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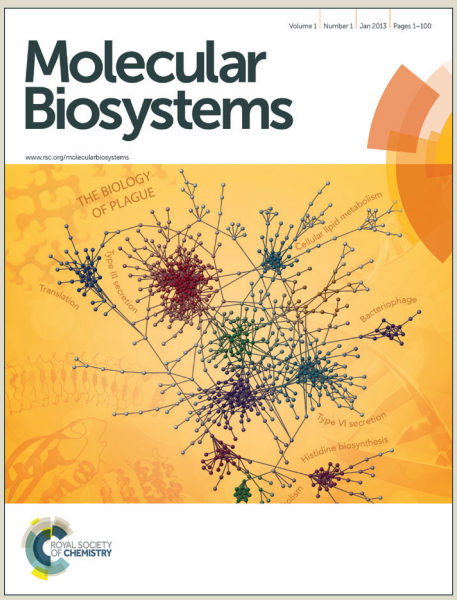

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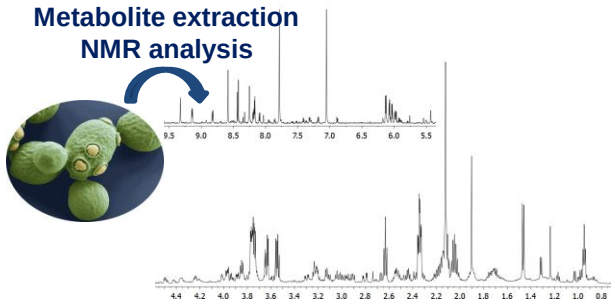

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COMMUNICATION

NMR analysis for budding yeast metabolomics: a rapid method for sample preparation

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Here we propose the optimization of a rapid and reproducible protocol for intracellular metabolite extraction from yeast cells and their metabolic profiling by ^{1}H-NMR spectroscopy. The protocol reliability has been validated through the comparison between the metabolome of cells in different phases of growth or with different genetic backgrounds.

Introduction

Saccharomyces cerevisiae is one of the most intensively studied eukaryotic model organisms in molecular and cell biology.[1] Thanks to its relative simplicity and easy handling, it has been widely used to elucidate fundamental aspects of cellular processes such as cell signalling, cell cycle and metabolism and for the study of several human diseases, such as cancer, neurodegenerative diseases and metabolic disorders.[2,3] Therefore, yeast metabolic profiling is gaining more and more importance to understand disease processes and metabolomics -the systematic analysis of large numbers of metabolites- has become a global analysis method complementary to transcriptomics and proteomics. Nevertheless, when the analysis of yeast intracellular metabolites has to be performed, a very carefully set up of the protocol for the extraction of intracellular metabolites is needed, since yeast cells present a cell wall whose composition and resistance to disruption can be modulated by several factors including growth conditions, the growth phase and genetic background. Several different methods of extraction of intracellular metabolites and analysis have been proposed,[4–8] but there is no consensus on their efficiencies in the literature. The different methods vary for (1) quenching procedures, (2) the method to break yeast cells and (3) the extraction temperature. In addition, most of the proposed techniques are based on the use of mass spectrometry (MS) as detection system. However, ^{1}H nuclear magnetic resonance (NMR) spectroscopy represents a rapid, non-destructive, high throughput method for the analysis of metabolites, that requires minimal sample preparation.[9] It allows both metabolite identification, without the need of their physical separation, and quantification, due to the signal intensity dependence on the number of identical nuclei. For quantification, a crude sample is mixed with a reference compound, such as 3-trimethylsilylpropane-1-sulfonic acid (DSS), at a known concentration, without additional steps for sample preparation or calibration curves for each individual component, as required for example for MS. NMR detects all metabolites present at concentrations larger than the instrument limit of detection and does not require specific chemical treatments of analytes, such as the derivatization needed in MS analysis. The main NMR drawback is its relatively low sensitivity, although improvements can be easily achieved by the application of higher magnetic fields (600 MHz spectrometer or higher), longer analysis times and the use of cryo-probes.[10] Nowadays the detection limit for ^{1}H NMR spectroscopy is of about 1 μM.

In this communication we describe a rapid protocol for intracellular metabolite extraction from yeast cells and their metabolic profiling by NMR spectroscopy.

Optimisation of the intracellular metabolite extraction protocol

We tried different experimental conditions based on the use of ethanol as solvent employed to extract intracellular metabolites: boiling ethanol (80°C), largely employed in the past literature,[5] was compared with ice-cold ethanol, in the presence or absence of glass beads shaking.

