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A fast method for the quantification of methylamine in fermentation broths by gas chromatography

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Abstract

The objective of this study was to develop a method for the quantitative analysis of the methylamine concentration in fermentation broths of *Hyphomicrobium zavarzinii* ZV 580 cultures. For this purpose an established method for the quantification of free amino acids in such matrices was adapted and validated. The detection limit was 10 μM, the calibration curve showed good linearity ($R^2 = 0.9998$) in the concentration range between 0.1 and 8 mM. The standard deviation of the injection-to-injection reproducibility ($n = 10$) of the retention coefficient was <1%, that of the peak area <5%. In case of the sample-to-sample reproducibility ($n = 8$), the standard deviation was <5% for the retention coefficient and <10% for the peak area. The validated method was successfully applied for monitoring a fed-batch bioprocess (starting volume: 8 L, initial methylamine hydrochloride concentration: 10 mM) producing a dye-linked formaldehyde dehydrogenase in *H. zavarzinii* ZV 580.
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1. Introduction

The reliable quantification of small, linear, aliphatic amines such as methylamine is of interest in various settings. Such amines, many of which are known to be hazardous to human health, are, e.g. found in (waste) water [1,2], decaying foodstuffs such as fish [3], but also in human urine (as metabolic products) [2,4–6]. They are intermediates in the chemical and pharmaceutical industries and are emitted by a number of anthropogenic sources. Our research group has recently been confronted with the necessity for a reliable quantification of methylamine in a biotechnical context [7]. The organism *Hyphomicrobium zavarzinii* ZV 580 can be grown on various C1-sources including methanol, but when grown exclusively on methylamine (provided as methylamine hydrochloride) produces a formaldehyde dehydrogenase that is of particular interest for the development of robust and reliable formaldehyde biosensors. The organism shows complex growth kinetics and a suitable bioprocess control algorithm ideally requires close monitoring of the C-source concentration, i.e. here the methylamine hydrochloride concentration. The indicated case differs slightly from the above-mentioned established methylamine quantification challenges in that the methylamine in not present in trace but rather in fairly substantial amounts (up to 10 mM) in the bioreactor and that the speed of the analysis and the day-to-day reproducibility are very important for efficient process monitoring.

The analysis of small aliphatic amines in aqueous solution presents a challenge. Such amines are very polar and hence dissolve well in water. In fact, volatile amines are usually entrapped in aqueous (acidic) solution. Sample preparation via extraction, a typical sample preparation method for analysis by chromatography, can thus be difficult. In principle, however the quantitative analysis of such molecules is possible by gas or liquid chromatography. Analysis by liquid chromatography usually takes much longer than by gas chromatography and a derivatisation step is normally required for detection. Analysis by gas chromatography is quicker, but unless derivatised such amines can only be analysed when packed or thickly coated columns are used [8–10]. Derivatisation also reduces the tendency for peak tailing and memory effects that are otherwise often observed in the case of small amines due to the pronounced hydrophilicity and hydrogen-bounding tendency of these strongly basic substances [11,12].

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Among the various derivatisation chemistries suggested for such amines [e.g. also 1,2,6], derivatising with chloroformates such as isobutyl chloroformate [5] seems to have advantages in terms of speed and specificity [4,5,12,13]. The reaction takes place spontaneously at room temperature and converts the amines into carbamates, which can usually be analysed well by gas chromatography. In order to react, the amine should be present in unprotonated form. Chloroformate derivatisation has, e.g. been used for analysis of small aliphatic amines in environmental samples and human urine [4]. In case of very small amines, isobutyl chloroformate is usually preferred as derivatising agent [e.g. 5]; smaller chloroformates such as ethyl chloroformate have been used successfully in the case of larger amines and diamines such as 1,6-hexamethylenediamine [4]. In the latter case both amino groups are brought to reaction.

A similar approach, i.e. the use of alkyl chloroformates as derivatising agents, has also been suggested for the analysis of amino acids [14–16]. In this case both the amino group and the carboxylate group react with the chloroformate, in the latter case followed by a decarboxylation reaction. The derivatisation reaction is rapid and takes place at room temperature. Moreover, a commercial kit is available (EZ:faast, Phenomenex), which uses propyl chloroformate for derivatisation and which yields reliable and reproducible results in the case of amino acids from serum, urine, tissue samples, protein hydrolysates, food stuffs, and fermentation broths. The procedure is based on a solid phase extraction step followed by derivatisation and liquid/liquid extraction; derivatised analytes are then analysed by gas chromatography with FID detection. The solid phase extraction removes putative interfering compounds in the sample matrix such as proteins and urea. Total sample preparation takes about 10 min and analysis is performed in less than 10 min. Compared to established HPLC methods for amino acid analysis, this is a considerable reduction in analysis time.

