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Determination of intermediary metabolites in *Aspergillus niger*

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Abstract

Procedures are described to recover and determine intermediary metabolites in *Aspergillus niger*. The methods involve rapid quenching of metabolism by direct transfer of a culture sample from a bioreactor to a buffered solution containing 60% methanol at −45°C. Metabolites do not leak from the cells when this sampling technique is used. Following quenching, the mycelium is collected by filtration and washed if required. Extraction of metabolites at −20°C and neutral pH using chloroform to permeabilise the cells prevents degradation of labile metabolites. An internal standard (xylitol) is used to correct for extraction recovery which was approximately 80%. To calculate actual intracellular concentrations, the intracellular volume has been determined. A value of 1.2 ml/g of dry weight was obtained for the growth conditions used. This method is suitable to determine the levels of glycolytic intermediates in *Aspergillus niger* mycelium. Although the measured levels were, in general, similar to values published previously and obtained by various other methods, the advantage of the procedure described here is that all metabolites can be assayed in a single extract.

Keywords: Glycolysis; Metabolite; Method; Filamentous fungus

1. Introduction

In studies on metabolic regulation, often the approach is taken to just investigate the kinetic and regulatory characteristics of enzymes in vitro. However, the physiological relevance of regulatory mechanisms deduced from experiments with isolated enzyme systems can only be assessed when the in vivo concentrations of substrates, products and effectors are also known. In other words, knowledge about the concentrations of intermediary metabolites is required to determine the factors that control metabolic fluxes in vivo.

The methods used for sampling of biomass and extraction of metabolites from the cells should be designed in such a way that the experimental values obtained for metabolite levels reflect those truly existing in vivo [1,2]. For instance, the turnover of intermediary metabolites is usually high. Thus, an important requirement of such a method is that cellular metabolism is quenched instantaneously upon sampling. In addition, the conditions used for extraction should not lead to conversions of the metabolites of interest. Recently an elegant method that satisfies these conditions was developed for *Saccharomyces cerevisiae* [3]. These experiments involve rapid quenching of metabolism by spraying yeast cells in a cold methanol–water mixture and extraction of metabolites from the cells at neutral pH.

Although determination of intermediary metabo-

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lites has been frequently reported for yeast, little is known about metabolite levels in filamentous fungi. This is surprising in view of the biotechnological relevance of filamentous fungi in the production of primary and secondary metabolites and proteins. Regarding primary metabolites, a well known example is citric acid production by *Aspergillus niger*. A high rate of citric acid accumulation by this fungus depends on a high glycolytic flux [4]. By investigating the factors which control the glycolytic flux, rational optimisation of the process may be obtained. To this end and to facilitate studies on metabolic regulation in filamentous fungi in general, we developed a methodology for the determination of intermediary metabolites in *Aspergillus niger* based on the procedures described for yeast. In this report, the method and some experiments that establish the validity of the results are described.

2. Materials and methods

2.1. Materials

HPLC-grade methanol was used since methanol of technical quality occasionally gave turbid solutions when mixed with water. Other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany). Fructose 2,6-diphosphate and sulphate-free preparations of aldolase, triosephosphate isomerase, glycerol-3-phosphate dehydrogenase and pyrophosphate-dependent phosphofructokinase used in the kinetic determination of fructose 2,6-diphosphate were purchased from Sigma (St. Louis, MO, USA). All other enzymes and biochemicals were obtained from Boehringer (Mannheim, Germany).

