Appl Microbiol Biotechnol (2007) 77:779–788
DOI 10.1007/s00253-007-1218-z

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Development of a fed-batch process for the production of a dye-linked formaldehyde dehydrogenase in *Hyphomicrobium zavarzinii ZV 580*

Valérie Jérôme · Markus Hermann · Frank Hilbrig · Ruth Freitag

Received: 27 April 2007 / Revised: 18 September 2007 / Accepted: 18 September 2007 / Published online: 16 October 2007

Abstract The dye-linked formaldehyde dehydrogenase (dlFalDH) from *Hyphomicrobium zavarzinii ZV 580* processes formaldehyde in a highly selective manner and without need for NAD(P). The enzyme thus has considerable potential for technical applications if the difficulties associated with its efficient production can be resolved. In this contribution, a fed-batch bioprocess is developed, which improves both the biomass production of *H. zavarzinii ZV 580* (from 0.6 to 2 g l^{-1} dry mass) and the specific dlFalDH production (from 0.1 to 0.3 units g^{-1} biomass), resulting in an overall improvement of the productivity by more than an order of magnitude compared to the previously reported process (Klein et al., Biochem J 301:289–295, 1994). In particular, the process uses an automated feeding strategy controlled via the dissolved oxygen concentration. In addition, our results show that the growth of *H. zavarzinii ZV 580* is rather sensitive toward increasing salt concentration in the culture medium. Growth is also inhibited by the presence of surfactant-based antifoam reagents. Adjustment of the pH via the addition of methylamine instead of NaOH, on the other hand, leads to an increase in biomass yield.

Keywords *Hyphomicrobium zavarzinii* · Dye-linked formaldehyde dehydrogenase · Fed-batch culture · Fermentation optimization · Process development

V. Jérôme · M. Hermann · F. Hilbrig · R. Freitag (✉)
Chair for Process Biotechnology, University of Bayreuth, 95440 Bayreuth, Germany
e-mail: bioprozesstechnik@uni-bayreuth.de

Introduction

The Gram-negative bacterial strain *Hyphomicrobium zavarzinii ZV 580* has first been described in 1955 (Zavarzin 1955) and elicited moderate interest since then (e.g., Matzen and Hirsch 1982; Moore and Duxbury 1981; Zavarzin 1960). *H. zavarzinii ZV 580* is a 'slow growing' methylotrophic bacterium, (Hirsch and Conti 1964b) most noted for the facultative expression of a formaldehyde-oxidizing enzyme when grown on certain C1-sources. This enzyme belongs to the family of NAD(P)-independent aldehyde dehydrogenases. It is described as a tetramer, each subunit with a molecular weight of 54,000 carrying a—putatively covalently bound—cofactor. The molecular nature of this cofactor has not yet been elucidated but bears features of a protein-bound quinone (Klein et al. 1994), hence the name 'dye-linked formaldehyde dehydrogenase' (dlFalDH) given to this particular enzyme.

dlFalDH produced by *H. zavarzinii ZV 580* when grown, e.g., on methylamine hydrochloride, differs from other dye-linked aldehyde dehydrogenases of methylotrophic bacteria by its affinity and specificity towards formaldehyde. This, together with the absence of NAD(P) as electron acceptor in the catalytic reaction, makes dlFalDH particular suitable for several technical applications, for example, in the biosensor field, if enough material can be produced in a cost efficient manner. However, to create a viable production process, the biomass and the specific dlFalDH yield of the organism have to be maximized. Expression of the enzyme in recombinant form is not straightforward due to the necessity of the cofactor.

To date, little effort has been made to optimize the cultivation parameters for *H. zavarzinii ZV 580* (Matzen and Hirsch 1982). The only previously established production process was based on a batch mode fermentation

without pH control and led to very low biomass yields (about 0.8 g l^{-1} dry weight, Klein et al. 1994). The situation is made worse by the fact that the cell structure of *Hyphomicrobium* and its method of reproduction are fairly complex (Zavarzin 1961). In particular, the life cycle of *Hyphomicrobium* sp. exhibits three different morphological states (swarmer, hyphal, and rosette) reflecting two processes, the formation of mother cells and the formation of daughter cells, occurring at the same time (Moore and Hirsch 1973a,b, Conti and Hirsch 1965; Zavarzin 1960, Poindexter 1964). Cells in the different stages of development may differ in their needs, rendering process development difficult. In this study, an improved fermentation process for *H. zavarzinii ZV 580* was developed, which is characterized by a more than threefold increase in both biomass and specific enzyme production.

Materials and methods

Materials Chemicals including minerals for the 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 "Institute für Allgemeine Mikrobiologie" (University of Kiel, Germany, accession number IFAM ZV-580, EMBL accession number Y14306).

Strain and 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 ethylenediaminetetraacetic acid (EDTA) and supplemented as indicated with either MeOH or methylamine hydrochloride (MA-HCl) as C1-source. The pH of the medium was adjusted to 7.0 before autoclaving. Bacterial growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}). Optical densities were related to the dry weight via a calibration curve prepared using cells obtained by centrifugation (11,700×*g*, 10 min, 4°C) from fully grown cultures at the end of the exponential phase. For this purpose, the bacteria were twice washed and re-centrifuged, then dried in pre-weighted glass tubes at 80°C to constant weight.