In this contribution we would like to show that this procedure can be adapted to the monitoring of the methylamine concentration in a fed-batch bioprocess and hence for the monitoring, if not yet the control of such processes.

2. Materials and methods

2.1. Materials

Chemicals including mineral salts for the culture media were from established suppliers such as Sigma–Aldrich and used as obtained. Millipore water was used for the preparation of all aqueous solutions. The strain *H. zavarzinii* ZV 580 was from the collection of the "Institut für Allgemeine Mikrobiologie" (University of Kiel, Germany) and a generous gift from Dr. Vorholt (INRA/CNRS, Castanet-Tolosan, France).

2.2. Microbial culture conditions

Bacteria were grown in a mineral medium containing 10 mM K_2HPO_4, 14 mM NaH_2PO_4, 10 mM $MgSO_4$, 15 mM $(NH_4)_2SO_4$, 4 μM $FeSO_4$, 1 μM $ZnSO_4$, 10 μM $CaCl_2$, 1 μM $CuSO_4$, 1 μM $CoCl_2$, 186 μM $(NH_4)_6Mo_7O_{24}$, 5 μM $MnCl_2$, and 4 μM EDTA. Culture media were supplemented as indicated with methylamine hydrochloride as carbon source (C-source). The pH of the culture medium as prepared was between 6.8 and 6.9. The pH was adjusted to 7.0 before autoclaving by adding a few drops of 1 M NaOH and verification via pH-electrode. Bacterial growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}) as described previously [7]. Cultivation of *H. zavarzinii* was done in a NLF22 bioreactor from Bioengineering (Wald, Switzerland) in the fed-batch mode. The cells were inoculated at an OD_{600} of 0.1. The cultivation temperature was 30 °C; a pH of 6.9 ± 0.1 was maintained by automatic titration with 2 M NaOH. The stirrer speed and the airflow were programmed in cascade with the dissolved oxygen (DO) as described previously [7].

2.3. Analytical methods

The methylamine hydrochloride concentration was analysed by adapting a protocol generally used to quantify amino acids in cell culture supernatant using the EZ:faast GC/FID free (physiological) amino acid kit (Phenomenex, Aschaffenburg, Germany). Briefly, the culture samples were clarified by centrifugation (13,000 × *g*, 4 °C, 10 min) and the supernatants were appropriately diluted in culture medium. Sample preparation was according to the manufacturer's instruction and involved a solid phase extraction (removal of proteins and other impurities), a derivatisation with propyl chloroformate, and an extraction into an organic solvent for additional purification. The sample was then ready for injection into the gas chromatograph. Norvaline was added to each sample as internal standard. For calibration and reproducibility studies fermentation supernatants were replaced with standard solutions of methylamine hydrochloride at concentrations between 0.1 and 10 mM. The gas chromatograph employed was a GC 6890N with FID detector and a 7683B injector, all from Agilent (Waldbronn, Germany). The column was a 10 m × 0.25 mm ZB-AAA fused-silica capillary column also from Phenomenex. Helium served as carrier gas (head pressure: 0.45 bar). Split injection (2 μL) was performed at an initial head pressure of 0.45 bar and a split-ratio of 1:15. The ChemStation software version Rev. B.01.03 was applied for computer-assisted processing of the chromatographic data.

3. Results and discussion

The goal of this research was to develop a method for the quantification of methylamine hydrochloride in cultures of *H. zavarzinii* ZV 580 based on an established method for the analysis of free amino acids in such matrices. The established assay uses propyl chloroformate for derivatisation of the amino (and carboxylic acid) groups prior to analysis by GC-FID. To our knowledge, such small alkyl chloroformates have never been used as basis for the analysis of small alkylamines, where contrarily to the situation with amino acids only an amino and no carboxylic acid end group is available for the reaction. It was thus investigated if and to what extent the various steps comprising the amino acid analysis protocol could be adapted to the analysis

Table 1
Conditions and results obtained for the analysis of glycine in Millipore water and cell culture medium

Sample	Temperature program	Retention time (min)	Retention coefficient[a]
10 mM glycine in Millipore water	110–320 °C at 32 °C/min, 1 min 320 °C	1.707	2.428
10 mM glycine in medium	40–320 °C at 32 °C/min, 1 min 320 °C	3.841	1.307
10 mM glycine in medium	40–220 °C at 32 °C/min, 1 min 320 °C	3.841	1.307
10 mM glycine in medium	40–220 °C at 16 °C/min, 1 min 320 °C	6.605	1.782

[a] Calculated as $k = (t_r - t_0)/t_0$, with t_r is the analyte retention time and t_0 is the elution time of an unretained tracer.

of methylamine hydrochloride as C-source in cultivations of *H. zavarzinii*.

