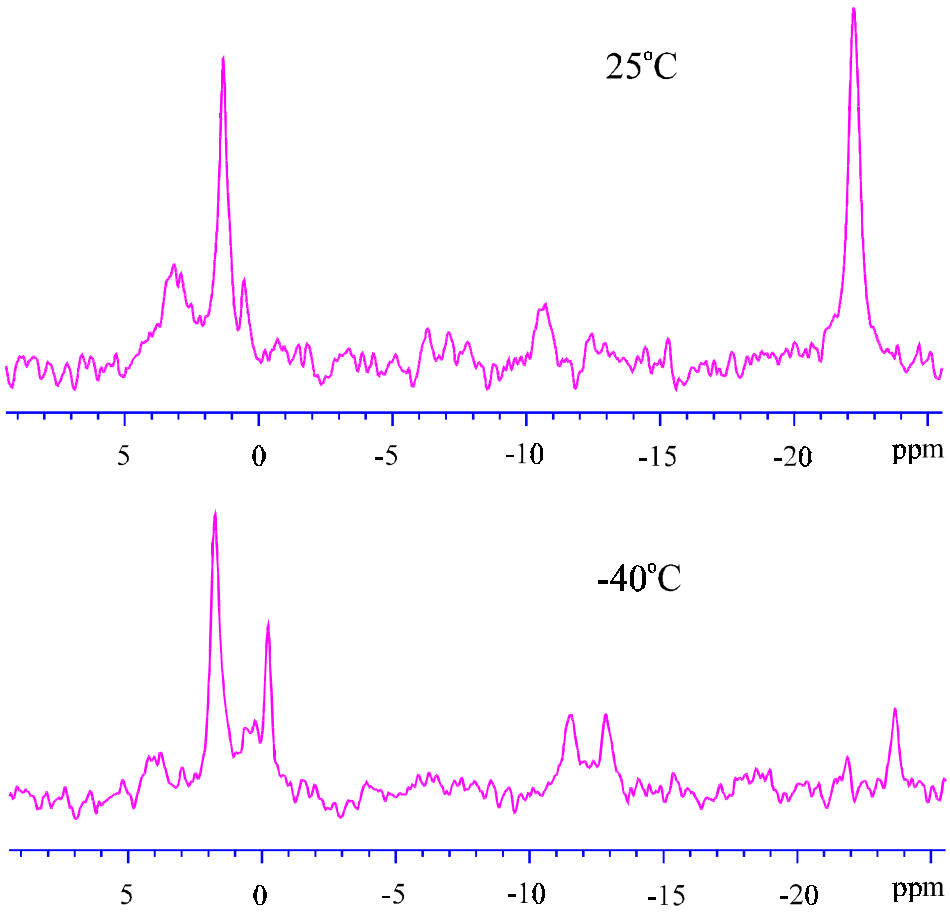

Figure 2: High resolution ^{31}P-NMR spectra of *Aspergillus niger* cells were recorded at -40 °C as well as at room temperature.

The highest single peak around 2 ppm corresponded to the intracellular inorganic phosphate, while several lower peaks between 3 and 5 ppm were formed by sugar monophosphates. In both diagrams the presence of di- and triphosphates could be

detected, however less characteristic polyphosphate peak around -22 ppm was recorded at low temperatures. In samples surrounded by methanol a peak close to 0 ppm was regularly found, representing the inorganic phosphate from the medium (pH=2.0),[10] which is left as a frozen film on the surface of the cells. No other peaks of inorganic phosphate could be detected. At this developmental stage *A. niger* mycelium contained no vacuoles and a relatively low number of mitochondria.

The effect of hypoxia on the chemical shift of ortho-phosphate peak that corresponded to apparent changes in intracellular pH value of *Aspergillus niger* cells was observed during growth in a fermenter (Figure 3).

