University of Applied Sciences Northwestern Switzerland
School of Life Sciences

Characterization of intracellular metabolites in microorganisms by liquid chromatography-mass spectrometry

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Rafael Sande

ABSTRACT

A powerful analytical platform for absolute quantification of 19 important intracellular metabolites from central metabolism of *B. subtilis* strains was developed and validated. The metabolites cover parts of glycolysis, tricarboxylic acid cycle, energy metabolism, and pentose-phosphate pathway. The developed method is characterized by a state of the art sample processing strategy, emphasizes the sustainable minimization of solvent consumption, and completely avoids the appearance of cell leakage for prokaryotic cells, which is a problem in classically used cold solvent quenching procedures. The method was applied for biotechnologically engineered *B. subtilis* strains used for fermentative production of Riboflavin (Vitamin B_2). A comparison of the intracellular metabolite concentration profiles during standard fermentations of a wild type *B. subtilis* strain and two engineered, different efficient Riboflavin producer strains resulted in substantial differences. Important achievements were that the results allowed disproving a hypothesis why the optimized strain has the ability to produce significantly more Riboflavin and gave evidence on a possible bottleneck in the strains. The application of the method as a fermentation quality control tool looks very promising.

CONCEPT

The developed method follows a state of the art sample processing strategy using a differential method. The concentrations of the total metabolites (intra- and extracellular) and extracellular metabolites are determined separately. The intracellular amount is calculated as the difference of both metabolite concentration measurements.

RESULTS

The following figure summarizes the developed/validated state of the art sample processing strategy for metabolism quenching and metabolite extraction of *B. subtilis* fermentation samples:

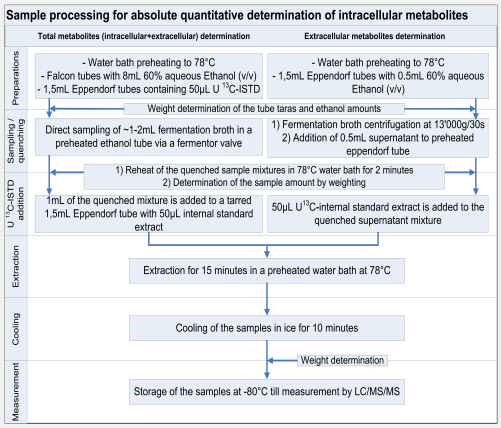


Fig. 1: The sample processing strategy starts with two parallel, independent routes for either determination of the total metabolites or extracellular metabolites only. By subtraction the intracellular metabolite amount can be determined. As internal standard a fully uniformly ^{13}C-labeled aqueous ethanolic *B. subtilis* extract was used.

The extracts were measured using a ion pair reversed phase HPLC-separation and MS/MS-quantification:

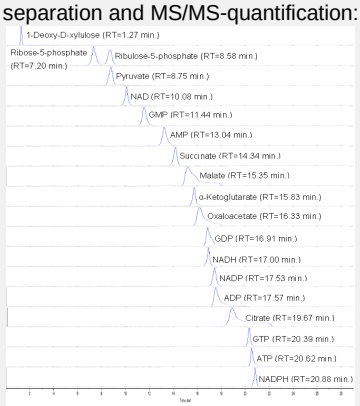


Fig. 2: The figure shows the separation of the 19 metabolites of interest in an aqueous standard mixture (concentration each ~1-3µg/mL).

Method characteristics:
- Phenomenex Synergi Hydro-RP-C18 column (150mm x 2.0mm; 4µm- 80Å)
- Phenomenex SecurityGuard Cartridge C18 (4*3.0mm)
- 10µL injection; Solvent A: 10mM Tributylamine+0.05% Formic acid (pH~3.4); Solvent B: Acetonitrile

Gradient:

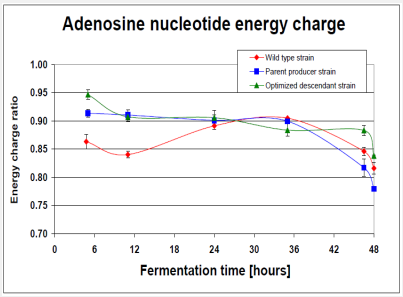
Time [minutes]	Solvent A [%]	Solvent B [%]	Flow [mL/min]
0.0	100.0	0.0	0.40
19.5	67.5	32.5	0.40
19.6	67.5	32.5	0.60
22.0	65.4	34.6	0.60
22.1	0.0	100.0	0.60
25.0	0.0	100.0	0.60

A major discovery of this thesis was the substantially different energy charge profiles of the different strains. The energy charge is defined as the ratio of the intracellular adenosine nucleotide concentrations:

$$\text{Energy charge} = \frac{0.5 * [ADP] + [ATP]}{[AMP] + [ADP] + [ATP]}$$

Equation 1: The energy charge ratio represents the energetic state of the cells. Concentrations used for calculation in mmol/L cell volume; no unit.

Fig. 3: The figure shows the energy charge profiles of a wild type, average Riboflavin producer- (parent), and high Riboflavin producer (optimized descendant) strain during 48 hours DSM standard fermentation process (process is published in Knorr et al., *Biochem. Eng. J.*, **2007**, 33, 263–274). The cellular energy charge significantly drops in the last hours of the fermentation for the wild type and average producer strains. The high producer shows an extended optimal energy balance and a timely delayed drop. The longer optimal production efficiency is responsible for a part of the higher Riboflavin yield.



Investigations of the developed method on the application as a fermentation quality control tool was done by a comparison of the intracellular metabolite profiles of the average producer strain in a correct accomplished fermentation run vs. a biologically contaminated fermentation run.

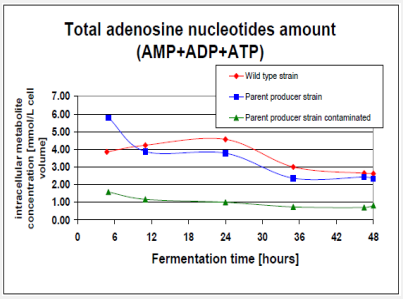


Fig. 4: The figure shows the sum of the total intracellular adenosine nucleotides (AMP, ADP and ATP) concentrations. The contaminated run led to a significantly changed metabolite profile which is not comparable to the wild type or the same strain during a correct fermentation run. Beside the total adenosine nucleotide concentration progression were also other significant indicators detected which can be used for the identification of fermentation quality control issues.

CONCLUSIONS

A powerful analytical platform was established based on a state of the art LC-MS/MS system. The method includes a basic metabolite catalog containing 19 metabolites from central metabolisms. The method will be used routinely for research purposes by DSM Nutritional Products and continuously improved, e.g. by addition of more metabolites and investigation of the applicability of the platform for new microorganisms.

DSM Nutritional Products
Wurmisweg 576
4303 Kaiseraugst

Supervisor FHNW: Prof. Dr. Götz Schlotterbeck
Supervisor DSM: Dr. Werner Bretzel
Expert: Dr. Markus Ehrat

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