3.1. Development of the analytical protocol

Of particular concern during method development was the question whether the methylamine derivative would be sufficiently retained on the column to distinguish it from other peaks likely to be produced by the samples, in particular the various solvent peaks and peaks caused by putatively present free amino acids/small peptides. Since glycine is the amino acid that is structurally most similar to methylamine, this amino acid was of particular concern. Subsequently the analysis of glycine (10 mM) was investigated in Millipore water and in *H. zavarzinii* culture medium. Based on the structure of the methylamine derivative and data found in the literature [4,5,14] we deduced that retention of our molecule of interest would be too low in the standard GC-protocol (32 °C/min from 110 to 320 °C, hold for 1 min at 320 °C). In order to optimise the separation, both the start and the final temperature of the temperature gradient as well as the gradient slope of the GC separation were therefore varied. In particular we reduced the start temperature to 40 °C. The final gradient temperature was adjusted to 220 °C in order to reduce analysis time when we found that the FID did not detect additional peaks beyond that temperature. Conditions and results of all experiments are compiled in Table 1. In all protocols a final step for 1 min at 320 °C was included to assure complete column regeneration. Compared to the standard GC-

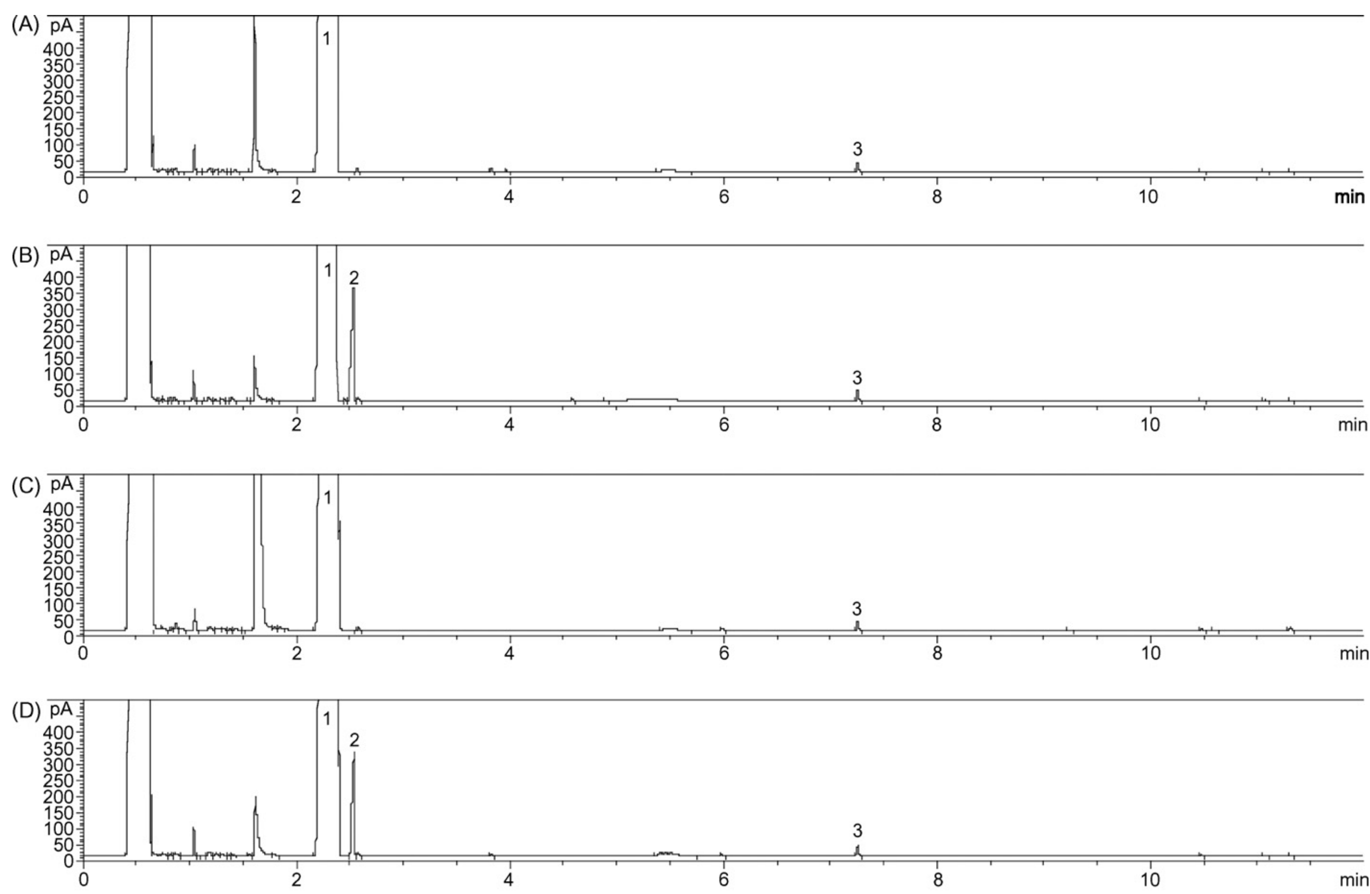

Fig. 1. Gas chromatograms of (A) the negative control (Millipore water); (B) Millipore water spiked with 10 mM methylamine hydrochloride; (C) the *H. zavarzinii* culture medium; (D) *H. zavarzinii* culture medium spiked with 10 mM methylamine hydrochloride. Temperature program: 40–220 °C with 16 °C/min, then 220–320 °C with 30 °C/min and 1 min 320 °C with constant gas flow of 1.5 mL/min. Peak (1) is the last signal of the solvent peaks; peak (2) is the methylamine derivate; peak (3) is the internal standard (propyl chloroformate derivate of norvaline).

Table 2
