DOLC-NMR:
Differential off-line LC-NMR analyses of nutrient-induced metabolome alterations in *S. cerevisiae* and their taste impact

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Abstract

Metabolome investigations by means of mass spectrometry are often limited in structure elucidation of unknown and new metabolites. A novel Differential Off-Line LC-NMR approach (DOLC-NMR) was developed to record and quantify nutrient-induced metabolome adjustments in *Saccharomyces cerevisiae*. Off-line coupling of preparative high performance liquid chromatography separation and ^{1}H-NMR spectroscopy supported by automated comparative NMR bucket analyses, followed by quantitative ^{1}H-NMR using ERETIC II has been successfully utilized to monitor significant quantitative changes in the metabolome of *S. cerevisiae* upon intervention with the aromatic amino acid *L*-tyrosine. Among the 33 metabolites identified by means of exact mass and 1D/2D-NMR experiments, glyceryl succinate, tyrosol acetate, tyrosol lactate, tyrosol succinate, and *N*-(1-oxoacyl)-*L*-tyrosine derivatives like *N*-(1-oxooctyl)-*L*-tyrosine have not been earlier reported as yeast metabolites. Depending on the chain length of the fatty acid, *N*-(1-oxooctyl)-, *N*-(1-oxodecanyl)-, *N*-(1-oxododecanyl)-, *N*-(1-oxomyristinyl)-, *N*-(1-oxopalmityl)-, and *N*-(1-oxooleoyl)-*L*-tyrosine imparted a kokumi taste enhancement above their human recognition thresholds ranging between 145 and 1432 µmol/L in a savoury model broth. Based on Carbon Modul Labelling (CAMOLA) and Carbon Bond Labelling (CABOLA) experiments using $^{13}C_6$-glucose as carbon source, biosynthesis pathways of the identified key metabolites could be described unequivocally. The aliphatic side chain of *N*-(1-oxooctyl)-*L*-tyrosine could be shown to be generated via de novo fatty acid biosynthesis from four C_2-carbon modules (acetyl-CoA) originating from *D*-glucose.

Introduction

Due to the wide application and its importance in foods production such as bread, wine, and beer, *Saccharomyces cerevisiae* has been one of the most investigated micro-organisms in the past 60 years. Various imaging techniques based on mass spectrometry and NMR spectroscopy were used to monitor stress-induced metabolome alterations and enabled the identification of stress markers. Salt stress, for example, has been reported to be responsible for the increase of trehalose levels to counteract osmotic pressure, whereas concentration levels of mono- and disaccharides depleted upon ethanol stress. Furthermore, different aroma and taste characteristics of fermented beverages are determined by utilizing different amino acids. Aliphatic branched-chain amino acids like *L*-leucine, *L*-isoleucine, and *L*-valine were found to be responsible for the typical aroma of *S. cerevisiae* fermented beverages. Upon entering the Ehrlich pathway, various fusel alcohols were provided by the yeast upon fermentation of different amino acids. [1]

Metabolomic studies on yeast were performed by analysing the metabolite profile emitted by the microorganism into the supernatant during the fermentation process.

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Although LC-MS has been often used for observing metabolite profiles because to its high resolution and rather low limit of detection, the unambiguous identification of important key metabolites undergoing an alternation in their concentration level upon an intervention becomes often a big challenge. Analyses of the whole extract by means of ^{1}H NMR spectroscopy seems to be promising as it can supply direct structure information as well as quantitative data of unknown metabolites. However, the low resolution and signal overlapping in ^{1}H-NMR spectroscopy limits the comprehensive analyses of complex natural extracts. Therefore, liquid chromatographic or SPE pre-separation has been described to simplify complex crude mixtures, such as faeces and urine, and to raise the resolution and decrease the complexity of one-dimensional ^{1}H NMR spectroscopy in metabolome research. [2]

To investigate secondary metabolites from *L*-tyrosine in yeast, a novel differential off-line HPLC-NMR approach (DOLC-NMR) was established to monitor metabolite alterations in *S. cerevisiae*. [3] Before and after an intervention with the aromatic amino acid *L*-tyrosine, yeast supernatants were pre-separated using preparative HPLC prior to a comparative NMR buckets analyses to record relative concentration changes of key metabolites, followed by absolute quantitation via qHNMR using the ERETIC II protocol. [3] In addition, the biosynthesis pathways of newly characterized key metabolites have been monitored by means of ^{13}C-labeling experiments, namely, carbon module labelling (CAMOLA) and carbon bond labelling (CABOLA) with LC-TOF/MS- and ^{13}C-NMR-based analyses of isotopologue patterns. [4,5]