Shake flasks cultivations were done in 250 ml to 2 l Erlenmeyer flasks containing 50 to 400 ml of growth medium. Cultures were inoculated at an OD_{600} of 0.1 and incubated at 30°C and 135 rpm on a rotary shaker (Thermo Life Sciences, Egelsbach, Germany). In the 'fed-batch mode', the pH of the culture was estimated before each feeding and adjusted to pH 7.0 by addition of few drops of 2 M NaOH when necessary. For cultivation in the bioreactor (NLF22 bioreactor, Bioengineering, Wald, Switzerland), the cells were inoculated at an OD_{600} of 0.1 and initial culture volume of 7.0 l. The culture temperature was 30°C. The stirrer speed and the air flow were programmed in cascade with the dissolved oxygen (DO) in such a way that the set point of 30% oxygen saturation was maintained by changing the air flow linearly between 0 and 11.5 l min^{-1} and the stirrer speed between 92 and 200 rpm. While 100% airflow was adjusted for 100% output signal, the maximum stirrer speed was already adjusted for 50% output signal. For higher output signals, a constant stirrer speed of 200 rpm was adjusted. Unless indicated otherwise, a constant pH of 6.9±0.1 was maintained by automatic titration with 2 M NaOH.

Preparation of the cell-free extracts For the preparation of cell-free extracts, the fresh or frozen cells were resuspended in 50 mM Tris/HCl buffer, pH 7.4, containing 1 mg ml^{-1} lysozyme, 0.2 mM phenylmethanesulphonyl fluoride, 2.5 units ml^{-1} DNase, and 3 mM $MgCl_2$, followed by sonication (five times for 1 min) on ice. Cell debris was removed by centrifugation (16,060×*g*, 4°C, 30 min). Ammonium sulfate was added to the supernatant (40% saturation) and proteins that precipitated during a 60-min incubation at 4°C under constant stirring ('40% fraction' or 'P40') were collected by centrifugation (13,000×*g*, 4°C, 40 min). The pellet (representing the P40 fraction) was resuspended in a minimum volume of a 50 mM potassium phosphate buffer pH 8.0 ('P buffer'). The concentration of the ammonium sulfate in the supernatant was increased to 60% saturation, and after a further incubation for 60 min at 4°C, the precipitated fraction ('60% fraction' or 'P60') was collected by centrifugation as above and resuspended in a minimum volume of P buffer. The concentration of ammonium sulfate in the supernatant was increased to 80% saturation, and an '80% fraction' or 'P80' was collected analogously. The samples (P40, P60, and P80) were desalted using a Zeba desalt spin column (Pierce, Bonn, Germany), and the protein concentration in the samples was determined using the micro-BCA protein assay (Pierce) according to the manufacturer's instructions.

Analytical methods To determine the product titers during the production of dlFalDH by *H. zavarzinii ZV 580*, the spectrophotometric enzyme assay for formaldehyde dehydrogenase previously published by Borodina et al. (2000) was scaled down to the microtiter plate format ('redox cycling microassay'). This test relies on a redox reaction converting the substrate (formaldehyde) into formate, thereby releasing two electrons, which are transferred first to the dlFalDH cofactor (unidentified quinone), then to the mediator PMS (phenazine methosulfate), and finally to the

dye DCPIP (dichlorophenolindophenol) that becomes colorless upon reduction, leading to a decrease of the OD_{595}. For the analysis, 200 µg protein was assayed in 80 mM phosphate buffer, pH 7.0, containing 2 mM phenazine methosulfate, 0.2 mM dichlorophenolindophenol, and 10 mM formaldehyde. The assay was performed in flat-bottom microtiter plates (Greiner Bio-one GmbH, Frickenhausen, Germany) using 200 µl reaction mixture (optical path length, 5.9 mm; detection wavelength, 595 nm) with the temperature maintained at 30°C in a Genios Pro plate reader (Tecan, Crailsheim, Germany). The activity was calculated using an extinction coefficient of 21,000 M^{-1} cm^{-1} (Steyn-Parvé and Beinert 1958). One unit of enzyme activity is defined as the amount of enzyme required for reduction of 1 µmol of DCPIP per minute.