Conditions and results obtained for the analysis of methylamine hydrochloride (MA-HCl) in Millipore water and culture medium

Sample	Temperature programme	Retention time (min)	Retention coefficient
10 mM MA-HCl in Millipore water	40–220 °C at 16 °C/min, 1 min 320 °C	2.533	0.067
10 mM MA-HCl in medium	40–220 °C at 16 °C/min, 1 min 320 °C	2.535	0.066
5 mM MA-HCl in medium	40–220 °C at 16 °C/min, 1 min 320 °C	2.525	0.058
10 mM MA-HCl in medium	40–180 °C at 16 °C/min, 1 min 320 °C	2.535	0.069
10 mM MA-HCl in medium	40–180 °C at 12 °C/min, 1 min 320 °C	2.927	0.077
10 mM MA-HCl in medium	40–180 °C at 12 °C/min, 1 min 320 °C	2.928	0.071
10 mM MA-HCl in medium	40–180 °C at 10 °C/min, 1 min 320 °C	3.194	0.075

protocol these changes in the temperature protocol increased the retention time of the glycine derivative from 1.7 to almost 4 min, while the retention coefficient dropped from 2.5 to 1.3. Finally, the slope of the temperature gradient was reduced to 16 °C/min in order to improve the resolution of the separation, which increased the retention time of the glycine peak to 6.6 min, while the retention coefficient became 1.8.

The modified temperature program was then applied to the analysis of methylamine hydrochloride first in water and then in cultivation medium, Fig. 1. For conditions and results see Table 2. Using the previously established protocol (16 °C/min from 40 to 220 °C, 220 to 320 °C at 30 °C/min, hold for 1 min at 320 °C) a retention time of 2.5 min and a retention coefficient of 0.07 were obtained for methylamine hydrochloride in these experiments. In spite of this rather low retention coefficient, base line separation of the methylamine hydrochloride peak (peak 2 in Fig. 1) from the last eluting solvent peak (peak 1 in Fig. 1) was possible. Moreover, as expected, the mineral culture medium itself contained few interfering compounds. In order to improve the retention coefficient, experiments were performed testing shallower temperature gradients, Table 2. In particular ramps of 12 and 10 °C/min were investigated. This increased the retention times to 2.9 and 3.2 min, while the retention coefficients became 0.071 and 0.075.