Due to its physicochemical properties, ethanol is suitable for both cell wall disruption and polar metabolite extraction. Moreover, the extraction with boiling ethanol is one of the most popular procedures. This method is described as simple, fast, accurate and reliable;[5] nevertheless extraction with boiling solvents could be not suitable for metabolites that are not stable at high temperatures, among which glutamine, glutamate, glutathione, succinate and threalose have been reported.[11]

Moving from these evidences, we decided to verify the efficiency in intracellular metabolite extraction of a protocol based on the use of ice-cold ethanol, that, to the best of our knowledge, has never been tested. As a matter of fact, only cold methanol has been tried, providing good extraction yields; however, the reliability of the method has been reported as discussible by some authors.[4,11]

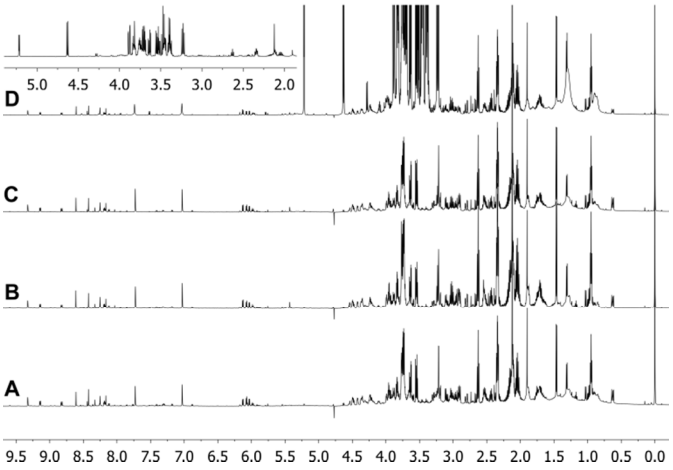

Fig. 1 ^{1}H NMR spectra of yeast cell extracts recorded at 600 MHz in phosphate buffer, pH 7.4, 25°C; A) boiling ethanol extraction (80°C); B) ice-cold ethanol extraction (4°C); C) ice-cold ethanol extraction with glass beads shaking (4°C); D) ice-cold ethanol extraction without cell washing (0.5x in intensity); the insert reports the region comprised between 5.7 and 1.8 ppm of the same spectrum (0.2x in intensity) and shows the great amount of glucose contaminating the sample when cell washing is omitted.

The comparison between ^{1}H spectra obtained from extraction performed vortexing cells in the presence of boiling (Fig. 1A) or ice-cold (Fig. 1B) ethanol clearly shows that the two procedures are very similar in terms of extraction efficiency and signal to noise ratio. These evidences suggests that the use of boiling ethanol can be easily avoided and substituted with ice-cold ethanol, thus preventing the thermal degradation of some metabolites.

Known the significant strength of the yeast wall, we also investigated the effect of cell mechanical breaking by glass beads shaking. Fig. 1C reports the spectrum obtained after extraction with ice-cold ethanol and sample vortexing in the presence of glass beads. Comparing spectra 1B and 1C, it can be noticed that the mechanical breakage does not increase the extraction efficiency. The procedure was repeated several times by increasing the number of breaking cycles, but no appreciable increase in extraction yield was observed (data not shown), suggesting that the cell treatment with ice-cold ethanol is sufficient to obtain a significant cell breakage.

In keeping with this result, all three extraction methods presented in Fig. 1 resulted in complete (>99%) cell membrane damage as assayed by propidium iodide staining, although cellular debries were only visible in the presence of glass beads (data not shown). Moreover, metabolite quantifications were comparable in samples extracted in ice-cold ethanol with or without glass beads, while some significant differences in the recovered amount of certain metabolites (i.e. glutathione, glutamate and histidine) were observed using boiling ethanol (Fig. 2), supporting the use of ice-cold ethanol to prevent thermal degradation of metabolites.

Although literature often reports a quenching step to arrest metabolic activity after cell harvesting,[6] this procedure is omitted in many studies[12,13] or is replaced by fast filtration[8,14] due to serious problems of metabolite loss caused by cell leakage and extracellular metabolite contamination. Therefore, all the experiments described above were performed after cell recovery by filtration followed by pellet washing with ice-cold water. As a matter of fact, in our experience washing of the cells is a very critical step for obtaining reproducible and reliable data. Figure 1D clearly shows that when the washing step is omitted, the sample is contaminated by a great amount of glucose and other metabolites present in the medium.