To collect additional information on the detected formaldehyde dehydrogenase activity in some of the samples, we also performed a redox assay in native polyacrylamide gel electrophoresis (PAGE; 8% gels, Tris/glycine buffer pH 8.3 according to Gallagher 1995), both in the presence and in the absence of formaldehyde. This procedure has the advantage that the enzymes giving positive results are first separated in the gel. Performing the assay in the presence and the absence of formaldehyde allows distinguishing between enzymes that reduce the redox coloring agent as follow-up to a specific formaldehyde oxidation and those that do not require the presence of formaldehyde for this reaction, i.e., most likely are not true formaldehyde dehydrogenases. In particular, the formaldehyde dehydrogenase (FalDH) activity in the gel was detected according to a protocol modified from Paz et al. (1991), employing nitroblue tetrazolium (NBT) as the redox coloring agent. Briefly, the gels were washed twice at 4°C with P buffer and then sequentially incubated at room temperature with: P buffer containing 50 µM $CaCl_2$ and 1 µM pyrroloquinoline quinone (PQQ) for 40 min ('reconstitution step' making up for potential loss of Ca^{2+} and/or the cofactor during sample processing) and 3.3 mM NBT for 60 min; pure P buffer for 5 min; 0.65 mM phenazine methosulfate (PMS) with or without 15 mM formaldehyde as substrate for 60 min. NBT and PMS stock solution were prepared in P buffer. If indicated, formaldehyde-responsive bands were cut out from the native gel and further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) using 10% gels. SDS-PAGE gels were stained by silver-stain (Koszelak Rosenblum 2002).

MA-HCl concentrations in the culture medium were analyzed by gas chromatography (GC 6890N with FID detector, Agilent, Waldbronn, Germany) using a protocol adapted from a generic protocol for the quantification of amino acids in cell culture supernatants based on the EZ:faast GC/FID free amino acid kit (Phenomenex, Aschaffenburg, Germany), as published elsewhere (Jérôme et al. 2007). Briefly, samples were clarified by centrifugation (13,000×*g*, 4°C, 10 min), and the supernatants were processed, pure or diluted 2- to 25-fold in mineral medium. For calibration and reproducibility studies, culture supernatants were replaced with standard solutions of MA-HCl at concentrations between 0.1 and 12 mM. The calibration curve was linear in the range of 0.1 to 8 mM (RSD≤5%). The analysis was carried out on a 10 m×0.25 mm ZB-AAA fused-silica capillary column (Phenomenex, Aschaffenburg, Germany), with the following temperature program: 40 to 180°C with 12°C min^{-1}, then 180 to 320°C with 30°C min^{-1}, and finally 1 min at 320°C. Helium was the carrier gas (head pressure, 0.45 bar). The ChemStation software version Rev. B.01.03 was applied for computer-assisted data processing.

Results

The aim of the bioprocess strategy described in this publication was to improve the biomass production of *H. zavarzinii ZV 580* cultured in the bioreactor and, in consequence, the overall dlFalDH enzyme production. For this purpose, various aspects were investigated.

Effect of media composition

To identify possible factors affecting the biomass production, series of shake flask cultures were performed. The cultures were inoculated at an OD_{600} of 0.1 in 50 ml mineral medium containing either 0.5% (*v*/*v*) MeOH or 10, 25, and 50 mM MA-HCl as C1-source. Cultures were run as batch or fed batch, in the latter mode, MeOH or MA-HCl

Table 1 Comparison of *Hyphomicrobium zavarzinii ZV 580* biomass obtained in the batch and fed batch mode and as a function of the C-source

C1-source	Batch mode (g l^{-1})	Fed-batch mode (g l^{-1})
MeOH (0.5% *v*/*v*)	1.2	2.2
10 mM MA-HCl	n.d.	1.4
25 mM MA-HCl	0.4	1.5
50 mM MA-HCl	0.6	1.2

Biomass yield (cell dry weight, g l^{-1}) determined after 78 h of culture at 30°C in shake flasks. In the fed-batch mode, the pH of the culture was measured before each feeding and corrected with 2 M NaOH if necessary.

were added again 48 and 72 h post-inoculation (same concentrations), and the pH was adjusted each time to 7.0 by the addition of few drops of 2 M NaOH. Growth rates and biomass production were monitored, Table 1. In the batch mode, cells stopped growing after 28 h (25 mM MA-HCl) or after 45 h [0.5% (*v*/*v*) MeOH; 50 mM MA-HCl]. Assuming that the C1-source has been completely metabolized, a substrate consumption rate of 2.3 mmol g^{-1} h^{-1} can be calculated for MeOH, while this rate is in the range of 2.0–2.4 mmol g^{-1} h^{-1} for MA-HCl. From the results compiled in Table 1, it becomes evident that, by running the culture in the fed-batch mode with pH adjustment, the biomass yield can be increased two- to threefold. A substrate limitation thus seems to be among the reasons for the previously observed low final biomass yields.

This was corroborated by the fact that a feeding strategy designed to further extend the growth phase (Fig. 1, see figure legend for details) further improved the overall biomass production up to a final value of 2.3 g l^{-1}. Concomitantly, the culture could be extended to 100 h. However, the specific growth rate could not be kept constant over the 100 h of cultivation, dropping from a μ_{max} of 0.12 between 10 to 32 h to a μ_{max} of 0.04 between 32 and 56 h, and finally a μ_{max} of 0.009 between 56 and 100 h. Subsequently, we will refer to these three growth phases as early, middle, and late phase.