Experimental

S. cerevisiae fermentation with/without L-tyrosine (Tyr1/Tyr0)

Dry yeast (*S. cerevisiae*, 460 mg of dried pellets; RUF, Quakenbrück, Germany) was mixed with water (200 mL), *D*-glucose (194 mmol/L) and *L*-tyrosine (12 mmol/L) were added, and the suspension was incubated for 96 h at 36°C under anaerobic conditions (Tyr^{1}). In addition, a control experiment (Tyr^{0}) was performed without the presence of *L*-tyrosine. Thereafter, the supernatants of $Tyr^{0/1}$ were separated from the yeast cells by filtration (0.45 μm, Sartorius Stedium Biotech GmbH; Göttingen, Germany) and freeze-dried and the residue obtained was used for chromatographic MPLC separation using a Spot Prep II (Gilson, Limburg, Germany) equipped with a preparative 250 × 21.2 mm, 5 μm, PhenylHexyl Luna column (Phenomenex, Aschaffenburg, Germany) to collect a total of 34 fractions in 1 min intervals. [3] After concentration of each fraction in vacuum by means of a HT-12 evaporation system (Genevac Limited, Ipswich, UK), the individual fractions collected from Tyr^{1} and Tyr^{0}, respectively, were dissolved in D_2O or MeOD-d_4 (Euriso-Top, Gif-sur-Yvette, France) for NMR analyses.

Stable isotope labelling experiments

To perform a CAMOLA experiment, a mixture of dry yeast (460 mg of dried pellets), *D*-glucose (97 mmol/L), $^{13}C_6$-glucose (97 mmol/L; Cambridge Isotope Laboratories, Inc., Andover, MA, USA), and *L*-tyrosine (12 mmol/L) in water (200 mL) was incubated for 96 h at 36 °C under anaerobic conditions. After fermentation, the supernatant was obtained by filtration and, then, directly used for UPLC-ESI-TOF/MS (Waters Synapt G2S HDMS; Waters, Manchester, UK, coupled to an Acquity UPLC core system; Waters, Milford, MA, USA) analyses.

For the CABOLA experiment, a mixture of dry yeast (460 mg of dried pellets), *D*-glucose (184.3 mmol/L), $^{13}C_6$-glucose (9.7 mmol/L), and *L*-tyrosine (12 mmol/L) in water (200 mL) was incubated for 96 h at 36 °C under anaerobic conditions. Purified

metabolites from the supernatants were taken up in D_2O or MeOD-d_4 and analysed by means of ^{13}C-NMR spectroscopy to analyse $^{n}J_{13C\text{-}13C}$ coupling patterns of the isolated target fermentation products.

Nuclear Magnetic Resonance spectroscopy (NMR)

^{1}H/^{13}C-NMR experiments were performed on a Bruker AVANCE III 500 MHz system equipped with a cryo-TCI Probe at 300 K (Bruker, Rheinstetten, Germany). The collected HPLC fractions were dissolved in D_2O (1 mL), and aliquots (540 µL) were then mixed with an aliquot (60 µL) of the NMR buffer (phosphate buffer, pH = 7) solution prior to the measurement. The more hydrophobic fractions 20−34 were taken up in MeOD-d_4 (1 mL) and aliquots (600 µL) used for NMR analyses. ^{1}H NMR spectra were acquired using the Bruker standard water suppression pulse sequence (noesygppr1d). The 90° pulse length (P1), PL9, and O1 were adjusted individually on each sample and spectra were acquired using 16 scans (NS) and 4 prior dummy scans (DS) and collected into 64K data points using a spectral width of 10273.97 Hz. The relaxation time (T1) was set to 20 s based on the longest relaxation time of a signal of interest.