Table 3
Retention times of methylamine hydrochloride (8 mM) as well as the standard mixture of free amino acids (1.6 mM) prepared in *H. zavarzinii* culture medium; conditions as indicated in the legend of Fig. 2

Compound	Retention time (min)
Methylamine hydrochloride	2.914
Alanine	7.869
Sarcosine	8.059
Glycine	8.180
α-Aminobutyric acid	8.501
Valine	8.781
β-Aminoisobutyric acid	8.964
Internal standard (Norvaline)	9.147
Leucine	9.383
Allo-isoleucine	9.443
Isoleucine	9.526
Threonine	10.071
Serine	10.162
Proline	10.338
Asparagine	10.609
Thioproline	11.476
Aspartic acid	11.998
Methionine	12.037
Hydroxyproline	12.293
Glutamic acid	12.473
Phenylalanine	12.647
α-Aminoadipic acid	13.061
α-Aminopimelic acid	13.392
Glutamine	13.466
Ornithine	13.926
Glycyl-proline	13.975
Lysine	14.207
Histidine	14.409
Hydroxylysine	14.592
Tyrosine	14.702
Proline-hydroxyproline	14.936
Tryptophane	15.013
Cystathionine	15.490
Cystine	15.719

For the subsequent experiments the following method was used as a compromise between speed and resolution of the analysis: temperature gradient from 40 to 180 °C at 12 °C/min, then 180 to 320 °C with 30 °C/min and 1 min at 320 °C with constant gas flow of 1.5 mL/min. Injector and FID temperatures were 250 and 320 °C, respectively. With this protocol methylamine hydrochloride (10 mM) was quantified in *H. zavarzinii* culture medium, culture medium spiked with 0.2 mM glycine and culture medium spiked with a standard solution containing 1.6 mM of the amino acids listed in Table 3. Table 3 also lists the retention times of the derivatised amino acids under the indicated conditions. The chromatograms are shown in Fig. 2. In all cases the methylamine hydrochloride peak is reproducibly obtained. In addition the peak is well separated from both the solvent and the amino acid peaks. Quantification of methylamine hydrochloride in bioprocesses using this molecule as C-source appears hence feasible.

3.2. Method calibration

As preparation for bioprocess monitoring, the method was calibrated using cell culture media samples spiked with different amounts of methylamine hydrochloride. The pre-set upper limit of interest in our case was 10 mM, i.e. the initial nutrient level set in the cultivations. Under the defined analytical conditions, a detection limit of 10 μM (ca. 20 μg/L) was determined (S/N > 3). Interestingly, this is approximately twice as high as the detection limit found by Skarping et al. [4] in the case of 1,6-hexamethylenediamine derivatised with ethyl chloroformate. Subsequently a calibration curve was prepared in