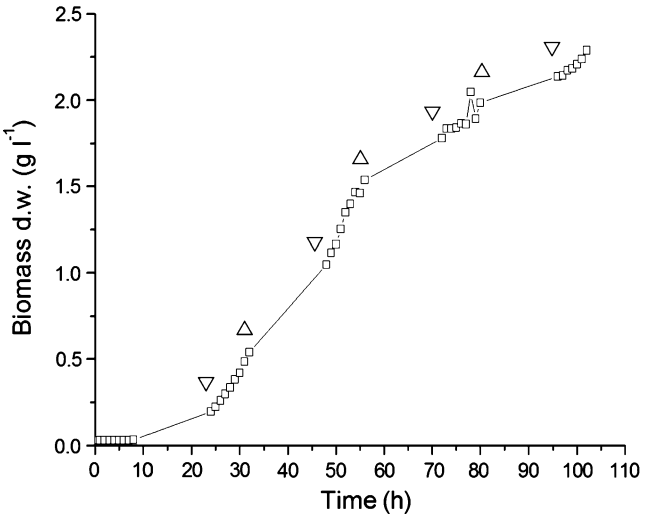

Fig. 1 Culture of *Hyphomicrobium zavarzinii ZV 580* in shake flask. The cells were inoculated at an OD_{600} of 0.1 in 50 ml culture medium containing 10 mM MA-HCl. After 24, 32, 48, 56, 72, 80, and 96 h, the culture was manually supplemented with MA-HCl to a final concentration of 25.5 mM (*triangle*) or 38.6 mM (*inverted triangle*). Before each addition, the pH of the culture was measured and adjusted manually to pH 7.0 with 2 M NaOH, if necessary. 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*: 10 to 32 h, μ_{max}=0.12 h^{-1}; 32 to 56 h, μ_{max}=0.04 h^{-1}; 56 to 100 h, μ_{max}= 0.009 h^{-1}

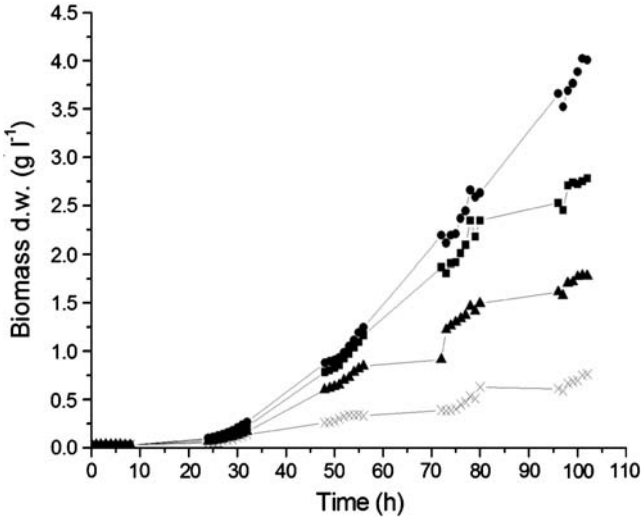

Fig. 2 Inhibition of cellular growth of *Hyphomicrobium zavarzinii ZV 580* by increasing amounts of NaCl. The cells were inoculated (shake flasks) at an OD_{600} of 0.1 in 50 ml culture medium containing 0.5% (*v*/*v*) MeOH as C1-source. The cultures were supplemented with 0 mM (*circle*), 10 mM (*square*), 25 mM (*triangle*), 50 mM (plus symbol) NaCl. After 48, 72, and 96 h, the culture were supplemented with MeOH to a final concentration of 0.5% and 10, 25, or 50 mM NaCl. If necessary, the pH of the culture was adjusted to 7 using 2 M NaOH

Several media components may also exert an inhibitory influence on biomass production. Variations observed when repeating these and other shaker flask cultivations of *H. zavarzinii ZV 580* run in the fed-batch mode suggested that high salt concentrations might inhibit cell growth (data not shown). To investigate the salt tolerance of *H. zavarzinii ZV 580*, we cultivated the cells on MeOH (0.5% *v*/*v*) as C1-source in the fed-batch mode with pH correction and added 10, 25, or 50 mM NaCl to the growth medium at *t*=0 h and also at each feed. For results and details, see Fig. 2. The cells were fed three times leading to final NaCl concentrations in the growth medium ranging from 40 to 250 mM at the end of the culture (*t*=102 h). As displayed in Fig. 2, the upper limit tolerated by the cells under these conditions seems to be 50 mM NaCl. Higher concentrations drastically inhibited cell growth. As a possible means to reduce the salt accumulation in the fermentation broth during the fed-batch culture, methylamine (MA) instead of NaOH was used for pH correction. Two types of shake flasks cultures were done in parallel correcting the pH either with NaOH or with MA. In both cases, the cultures were supplemented four times with MA-HCl as C-source (0.5 mmol per feed), Fig. 3. Even considering the standard deviation of the individual measurement points (*n*=3), pH adjustment with MA led to a significant increase in biomass at the end of the culture compared to a culture where NaOH was used.

Foaming was observed in several of the shake flask cultures and would have made the overnight cultivation in the bioreactor hazardous without proper counter measures.