2.2. Strains and culture conditions

Aspergillus niger NW131 (*cspA1, goxC17*) was used in all experiments. Conidiospores were harvested from complete medium [5] solidified with 1.5% (w/v) agar and containing 50 mM glucose. Mycelia were cultured for 48 h at 30°C in a defined medium containing 0.5 g KH_2PO_4, 2.5 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 6.5 mg $FeSO_4 \cdot 7H_2O$, 1.3 mg $ZnSO_4 \cdot 7H_2O$, 0.15 ml polypropyleneglycol and 140 g of glucose per litre using 3-litre fermenters (Applikon, Schiedam, Netherlands). After 48 h of culturing, the mycelia were in the late exponential growth phase. The culture pH was not regulated and decreased from an initial value of 3.5 to approximately 2 at the moment of sampling. Cultures were sparged with 0.2 vvm air, while addition of pure oxygen was used to keep the dissolved oxygen tension above 50% air saturation.

2.3. Sampling of mycelium

A 25-ml sample (containing approximately 200 mg of mycelial dry weight) was transferred directly from the fermenter vessel into 100 ml of a solution containing 60% (v/v) methanol and 200 mM triethanolamine, pH 7.3, which was precooled to −45°C in a cooling bath (B. Braun Biotech, Melsungen, Germany). Following sampling, the sample temperature was approximately −20°C. The residence time in the sample tubing (i.d., 5 mm; total length, approximately 1 m) was approximately 1 s. A short residence time is important to prevent changes in the conditions as present in the bioreactor during sampling. After 5–10 min of further cooling in a stirred ethanol bath at −45°C, mycelia were collected by vacuum filtration on a sintered glass funnel (pore size 40–100 μm) and washed 3 times with 100 ml of a solution containing 50% (v/v) methanol and 50 mM triethanolamine, pH 7.3, precooled to −45°C. Mycelium samples were either extracted immediately or frozen in liquid nitrogen and stored at −70°C.

2.4. Extraction of metabolites

Unless stated otherwise, all further manipulations were performed at −45°C. A mycelium sample obtained as described above was transferred to a tube and resuspended in 6 ml of methanol. To each tube, 15 μmol xylitol was added as an internal standard. Following addition of 10 ml of chloroform and mixing, samples were incubated for 30 min to allow permeabilisation of cells and denaturation of enzymes. Subsequently 4 ml of 5 mM triethanolamine, pH 7.2, kept at 0°C, was added, followed by vigorous mixing and a further 10-min incubation to cool the samples. To accomplish efficient extraction of metabolites from the cells the tubes were shaken

for 30 min at −20°C (in a commercial type freezer) at 1800 rpm on an IKA Vibrax VXR shaker (Janke and Kunkel, Staufen, Germany) using a universal holder. The water–methanol and chloroform phases were separated by centrifugation at 5000×*g* for 5 min at −20°C (mycelium collects at the interface). The water–methanol phase (supernatant) containing the extracted metabolites was taken off and transferred to another tube. The mycelium was then extracted a second time by adding 2 ml of methanol, mixing, and 2 ml of 5 mM triethanolamine pH 7.2 (0°C) followed by vigorous mixing. Following centrifugation, the supernatant fraction was added to the first extract. To the combined extracts, 15 ml of diethylether was added. After mixing and centrifugation at 1000×*g* for 5 minutes at −20°C, the diethylether was removed. Samples were then frozen in liquid nitrogen and concentrated from approximately 12 to 1.5 ml by evaporation under vacuum using a Speedvac (Savant Instruments, Hicksville, NY, USA) without additional heating. The exact volume of the extracts was then determined and the extracts were centrifuged briefly at 4°C to remove possible insoluble material.

The extracted mycelium was collected and lyophilised to determine the dry weight of the sample. The dry weight of a mycelium sample did not decrease significantly by extraction.

Xylitol, used as internal standard, was measured with high performance anion-exchange chromatography (Dionex, Sunnyvale, CA, USA) using isocratic elution with 0.48 M NaOH on a CarboPac MA1 column.

2.5. Determination of metabolites

Analyses were performed on a Cobas Bio (Roche, Basel, Switzerland) autoanalyser that was connected to a computer for data logging. The data-logging and data-analysis programs were those used by De Koning and Van Dam [3].