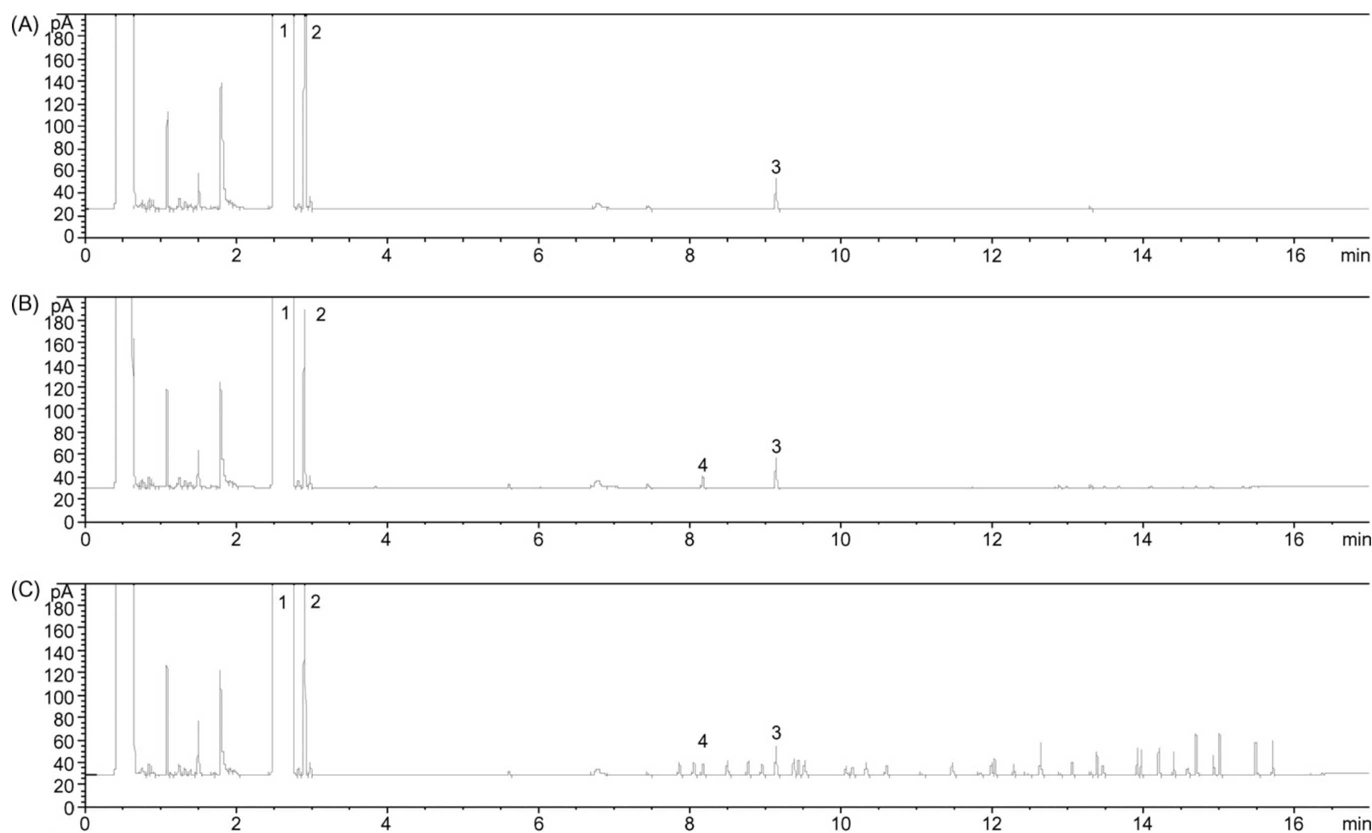

Fig. 2. Gas chromatograms of (A) a sample containing 8 mM methylamine hydrochloride in *H. zavarzinii* culture medium, (B) a sample containing 8 mM methylamine hydrochloride and 0.2 mM L-glycine in *H. zavarzinii* culture medium, and (C) a sample containing 8 mM methylamine hydrochloride and a mixture of amino acids (1.6 mM) also prepared in *H. zavarzinii* culture medium. Temperature program: 40–180 °C with 12 °C/min, then 180–320 °C with 30 °C/min and 1 min at 320 °C with constant gas flow of 1.5 mL/min. Peak (1) is the last of the solvent peaks, peaks (2) and (4) are the signals for the methylamine and L-glycine derivates, respectively. Peak (3) is the derivate of norvaline (internal standard). The retention times of all analytes are compiled in Table 3.

H. zavarzinii culture medium for 10 methylamine hydrochloride concentrations between 0.1 and 10 mM. Each concentration was measured three times starting from the beginning of the sample preparation protocol. In addition, samples containing 0.1, 1.0, 8.0, and 10.0 mM methylamine hydrochloride were both repeatedly injected (10 times) from a single aliquot for the determination of the injection-to-injection reproducibility (Table 4). For the same concentrations eight individual samples were prepared in parallel in each case for determination of the sample-to-sample reproducibility (Table 5).

The calibration curve showed very good linearity between 0.1 and 8 mM methylamine hydrochloride, Fig. 3, with $R^2 = 0.9998$. We presume that the non-linearity observed at concentrations higher than 8 mM is due to beginning saturation of the solid phase used for the extraction step in the sample preparation protocol. When the effect of the sample volume was investigated for the corresponding amounts (the supplier of the kit suggests sample volumes between 50 and 200 μL), a sample volume of 100 μL was found to give the best results in terms of reproducibility. Moreover, such a volume allows standardizing the

Table 4
Injection-to-injection reproducibility ($n = 10$ for each concentration) for the methylamine hydrochloride (MA-HCl) quantification

Concentration (MA-HCl) (mM)	Peak area	
	Mean (a.u.)	R.S.D. (%)
0.1	6.04	2.56
1.0	59.96	2.04
8.0	501.45	2.63
10.0	613.55	3.18

2 μL samples prepared in *H. zavarzinii* culture medium and containing the indicated amounts of methylamine hydrochloride were injected 10 times successively from a single aliquot. Temperature program: 40–180 °C with 12 °C/min, then 180–320 °C with 30 °C/min, hold 1 min at 320 °C; constant gas flow of 1.5 mL/min. R.S.D.: relative standard deviation; a.u.: arbitrary units.