The NMR buckets were calculated with Amix Viewer V3.9.13 software (Bruker, Rheinstetten, Germany). Each spectrum was referenced to TMSP (0.0 ppm). After checking the baseline offset and using the underground removal tool, the spectra were used to determine the buckets. Covering the chemical shift region from −1 to 11 ppm, the range of each bucket was set to 0.1 ppm. The area between 4.5 and 5 ppm was excluded from bucketing due to the water signal in the spectra. The calculation of the absolute integral value for each of the 115 buckets was performed successfully when the signal-to-noise ratio was >10. The noise was calculated in the region from 10 to 11 ppm, where no signals appeared. From the yeast fermentation with tyrosine (Tyr^1) and the control (Tyr^0), the corresponding buckets showing an integral ratio (Tyr^1/Tyr^0) of >2 or <0.5 were used for further analyses. Quantitative ^{1}H-NMR was performed using ERETIC 2 (Electronic REference To access In vivo Concentrations) based on the PULCON (PULse length based CONcentration determination) methodology. [6]

Sensory analyses for determination of taste threshold concentrations

Intrinsic taste threshold concentrations of the purified compounds were determined in Evian water using a duo test in triplicate analyses by a well-trained sensory panel (n = 14). Threshold concentrations for taste modulation effects were determined in a savoury model broth (pH 5.9, adjusted with 0.1% FA) consisting of sodium chloride (2.9 g/L), maltodextrin (6.4 g/L), monosodium *L*-glutamate (1.9 g/L) and an amino acid mix (0.38 g/L). The geometric mean of the last correct tasted and the first incorrect tasted concentration was calculated and taken as the individual threshold of each panellist. The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in two independent sessions.

Results and discussion

Differential off-line HPLC-NMR analyses

To overcome the challenge of overlaying signal, the fermentation batches Tyr^1 and Tyr^0 were separated by means of RP-HPLC into 1 min subfractions prior to ^{1}H-NMR analyses to increase spectral resolution of the key metabolites. The obtained NMR buckets from fraction 3-34 were used for statistical analyses to visualize relative concentration ratios in the metabolite signature, expressed as the ratio (Tyr^1/Tyr^0) of the signal integrals of the corresponding metabolites (Figure 1).

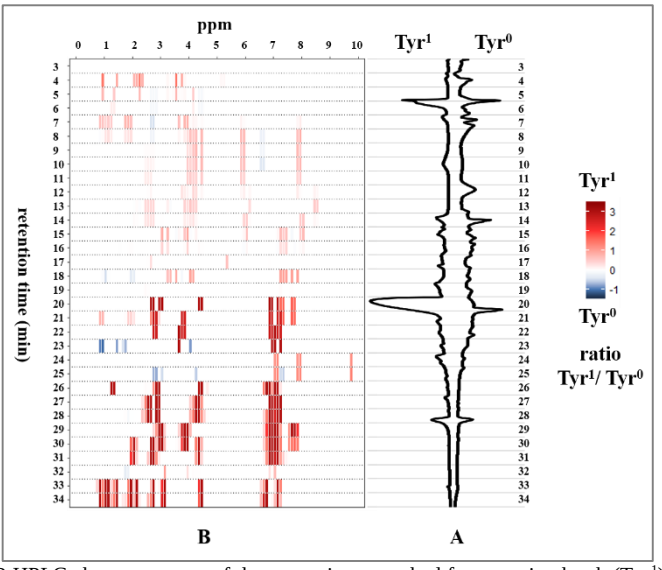

Figure 1: (A) RP-HPLC chromatograms of the *L*-tyrosine perturbed fermentation broth (Tyr^1) and the control approach without *L*-tyrosine (Tyr^0). (B) Differential NMR bucket analyses of fractions 3−34 showing the relative integral ratios (Tyr^1/Tyr^0) and chemical shifts of influenced metabolites.

The first-eluting fractions 3−15 showed only marginal differences in relative metabolite concentrations between the broths Tyr^0 and Tyr^1. Among the identified metabolites, many primary metabolites playing a key role in energy management and growth of the microorganism like intermediates of the citrate cycle such as succinic acid (fractions 8−10; **2**, Figure 3), malic acid (fraction 5), fumaric acid (fractions 7−9) were to be found. Furthermore, amino acids such as leucine (fraction 6/7), isoleucine (fraction 6/7), phenylalanine (fraction 15/16), degradation products of *D*-glucose like glycerol (fraction 4/5; **3**) and lactic acid (fractions 4−6; **4**), and the nucleotides adenosine (fractions 11−15), guanosine (fractions 14−16) and uridine (fractions 8−12) were significantly influenced by the nitrogen source. Additionally, the ester glycerol succinate (**5**), identified in fractions 12−14, has been found for the first time as a metabolite of *S. cerevisiae*.