Metabolites were determined essentially as described by Bergmeyer [6] in 50 mM triethanolamine, 5 mM $MgCl_2$, pH 7.6, unless stated otherwise. Where possible, a number of metabolites were assayed in one sample aliquot in the order given.

Glucose 6-phosphate (G6P), fructose 6-phosphate (F6P) and ATP were determined in the presence of 0.5 mM $NADP^+$ by addition of 0.3 U/ml glucose-6-phosphate dehydrogenase, 2.8 U/ml phosphoglucose isomerase and 2.2 U/ml hexokinase+1 mM glucose, respectively.

Dihydroxyacetonephosphate (DHAP), glyceraldehyde 3-phosphate (GAP) and fructose 1,6-diphosphate (F1,6DP) were determined in the presence of 0.2 mM NADH by the addition of 0.7 U/ml glycerol-3-phosphate dehydrogenase, 40 U/ml triosephosphate isomerase and 0.1 U/ml aldolase, respectively.

Pyruvate, phosphoenolpyruvate (PEP), 2-phosphoglycerate (2PG) and 3-phosphoglycerate (3PG) were determined in the presence of 0.2 mM NADH and 100 mM KCl by addition of 1.1 U/ml lactate dehydrogenase, 0.8 U/ml pyruvate kinase+0.8 mM ADP, 3.2 U/ml enolase and 20 U/ml phosphoglycerate mutase+40 μM 2,3-diphosphoglycerate, respectively.

ADP and AMP were determined in the presence of 0.2 mM NADH, 100 mM KCl and 1.1 U/ml lactate dehydrogenase by addition of 0.8 U/ml pyruvate kinase+0.4 mM PEP and 5.6 U/ml myokinase+0.2 mM ATP, respectively.

NADH was determined in the presence of 0.5 mM DHAP by the addition of 0.7 U/ml glycerol-3-phosphate dehydrogenase. NAD^+ was determined in 4× diluted glycine buffer reagent (Sigma, no. 332-9) in the presence of 250 mM ethanol by the addition of 36 U of alcohol dehydrogenase per ml.

Fructose 2,6-diphosphate (F2,6DP) was determined using a kinetic assay as described by Van Schaftingen et al. [7]. The assay mixture contained 50 mM Tris pH 8.1, 2.5 mM $MgCl_2$, 0.2 mM NADH, 0.75 mM fructose 6-phosphate, 17 mM glucose 6-phosphate, 0.05 U/ml pyrophosphate-dependent phosphofructokinase, 0.5 U/ml aldolase, 25 U/ml triosephosphate isomerase, 2.2 U/ml glycerol-3-phosphate dehydrogenase and 1 mM sodium pyrophosphate. Samples containing 0–20 nM fructose 2,6-diphosphate were included in measuring series to calculate the fructose 2,6-diphosphate content of the metabolite extracts.

2.6. Intracellular volume

The intracellular volume was determined as described for *Neurospora crassa* [8] using xylitol as a

probe. Solid xylitol was added to a 25-ml culture sample to a final concentration of approximately 50 mM. After 10 min of incubation, the mycelium was collected on a sintered glass funnel. A sample was taken from the filtrate to determine the exact starting concentration of xylitol (concentration F1) by high performance anion-exchange chromatography. Following measurement of the wet weight (WW) of the mycelium, the pellet was resuspended in 50 ml of water and incubated for 10 min. After another filtration step the dry weight (DW) of the mycelium was determined after lyophilisation and the xylitol concentration was measured in the second filtrate (concentration F2). The intracellular volume was calculated as ml/g dry weight according to the following equation in which WW and DW are in grams and using a value of 1 g/ml for the density of water:

Intracellular volume =

$$(WW - DW - 50*F2/F1)/DW$$

3. Results

3.1. Determination of metabolites

With the methods employed, most glycolytic metabolites could be measured without difficulty in *Aspergillus niger* extracts. A few exceptions were glyceraldehyde 3-phosphate, which is unstable and decomposes rapidly, and 2-phosphoglycerate and NADH, for which the levels were always very low in our *Aspergillus niger* samples.