Table 5
Sample-to-sample reproducibility ($n = 8$ for each concentration) for the methylamine hydrochloride (MA-HCl) determination

Concentration (MA-HCl) (mM)	Peak area	
	Mean (a.u.)	R.S.D. (%)
0.1	5.24	6.47
1.0	55.35	4.63
8.0	446.42	9.33
10.0	553.66	6.50

Samples containing the indicated concentration of methylamine hydrochloride ($n = 8$ each) were derivatised independently and analysed. Temperature program: 40–180 °C with 12 °C/min, then 180–320 °C with 30 °C/min, hold 1 min at 320 °C; constant gas flow of 1.5 mL/min. R.S.D.: relative standard deviation; a.u.: arbitrary units.

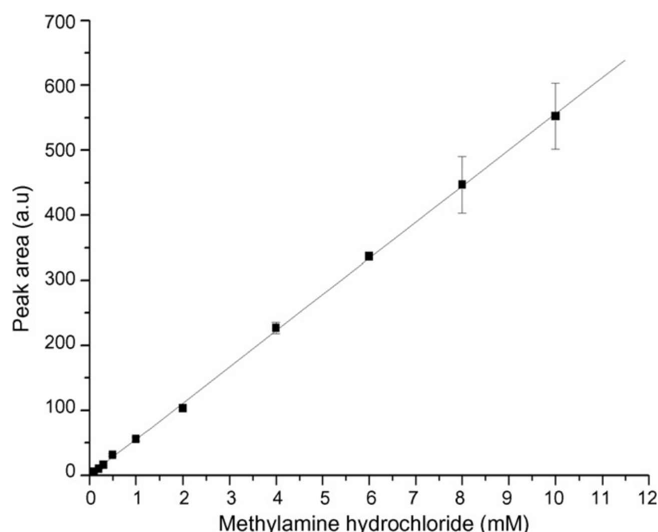

Fig. 3. Calibration curve for the evaluation of the methylamine hydrochloride concentrations in cultures of *H. zavarzinii*. Calibration was done in *H. zavarzinii* culture medium spiked with 0.1–10 mM methylamine hydrochloride. Each concentration was measured in triplicate starting from the derivatisation reaction. The trend line is represented as a solid line with $R^2 = 0.9998$.

time required to treat the sample. Samples with a concentration above 8 mM were thus diluted in culture medium rather than added at a reduced sample volume to allow the standardisation of the sample volume in all cases.

In the injection-to-injection reproducibility studies we found a standard deviation below 1% for the retention coefficient. A standard deviation below 3% was found for the various peak areas (sample concentrations), Table 4. As to be expected, standard deviations were higher for the extremes (0.1 and 8 mM) of the calibration curve. The standard deviation for methylamine hydrochloride concentration (peak area) found during the repeated analysis of the 10 mM sample was 3.2%. Standard deviations of both the retention coefficient and the peak areas were higher for the individually processed aliquots, where the various sample preparation steps add to the observed standard deviation. Even under these circumstances the standard deviation for the retention coefficient was below 5% and that for the various peak areas (concentrations) below 10%. The latter are summarised in Table 5. However, a standard deviation of 10% for the concentration was considered admissible for our purpose, i.e. the *at line* nutrient monitoring in the bioprocess.

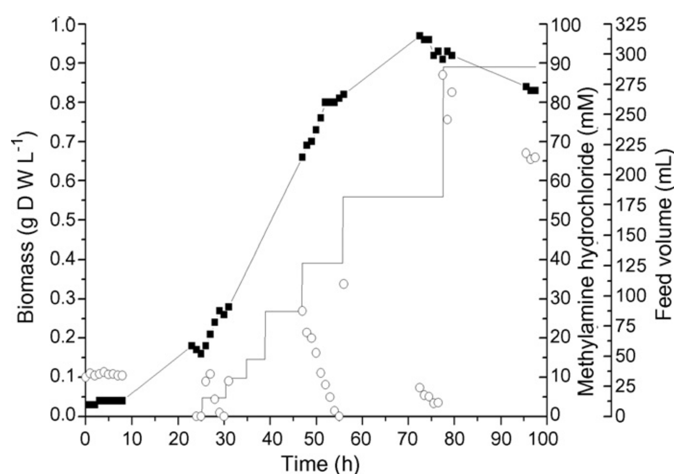

Fig. 4. Cultivation of *H. zavarzinii* in the bioreactor with automatic feeding. The cells were inoculated at an OD_{600} of 0.1 in 8 L culture medium containing 10 mM methylamine hydrochloride as C-source. The culture was run in the fed-batch mode, the pH was maintained at 6.9 ± 0.1 by automatic titration with 2 M NaOH. The biomass production is displayed by black squares, the methylamine hydrochloride concentration in the liquid by white circles, and the feed volume addition by solid line.