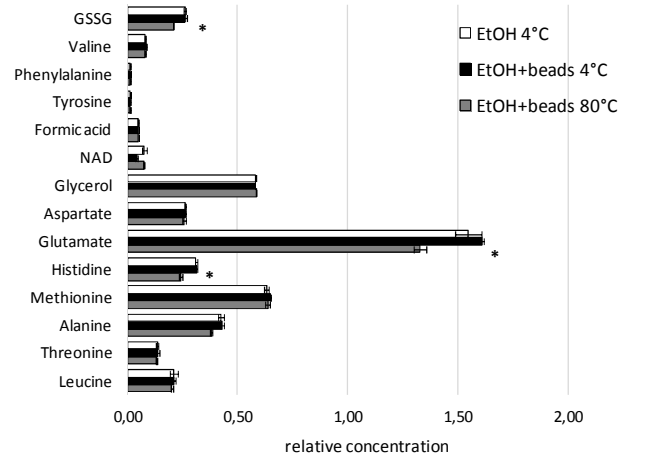

Fig. 2 Metabolite concentrations of wt cells during exponential phase of growth obtained with the different extraction methods: ice-cold ethanol, ice-cold ethanol with glass beads and boiling ethanol with glass beads. * p<0.05

To assess the usefulness of our protocol for NMR metabolic profiling of yeast we identified the majority of the metabolites present in our extracts; relative assignments are reported in Fig. 3 and Table 1.

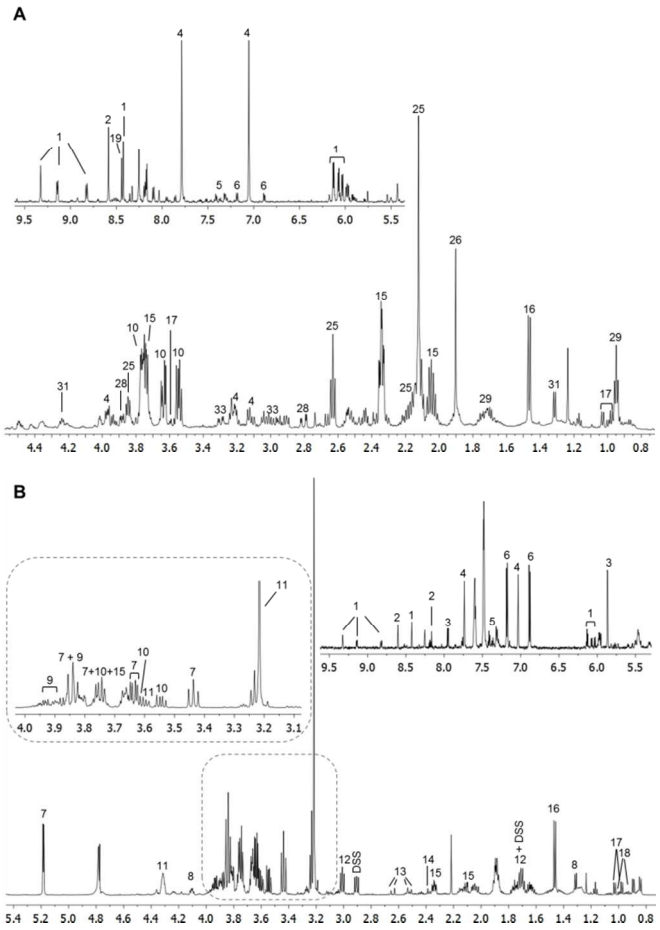

Fig. 3 1H NMR spectra of intracellular metabolites extracts recorded at 600 MHz in phosphate buffer, pH 7.4, 25°C; metabolites were extracted from yeast cells growing in exponential (A) or stationary (B) phase. The correspondence between peak numbering and metabolite assignments is shown in Table 1.

Table 1. Metabolite assignments and chemical shift of distinguishable peaks.