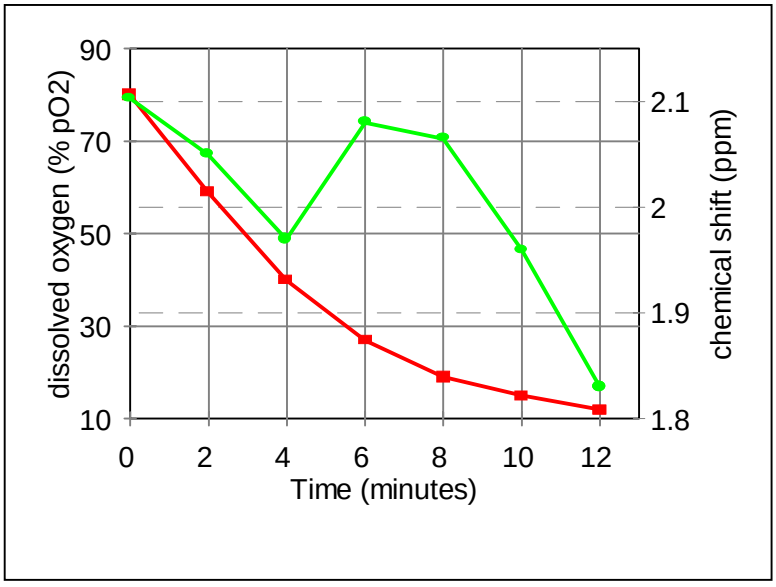

Figure 3: After interrupted aeration the amount of dissolved oxygen tension in the fermentation broth (■) is decreasing. Concomitantly a chemical shift of ortho-phosphate peak (●) in *A. niger* cells could be observed by ^{31}P-NMR which corresponds to the changes of the apparent intracellular pH value.

After 50 hours about 0.6 g of dry weight mycelia were present per 1 liter of substrate and the amount of dissolved oxygen reached about 80% saturation. The first sample for measurement was taken just before the air supply to the fermentation broth was terminated and subsequently the samples were collected in two-minute intervals.

Simultaneously, the amount of dissolved oxygen was recorded. As shown in figure 3, the oxygen was rapidly consumed by the cells and within the next 12 minutes its value dropped to about 10% of saturation. By monitoring the chemical shifts of ortho-phosphate peaks in subsequently taken samples of mycelia the changes in the apparent intracellular pH could be determined. The results show that pH value in the cells rapidly dropped for about 0.3 units during the first 4 minutes, followed by an alkalization to nearly the original level. Later intracellular pH continued to decrease (Figure 3). A transient recovery after the initial acidification was observed in all experiments conducted and it happened at approximately 4 to 8 minutes after the air supply was stopped. Unfortunately, the amount of biomass in separate fermentation at the time of sampling differed too much to allow statistical evaluation of results. Therefore, only the data of a single experiment are shown in Figure 3.

Discussion

The best way for collecting fungal samples would be to pour the fermentation broth directly into the cold buffered methanol and quench the metabolism instantly. However, under such conditions a substantial amount of medium containing inorganic phosphorus was frozen around the cells which was difficult to remove afterwards by washing. On NMR spectra of these samples a strong ortho-phosphate peak was regularly formed which often interfered with intracellular ortho-phosphate peak. Since no chemical shift of ortho-phosphate peak was observed between the samples poured directly into the methanol and samples kept at reduced temperature for about one minute it seemed to be reliable enough to collect the samples first in a cold Dewar flask and remove the medium rapidly by suction filtration. Anyhow, after submerging the cells in cold methanol solution, metabolism is expected to stop in less than a second.[5] Since further tests on the presence of possible intracellular metabolites in supernatants were negative, one can conclude that *A. niger* cells remained intact in the presence of methanol. This observation is in accordance with the findings of others that conducted similar tests on *A. niger* [6] and *S. cerevisiae* cells.[5] The increase of sugar components in the supernatants with time showed, that the remaining medium around the hyphae was frozen in cold methanol, very

slowly dissolved and mixed with buffered methanol. Actually, it seems that a film of frozen substrate is formed around the pellets that prevents a direct contact of methanol with the cell surface. Therefore, there was no need for washing the mycelium before quenching the metabolism with cold methanol. Even more, washing could be harmful: *S. cerevisiae* cells that were washed with cold methanol on a filter paper were reported to leak metabolites.[11]