3.3. Monitoring of the carbon source (methylamine hydrochloride) concentration in a fed-batch cultivation of H. zavarzinii

H. zavarzinii is known to produce a dye-linked formaldehyde dehydrogenase when grown on methylamine hydrochloride as the sole carbon source. This enzyme is of particular interest as a biocatalyst in technical applications. In general, growth of the organism is slow and follows a complex pattern. In our recent investigation of the bioprocess development [7] it became obvious that a successful fed-batch strategy can only be based on information regarding the substrate consumption rates. The developed assay was thus used for process monitoring, Fig. 4.

The cells were inoculated at an OD_{600} of 0.1 in 8 L culture medium containing 10 mM methylamine hydrochloride as carbon source. The culture was run in the fed-batch mode, the feed pump (5 M methylamine hydrochloride, flow rate: 32 mL/min) was activated three times for 0.5 min (after 25.5, 30.4 and 35.0 h of culture); two times for 1.3 min (after 39.0 and 47.0 h of culture); for 1.8 min after 56.0 h of culture; for 3.6 min after 77.5 h of culture. The pH of the culture was maintained constant at pH 6.9 ± 1 by automatic titration with 2 M NaOH. In Fig. 4 the biomass development is displayed by black squares, the

Table 6
Characteristic bioprocess data

Time of culture (h)	Specific growth rate, μ_{max} (h^{-1})	Methylamine hydrochloride consumption rate (mmol h^{-1})	Biomass yield (g DW (mol MA-HCl h)$^{-1}$)	Enzyme concentration
8–29	0.09	7.3	0.59	0.09 U/g WW (0.36 U/L)[a]
29–52	0.05	22.6	0.35	0.137 U/g WW (1.26 U/L)[b]
52–72.5	0.01	14.4	0.22	0.09 U/g WW (0.88 U/L)[c]

DW: dry weight; WW: wet weight; U: enzyme units.
[a] Determined after 29 h of culture.
[b] Determined after 52 h of culture.
[c] Determined after 72 h of culture.

methylamine hydrochloride concentration in the liquid by white circles, and the added feed volume by solid line.

Data concerning biomass, methylamine consumption and enzyme activity are compiled in Table 6. Based on the biomass data, the following specific growth rates were calculated from the slope of a plot ln N (cell concentration) against time t: 8–29 h, $\mu_{max} = 0.09\,h^{-1}$; 29–52 h, $\mu_{max} = 0.05\,h^{-1}$; 52–72.5 h, $\mu_{max} = 0.01\,h^{-1}$; 72.5–97.5 h, $\mu_{max} = -0.007\,h^{-1}$. The methylamine consumption rate on the other hand went through a maximum of 22.6 mmol h^{-1} between 29 and 52 h, followed by a 1.5-fold decrease (14.4 mmol h^{-1}) after 52 h of culture. At the same time was the biomass production per mol of C-source and hour reduced. The reason for this behaviour can only be speculated upon. Sufficient amounts of methylamine hydrochloride were still available even after 52 h of culture. It is possible that at this point the biomass concentration was so high that the C-source was primarily used for other processes than further cell division (e. g., swarmer motility, elongation of the hyphae, rosettes formation).

More importantly, high enzyme production was observed only during the period of high methylamine consumption. This is reasonable as this enzyme is necessary for the organism only for metabolising methylamine. A tight link between enzyme production and methylamine consumption is therefore likely. Managing a process for bioproduction of the formaldehyde dehydrogenase thus cannot be based on biomass data alone, but rather requires a reliable tool for the monitoring of the C-source consumption rate. In this context, the developed assay allowed us to develop and verify an automatic feeding strategy based on the methylamine hydrochloride concentration. It also allows us to precisely calculate, e.g. the biomass yield per mole C-source consumed and the consumption rate during the different growth phases, which further aided rational bioprocess development.

4. Conclusions

The reliable monitoring of low carbon number C-sources in the form of alkylamines is possible using the propyl chloroformate derivatisation chemistry. Using the outlined method an *at line* quantification of the C-source becomes possible with a standard deviation below 10% in the range of 0.1–8 mM of methylamine. With a total assay time of 40 min for drawing, preparing and analysing the sample, the method is fast enough for bioprocess monitoring in the case of slow growing organisms such as *H. zavarzinii* ZV 580.

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