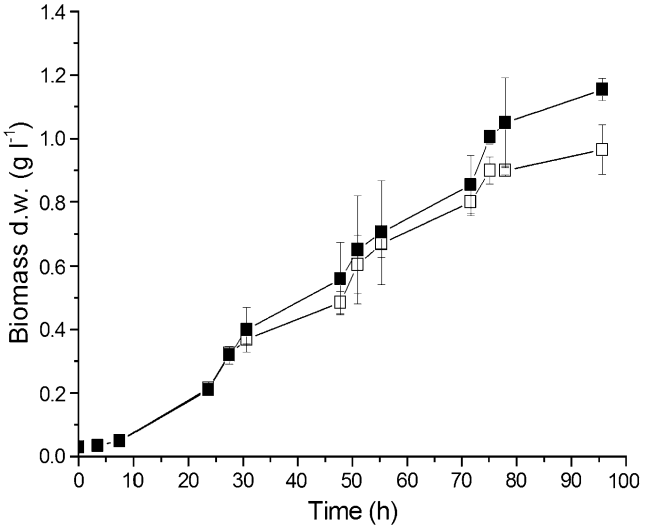

Fig. 3 Influence of the means of pH control (NaOH or methylamine) on the growth of *Hyphomicrobium zavarzinii ZV 580.* The cells were inoculated (shake flasks) at an OD_{600} of 0.1 in 50 ml culture medium containing 10 mM MA-HCL as C1-source. After 24, 32, 48, and 56 h, the cultures were supplemented with MA-HCl to a final concentration of 10 mM. Before each addition of the C1-source, the pH of the culture was measured and corrected with NaOH (*open square*) or MA (*filled square*) if necessary (data represent the mean values of the biomass of three independent experiments)

In preparation for scale-up of the cultivation by transfer to the bioreactor, the effect of two antifoam agents on *H. zavarzinii ZV 580* growth was studied, namely, the non-silicone polyether dispersion Sigma-A204 and the silicone polymer-based antifoam agent Sigma-A. The former was added to the culture from the start, and the latter was added when the cells reached the mid-exponential phase. Cultivations without antifoam addition were performed in parallel; results are compiled in Table 2. According to these results, as little as 0.5% (*v*/*v*) of Sigma-A204 in the growth medium already caused a 47% decrease in the final biomass, while the addition of Sigma-A at concentrations of up to 6 ppm had no significant effect on *H. zavarzinii ZV 580* growth. Increasing the concentration of Sigma-A beyond 6 ppm started to inhibit bacterial growth, reaching a 60% reduction in biomass production for 50 ppm Sigma-A compared to a parallel culture that lacked the antifoam agent.

Table 2 Effect of antifoam on *Hyphomicrobium zavarzinii ZV 580* biomass

Antifoam	Biomass (% decrease)
No addition	0
0.5% (*v*/*v*) Sigma-A204	47
3 ppm Sigma-A	5
6 ppm Sigma-A	0
13 ppm Sigma-A	15
25 ppm Sigma-A	50
50 ppm Sigma-A	60

Biomass (cell dry weight, g l^{-1}) determined after 27 or 100 h of culture in a medium containing the indicated amounts of antifoam Sigma-A204 or Sigma-A.

Fed-batch cultivation of *H. zavarzinii ZV 580* in the bioreactor

To scale up the process, the optimized fed-batch protocol developed for the shake flask was transferred to the bioreactor (starting volume 7 l) keeping all other experimental parameters as similar as possible, although the quantity of MA-HCl added to the culture after 72, 80, and 96 h of growth was somewhat increased (see figure legends for details). The bioreactor results are compiled in Fig. 4 and should be compared directly with those shown in Fig. 1 (corresponding shake flask culture). The three growth phases previously observed (Fig. 1) were also observed in the bioreactor (μ_{max} of 0.11 between 10 to 32 h; μ_{max} of 0.03 between 32 and 56 h; μ_{max} of 0.008 between 56 and 100 h). Moreover, cell growth was identical in both culture vessels over the first 55 h of culture. Afterwards, the two curves slightly diverged from each other, with the bioreactor yielding less biomass than the shake flask, although the specific growth rates were almost identical in the late phase (Fig. 4).