The results of ^{31}P-NMR measurements presented in figure 2 demonstrate that it is possible to obtain high-resolution spectra on frozen mycelium at –40 °C. The signals from the mycelium which is actually solidified do not significantly differ from those measured at room temperature and even the signal to noise ratio has a similar level in both experiments. The motion of frozen cells in liquid buffered methanol is sufficient to allow the application of high resolution spectroscopy. There is no need for magic-angle spinning technique[12] for experiments on intact frozen biological systems, as suggested previously.[13]

Yet, both spectra presented in Figure 2 differ in details. It is important to realise that the mycelium measured at +25 °C was not oxygenated during the experiment, so that severe metabolic changes occurred that are reflected in ^{31}P-NMR spectra.

A major drawback of taking spectra at –40 °C is a relative narrow shift of ortho-phosphate peak in frozen buffers with different pH values. According to the accuracy of chemical shift values, the peaks in spectra can vary for 0.030 ppm and correspondingly the accuracy of the apparent intracellular pH value can be adequately determined to the variance of 0.04 units.

It is important to realise that determination of apparent intracellular pH by ^{31}P-NMR technique is less accurate at –40 °C than at room temperature, where the chemical shift spans for nearly 2 ppm in the same pH range.[14] However, the biochemical processes that would occur during the measurement if the metabolism was not completely stopped could distort the results even more.

The fact that prolonged storage under the liquid nitrogen did not change the position of ortho-phosphate peak in the cells enabled collection of the samples from

different fermentations and subsequent measurement by the NMR instrument in a single day, which substantially reduced experimental time required.

Monitoring the chemical shifts of ortho-phosphate peak that corresponded to apparent intracellular pH values in fungal cells grown in a fermenter has clearly shown the effect of lowered oxygen tension on intracellular acidification, which was previously reported elsewhere.[1,15,16] When the air supply was stopped and the dissolved oxygen tension started to decrease in the fermentation broth, it caused immediate acidification in the cells. These results unequivocally show how rapidly the concentration of protons changes in the cells in response to a slight decrease of dissolved oxygen tension in the medium. One is forced to conclude, therefore, that the metabolism should be completely stopped before measurement, if undistorted results about intracellular pH of mycelium growing in the bioreactors are to be obtained.

Up to now, it was practically impossible to follow very rapid metabolic changes with *in situ* ^{31}P-NMR measurements, since a single accumulation of scans took at least 2 minutes. The described method enables much more frequent sampling from a fermenter where overall cultural conditions can be better controlled.

Conclusions

It has been shown that metabolism could be stopped immediately by immersing the mycelium from the fermenter into the cold buffered methanol at –40 ^{o}C. At low temperatures high resolution ^{31}P-NMR spectra could be obtained. The method therefore enables monitoring of chemical shifts of ortho-phosphate peak that correspond to changes in apparent intracellular pH during the long term growth of the cells in a fermenter which is extremely important for understanding the regulation of metabolism in productive micro-organisms.

Experimental

A. niger (MZKI A60) spores were harvested from 7-day-old wort agar slants and were suspended in 25 ml of sterile 0.1% Tween 80 solution. A suspension of spores was used to inoculate chemically defined medium as described previously.[7] Sterilisation was

done by pasteurization, which proved to be sufficient due to the low initial pH (2.5) of the medium.

For obtaining the mycelium at the desired physiological state fermentations were run in a glass stirred tank bioreactor (Infors, Bottmingen, Switzerland) with a 5-liter working volume. The medium was inoculated with approximately 10^9 spores. The temperature was kept at 30 °C, and the medium was aerated with 5 litres of air per minute. Dissolved oxygen tension was monitored by a sterilizable polarographic electrode (Ingold).