The lowest metabolite concentration that could be determined routinely in a concentrated extract was 2–3 μM, which is equivalent to approximately 0.02 μmol/g dry weight (the kinetic assay used for fructose 2,6-diphosphate allows determination of 10 nM F2,6DP in a concentrated extract). Standard deviations were generally 10–20% of the measured values, but were higher for metabolites that were present at low concentrations in the cells.

3.2. Sampling method

To quench cellular metabolism rapidly, a sample of an *Aspergillus niger* culture was transferred directly to a solution containing 60% methanol and 200 mM triethanolamine pH 7.3 precooled to −45°C. The use of cold methanol for quenching has been developed for yeast [2,3], but due to strong acidification of the culture medium by *Aspergillus niger* a buffered methanol solution had to be used in this case to prevent degradation of acid labile metabolites.

Following quenching, the mycelia were either collected by centrifugation or filtration, both at low temperature. When centrifugation was used to collect mycelia from the samples, the amounts of dihydroxyacetonephosphate and ATP extracted were 30–80% lower than the amounts obtained with filtration (data not shown). In addition, the amounts of ADP and AMP were higher with centrifugation. With the available equipment, a suboptimal temperature of −10 to −5°C could be maintained during centrifugation. Since the temperature could be kept lower during filtration (below −20°C) we interpreted the observed differences as a phosphatase activity that could be inhibited by extremely low temperatures. Therefore, filtration was used to collect mycelium.

Possible leakage of metabolites from the cells was checked by measuring the amount of the various metabolites both in the cells and in the filtrate directly following sampling and after a 30-min incubation of the sample at −45°C. None of the metabolites could be detected in significant amounts in the filtrates (Table 1). Furthermore, the metabolite levels in the cells were independent of the residence time at −45°C following quenching. Variation in the number of wash steps (0–5 times) also did not affect the metabolite levels (data not shown). These observations indicate that the metabolites determined did not leak from the cells after quenching of culture samples in 60% methanol at −45°C.

In addition, the constant level of metabolites in the cells verifies the effectiveness of the sampling method in sufficiently stopping metabolism.

3.3. Extraction method

Due to the filamentous morphology of the fungus, quantitative removal of the water–methanol phase after extraction was difficult. With two subsequent extraction steps an acceptable, but not quantitative, extraction recovery could be obtained. To correct for

Table 1
The effect of sampling of *Aspergillus niger* mycelium in 60% methanol at −45°C on metabolite levels

	Amount of intracellular metabolite (nmol/ml)[a]		Amount of extracellular metabolite (nmol/ml)[a]		
	Incubation time after sampling (min)		Incubation time after sampling (min)		Culture
	1	30	1	30	
G6P	7.1	7.4	0.1	0.1	0.0
F6P	1.8	1.7	0.2	0.0	−0.1
F1,6DP	1.3	1.3	0.3	0.6	0.1
F2,6DP[a]	37.0	38.0	0.0	0.0	0.0
DHAP	1.3	1.2	0.1	0.2	0.0
3PG	1.5	1.6	−0.1	−0.1	−0.3
2PG	0.0	0.0	0.5	0.4	0.1
PEP	0.3	0.3	0.4	0.3	0.2
Pyruvate	0.2	0.1	0.9	0.6	0.2
NAD^+	6.6	5.8	−0.5	−0.1	0.4
NADH	0.1	0.1	0.0	−0.2	0.1
ATP	17.1	17.1	−0.1	−0.5	−0.2
ADP	2.5	2.6	−0.1	0.2	0.1
AMP	0.5	0.5	−0.5	−0.6	−1.0