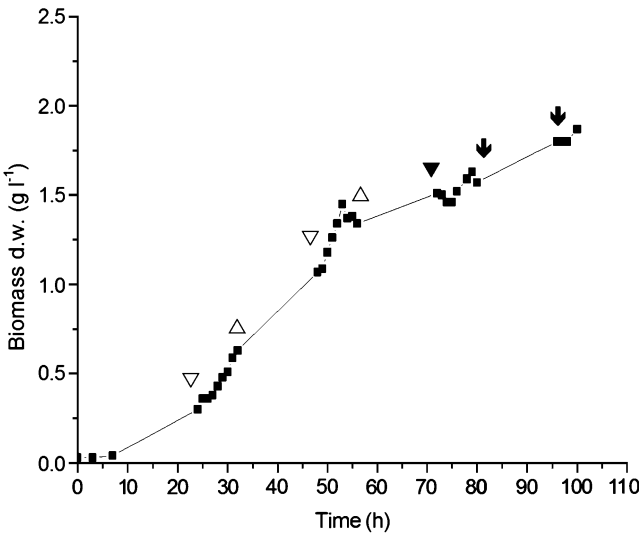

Fig. 4 Cultivation of *Hyphomicrobium zavarzinii ZV 580* in the bioreactor with manual feeding. The cells were inoculated at on OD_{600} of 0.1 in 7 l of culture medium containing 10 mM MA-HCl in a bioreactor. After 24, 32, 48, 56, 72, 80, and 96 h, the culture were manually supplemented with MA-HCl to a final concentration of 25.5 mM (*inverted open triangle*), 38.6 mM (*open triangle*), 55.7 mM (*filled triangle*) or 64.3 mM (*arrow down*). The pH of the culture was maintained constant at pH 7 by automatic titration with 2 M NaOH. 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*: 10 to 32 h: μ_{max}=0.11 h^{-1}, 32 to 56 h: μ_{max}=0.03 h^{-1}, 56 to 100 h: μ_{max}=0.008 h^{-1}

To improve the feeding schedule, additional experiments including the assessment of the MA-HCl concentration in the medium were performed. For this purpose, the substrate (MA-HCl) concentration was analyzed by gas chromatography. In parallel, we attempted to automate the feeding process. After a few bioreactor runs, it became obvious that the dissolved O_2 (DO) briefly jumped above 35% air saturation when the *H. zavarzinii ZV 580* cells stopped growing. As we assumed that the growth arrest reflects a C1-source limitation, we took advantage of this phenomenon to develop an 'on-demand' feeding for the culture in the bioreactor, activating the feeding-pump whenever the pO_2 values were above 35% air saturation. The feed-pump (flow rate, 30.4 ml min^{-1}) was set empirically to automatically run for 0.3 min (between t_0 and t_{40}) and for 0.6 min (between t_{40} and t_{55}) whenever DO≥35%. Data for biomass development and C1-source concentration are presented in Fig. 5. After 45 h of fermentation, we activated the feed pump twice manually for 1 min because the DO values consistently stayed below 35% (see legend to Fig. 5 for details). It can be seen that during the exponential phase the MA-HCl is quickly consumed, and the biomass yield corresponds to 0.029 g (dry weight) per mmol of MA-HCl. After 55 h of culture, the cell growth slowed down, although the C1-source was not depleted from the medium; the methylamine concentration was still above 20 mM. Growth stopped (plateau) after 75 h of culture, although the vitality of the bacteria was nearly 100% in colony forming assay (data not shown) and nutrients were still available. Under this feeding strategy, the biomass yield was 1.5 g l^{-1} demonstrating the potential of such a feeding strategy for the fermentation of *H. zavarzinii ZV 580*, although the fully automated 'on demand' feeding could only be achieved for the first 45 h of culture. Most importantly, this approach allowed us to increase the specific growth rate in the middle phase more than twofold.

Effect of culture condition on dlFalDH yield To determine the product titers during the production of dlFalDH by *H. zavarzinii ZV 580*, the redox cycling microassay was used to analyze the cell free extract (CFE) and fractions P40, P60, and P80 obtained by $(NH_4)_2SO_4$ precipitation. The results compiled in Table 3 show that the enzyme activity in the CFE is relatively weak. More importantly, we obtained positive results even in the absence of the mediator PMS in the assay mixture. The assay is hence not specific for FalDH activity in complex mixtures. In case of the protein fractions, protein fraction P40 showed almost no FalDH activity, while both P60 and P80 showed an enzymatic activity, which was an order of magnitude higher than for