In comparison to the first eluting polar metabolites, higher differences in metabolite concentrations between Tyr^0 and Tyr^1 were monitored in fractions 16−34 containing semi- and nonpolar compounds. The aromatic fusel alcohol tyrosol (fraction 21/22; **1**), degradation product of *L*-tyrosine via the Ehrlich pathway, was found in a 100-fold higher concentration in Tyr^1. In the further analyses three tyrosol esters, varying in the organic acid side chain, could be clearly identified, namely, tyrosol acetate (**6**) in fraction 30/31, tyrosol succinate (**7**) in fraction 27/28, and tyrosol lactate (**8**) in fraction 26. Neither Tyrosol succinate (**7**) nor tyrosol lactate (**8**) have been reported as a metabolite of *S. cerevisiae*. Moreover, p-hydroxyphenyl lactic acid (fraction 20) and p-hydroxybenzaldehyde (fraction 24/25) were identified as nitrogen-free *L*-tyrosine degradation metabolites. Finally, the amino acid amide *N*-(1-oxooctyl)-*L*-tyrosine (**9**) could be isolated from the Tyr^1 fractions 33/34. This amide is already known from *Escherichia coli* but has not yet been reported as a metabolite of *S. cerevisiae*. Furthermore, LC-MS/MS analyses revealed the presence of *N*-(1-oxoacyl)-*L*-tyrosine derivatives with acyl chain lengths of C_4, C_6, C_{10}, and C_{12} along with the predominating *N*-(1-oxooctyl)-*L*-tyrosine (**9**) homologue.

Absolute metabolite quantitation by ^{1}H NMR spectroscopy

A validated PULCON methodology using ERETIC II was applied to the NMR buckets of fractions 3−34 collected from Tyr^0 and Tyr^1. Using fraction 14 as an example (Figure 2), glycerol succinate (**5**), uridine and adenosine were quantitated using selected resonance signals that were baseline separated without showing no signal overlap, namely, 2.64 ppm (dd, 2H, H-(3′)) for glycerol succinate (**5**), 7.88 ppm (d, 1H, H-(6)) for uridine and 8.35 ppm (s, 1H, H-(2)) for adenosine, respectively.

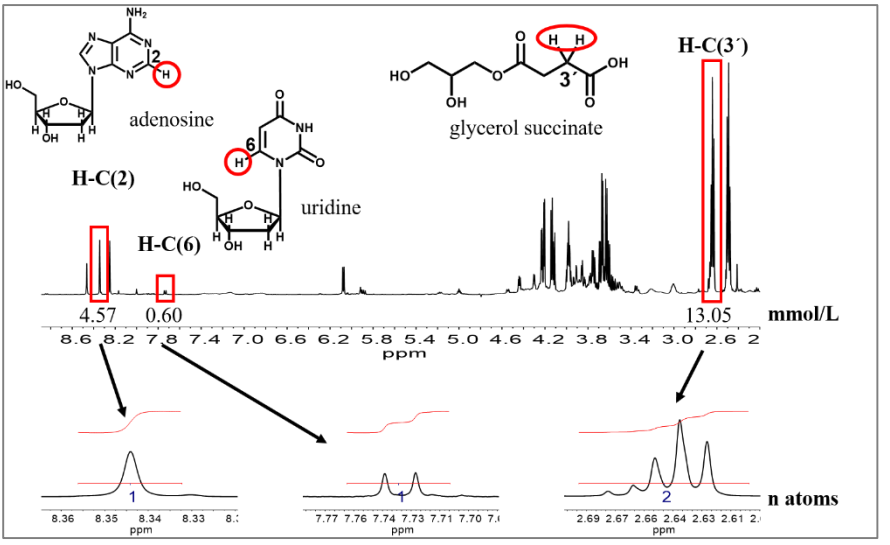

Figure 2: Quantitative ^{1}H NMR spectroscopy (noesygppr1d, 500 MHz, D2O, 300 K) of fraction 14 containing adenosine (H-(2), 8.34 ppm, 1H), uridine (H-(6), 7,75 ppm, 1H), and glycerol succinate (**5**; H-(3′), 2.64 ppm, 2H). Labelled signals were used for metabolite quantitation.

Sensory activity of metabolites

All detected *N*-(1-oxoacyl)-*L*-tyrosine derivatives showed a bitter intrinsic taste within a narrow threshold range from 343 to 647 µmol/L in Evian water (Table 1). The evaluation of the compounds in a spicy model broth demonstrated a clear kokumi enhancing activity of *N*-(1-oxooctyl)-*L*-tyrosine (9) above 1432 µmol/L (Table 1).