Assigned number	Metabolite	chemical shift (ppm)[a]
1	NAD	9.33 (s) 9.15 (d) 8.83 (d) 8.42 (s) 8.19 (m) 6.13 (d) 6.08 (d) 6.02 (d)
2	AMP derivate	8.6 (s) 8.17 (s)
3	UDP derivate	7.95 (d)
4	Histidine	7.8 (s) 7.05 (s) 3.96 (dd) 3.22 (dd) 3.12 (dd)
5	Phenylalanine	7.42 (m) 7.36 (m) 7.32 (d) 3.97 (dd) 3.29 (dd) 3.12 (dd)
6	Tyrosine	7.18 (d) 6.89 (d) 3.97 (dd) 3.13 (dd) 3.02 (dd)
7	Trehalose	5.18 (d) 3.85 (m) 3.75 (dd) 3.64 (dd) 3.44 (t)
8	Lactate	4.11 (dd) 1.32 (d)
9	Serine	3.94 (m) 3.83 (dd)
10	Glycerol	3.77 (m) 3.65 (dd) 3.55 (dd)
11	Glycerophosphocholine	4.31 (m) 3.6 (dd) 3.22 (s)
12	Lysine	3.7 (m) 3.00 (t) 1.87 (m) 1.71 (m) 1.45 (m)
13	Citrate	2.64 (d) 2.52 (d)
14	Succinate	2.39 (s)
15	Glutamate	3.74 (dd) 2.34 (td) 2.05 (m)
16	Alanine	1.47 (d)
17	Valine	1.03 (d) 0.98 (d)
18	Isoleucine	1.00 (d) 0.94 (t)
19	Formate	8.44 (s)
20	Uracil	7.53 (d) 5.79 (d)
21	Fumarate	6.5 (s)
22	Uracil-6-carboxylate	6.18 (s)
23	Thiamine derivate	5.46 (s)
24	Pyruvate	2.36 (s)
25	Methionine	2.63 (t) 2.12 (s)
26	Acetate	1.91 (s)
27	Ethanol	3.65 (q) 1.71 (t)
28	Aspartate	3.88 (dd) 2.80 (dd)
29	Leucine	3.71 (m) 1.69 (m) 0.95 (t)
30	Glucose	5.22 (d) 4.64 (d) 3.89 (dd) 3.83 (m) 3.73 (m) 3.52 (dd) 3.46 (m) 3.40 (td) 3.23 (dd)
31	Threonine	4.24 (m) 1.31 (d)
32	Phenylacetate	7.38 (m) 7.30 (m) 3.52 (s)
33	Glutathione ox	3.30 (dd) 2.96 (dd)

[a] chemical shifts are referred to DSS and multiplicities showed in brackets. Abbreviation: (s) singlet, (d) doublet, (t) triplet, (m) multiplet, (dd) double doublet, (td) triple doublet.

In particular, in Fig. 3A and 3B the ^{1}H NMR spectra of intracellular metabolites extracted from BY4741 wild type yeast cells in exponential and stationary phase of growth respectively are depicted. Overall, the unambiguously identification of about 30 metabolites was possible. Many of these compounds can be quantified; the accuracy of metabolite quantification has been easily improved through the application of a global spectral deconvolution (GSD) protocol that allows overlapping regions to be deconvolved and absolute integrals to be measured.[15]

For yeast cells metabolic analysis, the characterization of nutrients and metabolites up-take and secretion is also of great importance. For this reason we provide representative ^{1}H NMR spectra, and relative assignments, also for metabolites present in growth media collected for wt cells during exponential (Fig. S1- Supplementary Information) and stationary (Fig. S2 - Supplementary Information) phase of growth.

Metabolome analysis of yeast cells: case studies

Our protocol allows the analysis of the metabolic profile of yeast cells in a fast and very reproducible way and to get quantitative information on a considerable number of metabolites. To further validate this method, we applied our protocol to detect changes in the metabolic profile of yeast cells in two different growth phases: exponential phase and early stationary phase. As expected, after 2 days growth in synthetic medium, the metabolic profile of yeast cells is completely different from that of exponentially growing cells (Fig. 4A). For instance, trehalose, which is a typical quiescence marker and a key storage carbohydrate,[16] accumulates in stationary phase, as well as citrate and succinate, indicating that mitochondrial respiration took place in these cells. In addition major changes in many amino acids are evident, in keeping with the great rewiring of metabolism of cells in stationary phase (Fig. 4A).