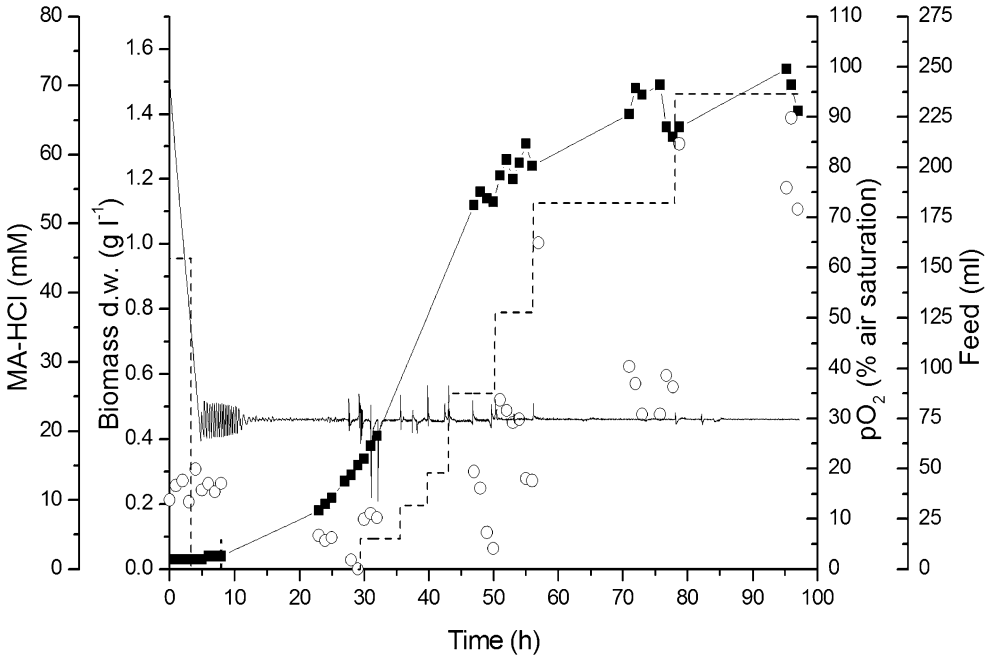

Fig. 5 Cultivation of *Hyphomicrobium zavarzinii ZV 580* in the bioreactor with automatic feeding based on the dissolved oxygen. The cells were inoculated at an OD_{600} of 0.1 in 7 l culture medium containing 10 mM MA-HCl as C1-source. The culture was run in the fed-batch mode, the feed (5 M MA-HCl) pump (flow rate, 30.4 ml min^{-1}) was activated for 0.3 to 1 min whenever the dissolved oxygen value surpassed 35%. The pH of the culture was maintained constant at pH 7 by automatic titration with 2 M NaOH. After 56 and 78 h of culture, the DO increased slightly but did not surpass 35% anymore. Each time, the culture was manually supplemented with 38 and 38.7 mM MA-HCl. The biomass production is displayed by *black squares*, the MA-HCl concentration in the fermentation broth by *white circles*, the dissolved oxygen tension by *solid line*, and the feed by *dashed line*. 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 to 28 h, μ_{max}=0.101 h^{-1}; 28 to 48 h, μ_{max}=0.07 h^{-1}; 48 to 100 h, μ_{max}=0.009 h^{-1}

Table 3 dlFalDH enzymatic activity determined by redox cycling assay in the protein fraction of the cell lysate obtained by ammonium sulfate fractionation

			Pre-incubation with PQQ and CaCl (reconstitution)	
Soluble fraction	− PMS	+ PMS	− PMS	+ PMS
CFE	n.d.	0.041	n.d.	n.d.
P40	0.01	0.00	n.d.	0.00
P60	0.32	0.46	0.18	0.64
P80	0.52	0.48	n.d.	0.48

The enzyme activity is expressed in units ml^{-1} in the redox cycling assay performed with 200 μg of protein.
CFE Cell-free extract

the CFE. While the activity in case of P80 was similar in the presence and the absence of PMS, in P60, the activity was significantly increased in the presence of PMS.

When the redox assay was performed in the native PAGE gels of the corresponding samples, we were not able to see any differences in the band patterns obtained for P60 and P80 in the presence and absence of formaldehyde (data not shown). It has been suggested (Schwartz AC, personal communication) that Ca^{2+} ions are required for the stabilization of the tetrameric structure of the dlFalDH from *H. zavarzinii ZV 580* and for its optimal functionality. The $(NH_4)_2SO_4$ fractionation might have led to a removal of the Ca^{2+} ions (formation of $CaSO_4$). Moreover, as the nature of the cofactor is not known, it cannot be excluded that the cofactor was removed from the protein during precipitation/sample processing. It has been speculated that PQQ might be the bound cofactor in the dlFalDH from *H. zavarzinii ZV 580*. This is the case as for the membrane-associated quinoprotein formaldehyde dehydrogenase from *Methylococcus capsulatus* bath (Zahn et al. 2001). Therefore, all protein fractions were once more investigated in native PAGE after pre-incubation ('reconstitution') for 45 min at 4°C, with 50 μM $CaCl_2$ und 1 μM PQQ. The results are included in Table 3, and the corresponding gels are shown in Fig. 6. Activity is indicated by dark bands. In addition, some lighter bands are observed in the gel. Such "negative" bands are observed for most gel-based redox assays of complex samples. The reason for the occurrence of such bands is at present still unclear; however, they do not present formaldehyde dehydrogenase activity.

Save for P40, two bands are seen in the high molecular weight range of both gels, i.e., in the absence and the presence of the substrate formaldehyde for all fractions and the CFE. This phenomenon is described in the literature as 'nothing dehydrogenase' (Rothe 1994) and is of no further concern. In addition, save for P40, one band is seen only in the presence of the substrate formaldehyde for all fractions and the CFE, indicated by a star in Fig. 6. Two additional bands (marked by arrows in Fig. 6) appear exclusively for fraction P60 in the presence of the substrate formaldehyde. All 'formaldehyde-depending' bands were subsequently cut out from the native gel and analyzed by SDS-PAGE. This analysis revealed (data not shown) that the two bands seen