Table 1: Human taste recognition thresholds in µmol/L of *N*-(1-oxoacyl)-*L*-tyrosine derivatives in Evian water (intrinsic bitter taste) and savoury model broth (kokumi enhancement) with a significance level $\alpha \leq 0.05$

Compound	*Bitter taste*	*Kokumi enhancement*
N-(1-Oxobutyl)-*L*-tyrosine (C_4)	647	n.d.
N-(1-Oxohexyl)-*L*-tyrosine (C_6)	343	n.d.
N-(1-Oxooctyl)-*L*-tyrosine (C_8)	631	1432
N-(1-Oxodecanyl)-*L*-tyrosine (C_{10})	627	537
N-(1-Oxododecanyl)-*L*-tyrosine (C_{12})	480	145
N-(1-Oxomyristyl)-*L*-tyrosine (C_{14})	672	160
N-(1-Oxopalmitylyl)-*L*-tyrosine (C_{16})	627	183
N-(1-Oxooleoyl)-*L*-tyrosine ($C_{18:1}$)	446	217

A prolongation of the length of the fatty acid side chain reduced the kokumi recognition thresholds concentrations to 145−217 µmol/L as for the C_{12} to $C_{18:1}$ homologues. A shortening of the chain length (C_4 and C_6) eliminated the kokumi activity.

Biosynthetic pathway analyses using 13C-labelling experiments

The analyses of the biosynthetic pathways (Figure 3) of identified metabolites were performed by means of two different ^{13}C-labelling experiments. The added glucose was diluted with 5% (CABOLA) and 50% (CAMOLA) $^{13}C_6$-glucose, respectively, prior to yeast fermentation in the presence and absence of *L*-tyrosine to monitor the joint transfer of several ^{13}C atoms *en bloc* into the target metabolite. Supported by these experiments it could be clearly shown that the aliphatic side chain of *N*-(1-oxooctyl)-*L*-tyrosine (**9**) is generated via *de novo* fatty acid biosynthesis from four C_2-carbon modules (acetyl-CoA). In addition, the ^{13}C signatures of the secondary metabolites tyrosol acetate (**6**), tyrosol lactate (**8**), tyrosol succinate (**7**), and glyceryl succinate (**5**) beside the primary intermediates glycerol (**3**), succinic acid (**2**) and lactic acid (**4**) were investigated. The labelling experiments showed the ability of *S. cerevisiae* to form tyrosol (**1**) via the Ehrlich pathway from *L*-tyrosine or *de novo* via the Shikimi pathway from *D*-glucose.

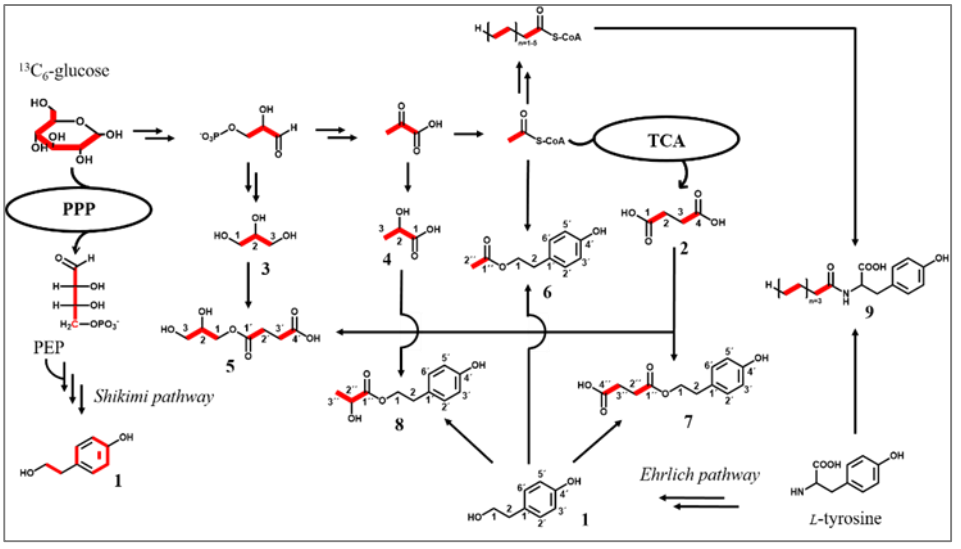

Figure 3: Biosynthesis pathways of key metabolites originating from *D*-glucose and *L*-tyrosine (TCA: tricarboxylic acid cycle; PPP: pentose phosphate pathway; PEP: phospho enol pyruvate). Bold lines indicate intact carbon bonds originating from ^{13}C-glucose as observed by the CABOLA and CAMOLA experiment.

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