Samples, about 250 ml of fermentation broth, were poured into a 500 ml stainless steel Dewar flask which was previously cooled by liquid nitrogen. Mycelium was collected by suction filtration through the stainless steel filter support grid (Millipore) with pore dimension of 100 μm. About 2-3 grams of wet weight mycelium was not washed, but immediately submerged into 100 ml of 40% (v/v) 100 mM imidazole buffer (pH=7.0) and 60% (v/v) methanol kept at –40 °C by a circulating ethanol bath (Ultra kryomat RUL-80-D, Lauda, Koenigshofen, Germany). The overall sampling procedure was completed in less than 30 seconds. Finally, buffered methanol was rapidly removed by suction filtration, frozen mycelium placed in a cryo-vial and stored under the liquid nitrogen. Alternatively the fermentation broth was poured directly into about 500 ml of buffered methanol at –40 °C and subsequently the frozen mycelium collected by suction filtration.

To check whether the cells were kept intact or cell membranes became permeable in the presence of cold methanol, the supernatant was tested for the presence of intracellular metabolites by means of liquid chromatography. 10 g of wet weight of mycelium was incubated at –40 °C in 100 ml of buffered methanol. Aliquots of 1 ml supernatant were taken at 30 minutes intervals. Methanol was subsequently evaporated at 60-70 °C, the sample re-dissolved in 100 μl of 5 mM H_2SO_4 and applied to HPLC system (Knauer) with an Aminex HPX-87H column (BioRad) as described previously.[8] Detection was done by means of refractometry and UV at 214 nm.

^{31}P-NMR spectroscopy was accomplished on a Varian Unity Plus-300 spectrometer (Palo Alto, USA), operating at 121.384 MHz. For a typical spectrum 500 scans were

accumulated using 45° pulses with a repetition time of 0.585 s. Chemical shifts were related to 85% H_2PO_4 (0 ppm) contained in a capillary tube. Condensed, cooled mycelium was placed in 10 mm NMR tube and poured with cooled buffered methanol. Finally 10% of D_2O was added. During the spectroscopy the sample was kept at -40 ± 0.1 °C by ventilating the sample with nitrogen pre-cooled on dry ice.

For taking spectra at +25 °C the cells were kept in 40 mM water solution of imidazole buffer pH=7.0 and 10% D_2O only, without methanol. During the measurement cells were not aerated.

Calibration curves have been done in water solution containing 40 mM of imidazole buffer, 0.5 mM EDTA, 50 mM NaH_2PO_4 and 100 mM KCl as recommended previously.[9] After the pH was determined at 22 °C with a pH meter (Knick, Berlin, Germany) and combined pH electrode (Ingold, Urdorf, Switzerland) the solution was frozen with liquid nitrogen and pulverised in a mortar. Frozen, pulverised buffer was submerged into the cold (-40 °C) 60% methanol with 40 mM imidazole and D_2O and placed into the NMR tube. Spectra were also recorded at –40 °C as well. To make the calibration curve under liquid conditions calibration buffer was mixed with methanol (60% v/v) cooled to –40 °C and measured.

For monitoring the influence of hypoxia on the changes in the apparent intracellular pH values, the air supply was stopped after 50 hours of incubation. Mycelia were collected in 2 or 3 minute intervals and prepared for NMR spectroscopy. Simultaneously the amount of dissolved oxygen was recorded polarographically by a O_2 electrode (Ingold, Urdorf, Switzerland) and data stored in a computer.

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References and Notes

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Povzetek

Do sedaj je bilo težko natančno določiti intracelularni pH pri filametoznih glivah, ki so rastle v velikih bioreaktorjih. Da ne bi prišlo do spremembe pH_i vrednosti med pripravo vzorcev, oziroma med samo meritvijo P^{31} jeder v NMR magnetu, smo razvili novo metodo, kjer smo metabolizem ustavili neposredno po tem, ko smo celice vzeli iz gojišča, in sicer tako, da smo jih potopili v ohlajen metanol pri –40 °C. Nizko temperaturo smo vzdrževali tudi kasneje med snemanjem v NMR magnetu. Ker so bile zamrznjene celice obdane s tekočim metanolom, so se molekule s fosforjem gibale v zadostni meri, da je lahko nastal NMR spekter visoke ločljivosti. Z uporabo nove metode smo uspeli zasledovati spreminjanje pH vrednosti v celicah med hipoksijo, ki smo jo povzročili s prekinjenim prezračevanjem v fermentorju.