Fifty millilitres of an *Aspergillus niger* culture was sampled into 200 ml of a solution containing 60% (v/v) methanol and 200 mM triethanolamine (pH 7.3) precooled to −45°C. Mycelia were collected by filtration from 125-ml portions either directly (approximately 1 min after sampling) or following a 30-min incubation at −45°C and extracted as described in Materials and methods. The sample filtrates were concentrated under vacuum from 125 to 10 ml. Metabolites were assayed in extracts and filtrates as described in Materials and methods and expressed as nmol/ml culture to facilitate direct comparison between extracellular and intracellular concentrations. The column 'Culture' gives the metabolite concentrations present in culture filtrate at the time of sampling. The detection limits are 0.2 nmol/ml for extracts and 1.0 nmol/ml for sample filtrates and culture filtrate. Negative values can occur due to baseline drift.
[a]Fructose-2,6-diphosphate is given in pmol/ml.

extraction recovery xylitol was added at the start of each extraction as an internal standard. The recovery of xylitol was 81±4% (value±S.D.; $n=4$) when two extraction steps were performed. Introduction of a third extraction step improved the recovery slightly (89±3% (value±S.D.; $n=4$)), but increased the volume of the extract leading to a longer time required for concentration of the extract. Therefore we routinely applied two extraction steps and used xylitol as an internal standard.

A test was performed to check whether the use of low temperature was sufficient to block metabolism during extraction of the cells. For a number of glycolytic metabolites added at the start of an extraction the recovery was 73–81%, values similar to that obtained for xylitol (Table 2). This result indicates that not more than 10% and usually less, of the metabolites was lost due to conversions during extraction.

3.4. Intracellular concentrations of metabolites

To enable calculation of actual intracellular concentrations a conversion factor relating dry weight to the intracellular volume was determined. With an intracellular volume of 1.20±0.15 ml/g dry weight (value±S.D.; $n=13$) concentrations of glycolytic intermediates were calculated for *Aspergillus niger* cultured 48 h on a medium designed for citric acid production (Table 3).

4. Discussion

Knowledge about the intracellular concentrations of intermediary metabolites is essential when regulation of metabolic pathways is studied quantitatively. In this report, we describe a method to determine intermediary metabolites in *Aspergillus niger* which

Table 2
Recovery of metabolites from *Aspergillus niger* mycelium

	Amount of metabolite in control extract (nmol)	Amount of metabolite added (nmol)	Amount of metabolite in spiked extract (nmol)	Recovery of spiked metabolite (%)
F1,6DP	19	77	78	77
DHAP	4		6	
F6P	45	655	531	74
G6P	359		395	
2PG	3	90	69	73
3PG	42		51	
PEP	12		12	
NADH	2	97	80	80
NAD^+	122		132	
ADP	182	731	777	81
ATP	344		351	
AMP	62		66	
Xylitol	0	14,500	11,800	81

Fifty millilitres of an *Aspergillus niger* culture was sampled into 200 ml of a solution containing 60% (v/v) methanol and 200 mM triethanolamine (pH 7.3) precooled to −45°C. Following collection of the mycelium as described in Materials and methods the mycelium was resuspended in 12 ml methanol and the sample was split into two equal portions. Just before the start of extraction, a number of metabolites were added to one of the two portions. The other portion was used as a control sample. Subsequently metabolites were extracted from the spiked sample and the control sample and assayed as described in Materials and methods. The absolute amounts of metabolites obtained by extraction are given. In addition to the metabolites added, values for some compounds that can be formed from the added metabolites due to biochemical conversions are shown. Xylitol is used as an internal standard.