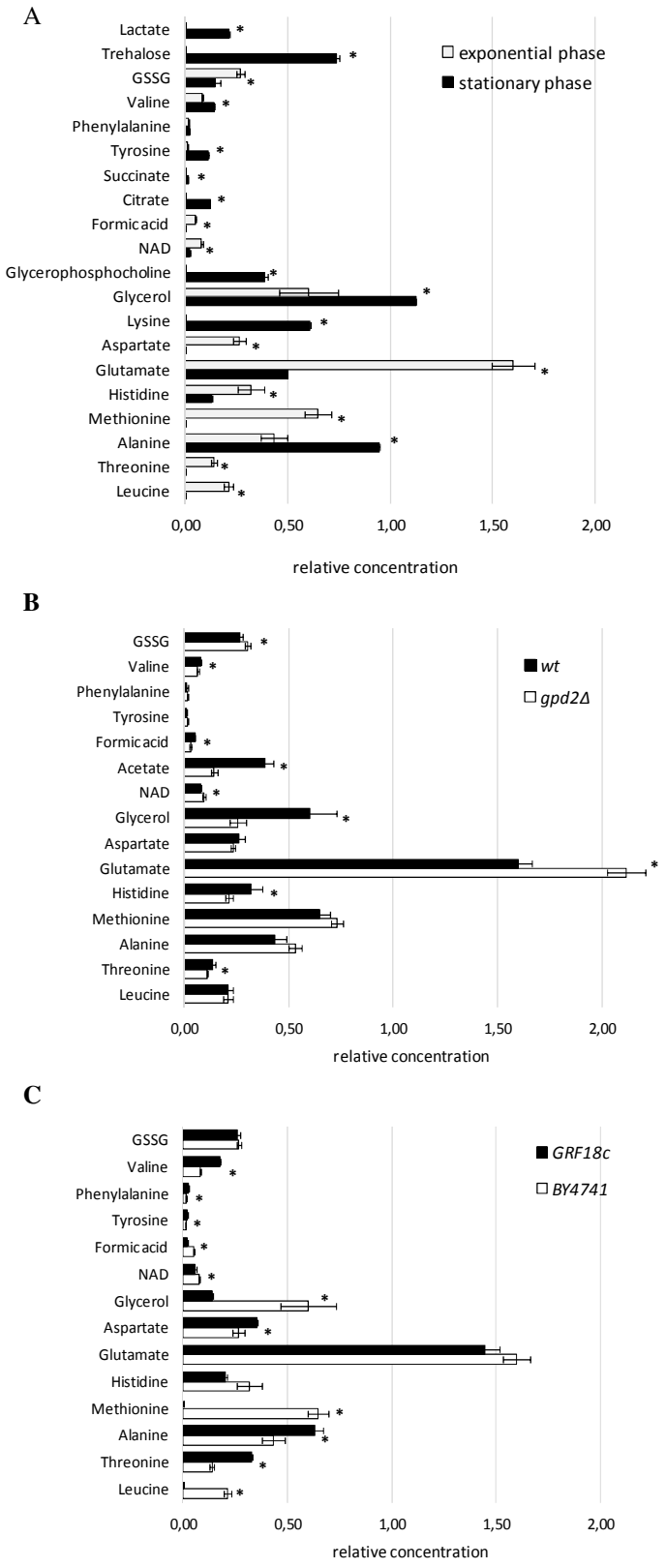

Fig. 4 Comparison of metabolite concentrations of (A) wt cells in exponential and in stationary phase of growth; (B) wt and *gpd2Δ* cells exponential phase of growth; (C)*BY4741* and *GRF18c* wt strains in exponential phase of growth . * p<0.05

To further validate our method, we investigated the changes in the metabolic profile of a mutant yeast strain with known, previously reported differences in its metabolic content. Therefore, we compared the metabolome of wt cells with that of *gpd2*Δ cells, which lack one of the two isoforms of the glycerol-3-phosphate dehydrogenase. Gpd2 catalyzes the NADH-dependent conversion of dihydroxyacetone phosphate to glycerol-3-phosphate and is required for growth under anaerobic conditions.[17] It was previously reported that cells lacking Gpd2 have half of glycerol and produce less acetate than a wild type strain.[18–20] Our NMR analysis validated those data, since we found that the wt/*gpd2*Δ ratio for intracellular glycerol and acetate are 0.43 and 0.37, respectively (Fig. 4B). Moreover, we found other metabolite variations in *gpd2*Δ cells, the most interesting being a higher content of glutamate, whose synthesis could be increased as a result of the lower glycerol production. A *gpd2*Δ strain also shows a statistically significant lower content of valine, histidine, threonine and of formic acid while presents a higher level of NAD^+ and oxidized glutathione (Fig. 4B). Finally, to confirm the reliability of our extraction method, we compared two different wild type strains, the auxotrophic BY4741 strain, already used in this work, and the prototrophic GRF18c, which is widely used in chemostat experiments. Although both strains are wild type, their metabolite content presents several quantitative differences (Fig. 4C), supporting the notion that the genetic background has a great impact on the cellular metabolism.

Conclusions

Here we described a fast and reproducible protocol for safe extraction of intracellular metabolites from yeast cells. It consists in two simple steps: (1) the rapid filtration of cells and their washing with cold water, to efficiently remove medium residual and (2) the metabolite extraction with ice-cold ethanol allowing to preserve thermal instable compounds. This method allows to obtain good yields, in terms of metabolite recovery, through a very rapid and easy procedure based on the use of very small amount of organic solvent, thus saving costs in terms of time and money and featuring, in addition, an eco-friendly procedure.

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Notes and references

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Electronic Supplementary Information (ESI) available: Materials and methods and Supplementary figures. See DOI: 10.1039/c000000x/

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