Table 3
Concentrations of glycolytic intermediates in *Aspergillus niger*

Metabolite	Intracellular concentration (mM)[a]
G6P	1.08±0.13
F6P	0.23±0.03
F1,6DP	0.25±0.03
F2,6DP[a]	6.70±0.70
DHAP	0.17±0.03
3PG	0.23±0.01
2PG	0.01±0.01
PEP	0.04±0.01
Pyruvate	0.04±0.01
ATP	2.59±0.22
ADP	0.47±0.06
AMP	0.09±0.03
NAD^+	0.83±0.12
NADH	0.01±0.01

Procedures for culturing *Aspergillus niger* and for sampling, extraction and determination of metabolites are given in Materials and methods. For the extracted metabolites, intracellular concentrations were calculated using a conversion factor of 1.2 ml/g dry weight. The values are given with standard deviations and are averages of 5 extracts.
[a]Fructose-2,6-diphosphate is given in nM.

is based on procedures developed for yeast [3]. The method involves rapid quenching of metabolism by sampling in cold methanol followed by extraction of metabolites from the cells at neutral pH. A few tests indicated that no changes in metabolite levels occurred due to leakage from the cells upon sampling, whereas changes due to conversions during sampling or extraction were minimal and not exceeding 10%. The tests described here were all performed on mycelia cultured for 48 h, but in other experiments the validity of the results was also established for both younger and older mycelia.

In pilot experiments the extraction method described here, using chloroform at neutral pH, was compared to procedures frequently described in the literature using either acid or alkaline treatments. For a number of glycolytic intermediates (e.g., F2,6DP, DHAP, PEP) the chloroform method appeared to be superior to both extraction with perchloric acid and KOH, since larger amounts of these metabolites were extracted. This is probably due to instability of these metabolites at both high and low pH.

Using fast quenching of metabolism by sampling in cold methanol, De Koning and Van Dam [3] described experiments in which they studied changes in the levels of glycolytic intermediates in yeast on a subsecond time scale. The morphology of filamentous fungi is likely to cause specific problems in such experiments due to high viscosity or non-homogeneous incubation mixtures. We did not test the possibilities to perform experiments with *Aspergillus niger* at the same time-scale. In this study, the method is used to determine steady-state levels of glycolytic intermediates in citric acid producing *Aspergillus niger*. No extensive analysis is presented here, but work is in progress using the techniques described to investigate the glycolytic flux in relation to citric acid production by this fungus. For most of the glycolytic metabolites determined here the intracellular concentrations obtained are similar to the few values published for citric acid producing *Aspergillus niger*. Values for fructose 2,6-diphosphate determined by Kubicek-Pranz et al. [9] are nearly identical to the values measured here. Führer et al. [10] determined NAD^+ and NADH and found high levels of NADH compared to our results. The extraction procedure used by these authors has been checked thoroughly and does not appear to give erroneous results. One reason for relatively high NADH levels might be anaerobic conditions during sampling, but otherwise the reason for the observed discrepancy remains obscure. Finally, the intracellular concentrations of fructose 6-phosphate, glucose 6-phosphate, ATP and AMP determined by Habison et al. [11] are lower than the values obtained in this study. This is, however, mainly due to the high value for the intracellular volume (2.54 ml/g dry weight [8]) assumed by these authors. When the two-fold difference in the values used for intracellular volume is taken into account, the differences in metabolite levels are minor. A range of values for intracellular volume of fungi is reported in the literature. For yeast, a frequently cited value is 3 ml/g dry weight [1], whereas values for filamentous fungi vary from 1 ml/g dry weight for *Claviceps purpurea* [12] to 2.5 ml/g dry weight for *Neurospora crassa* [8]. In our experience the intracellular volume to dry weight ratio for *Aspergillus niger* varies with growth conditions (e.g., a value of 2.3 ml/g dry weight was found for *Aspergillus niger* mycelium cultured in medium supplemented with trace metals). This is not surprising in view of the effects on morphology and cellular composition of *Aspergillus niger* caused by cultivation on a manganese-depleted medium (i.e., during citric acid production). Therefore it appears necessary to determine the intracellular volume of the mycelium for the experimental growth conditions one wants to use for the determination of metabolites.

Finally, it should be noted that the procedures described here may also be applied to studies on metabolic regulation in other filamentous fungi.

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