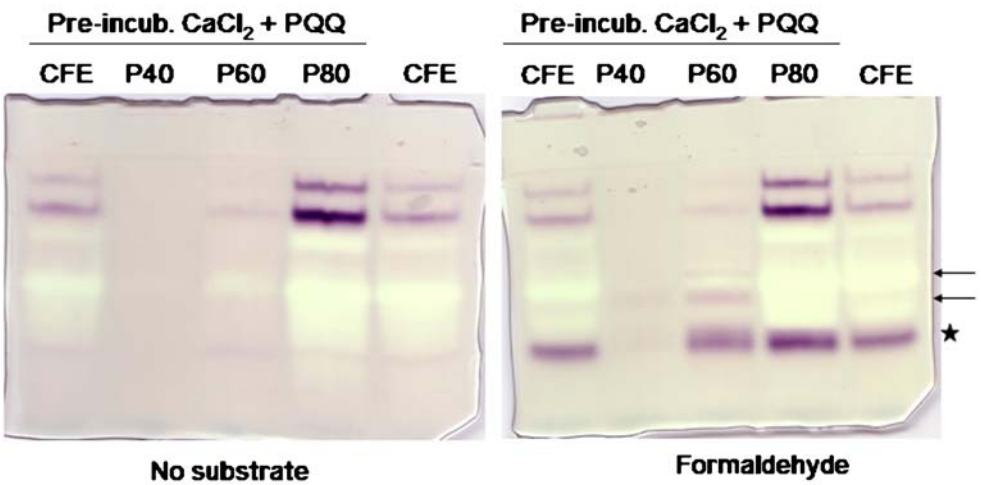

Fig. 6 Assay of dlFalDH activity in a native PAGE gel. Two hundred micrograms of protein/well were loaded on 8% native PAGE at 4°C (10 mA/gel of constant current). After the run, the gel was washed twice with phosphate buffer pH 8.0 before being incubated sequentially in reconstitution solution (50 μM $CaCl_2$, 1 μM PQQ; 40 min), NBT solution (60 min), phosphate buffer pH 8.0, and PMS with or without formaldehyde as substrate (60 min). All solutions were prepared in phosphate buffer pH 8.0. *Dark bands* represent the formazan precipitate, which reflects the desired enzymatic activity

exclusively in fraction P60 (indicated by arrows in Fig. 6) contain proteins or protein subunits with a molecular weight in the range of 45 to 55 kDa, which correlates with the expected size of the dlFalDH monomer (Klein et al. 1994). The lower band seen in all fractions save P40 (indicated by a star in Fig. 6) contains proteins with a molecular weight in the range of 10 to 14 kDa. Most likely, this band contains cytochrome proteins, which are known to react with formaldehyde in redox-cycling assays (Zahn et al. 2001). This result incidentally also demonstrates that the redox cycling assay when performed on non- or only partially purified cell extracts can only give an estimation of the formaldehyde dehydrogenase activity. Unspecific cross-reactivity can never be excluded. Subsequently, the redox cycling microassay (microtitre plate redox assay) was repeated for the reconstituted fractions P60 and P80, Table 3. Of all fractions, only P60 showed an increase in activity measured after reconstitution compared to the activity determined without reconstitution. The data presented above strongly suggest that fraction P60 contains the dlFalDH.

'Volumetric' productivity is an important index for evaluating the efficiency of a bioprocess and can be defined as product produced per unit biomass. The effect of the culture conditions on the dlFalDH production was analyzed by determining the dlFalDH activity in the reconstituted P60 fraction at the end of the fermentation by the redox cycling microassay, and the quantity of enzymatic units present per gram biomass was calculated. As shown in Table 4, even in the shake flasks, the fed-batch mode, in particular in the case of 'fed-batch 2', can almost double the productivity compared to the batch mode. The initial C1-source concentration in the medium and the concentration and time course of the feeding also influence the overall productivity of the cells. A comparison of the fed-batch shake flask with the fed-batch bioreactor data shows that, in spite of the somewhat lower biomass titers, the productivity in the bioreactor was considerably higher. The method used for feeding (manual or automatic) was not of much influence in regard to the dlFalDH production. Most importantly, when the productivity was determined as a function of the fermentation time, in particular after 29, 53, 77, and 97 h of fermentation in the bioreactor (Fig. 5), values obtained after 29 and 53 h (0.27 U g^{-1} for both time points) were in the same order of magnitude as before (Table 4), while somewhat lower values were determined after 77 and 97 h (0.18 and 0.16 U g^{-1}, respectively).

Discussion

H. zavarzinii ZV580 is known to grow on various C1-sources including methanol (MeOH) and methylamine hydrochloride (MA-HCl), albeit typically only to rather low cell densities. The previously published protocol (bioreactor without pH control) for growth on MA-HCl as C-source yielded approximately 0.8 g l^{-1} dry weight at the end of the exponential phase (Klein et al. 1994), which can be considered typical. Such a weak biomass production might be due not only to growth limitation because of nutrient limitation and/or acidification of the fermentation medium by by-products released during the culture, but also to the

Table 4 Comparison of dlFalDH production as a function of the cultivation conditions

Culture mode and C-source feeding strategy	Shake flask	Bioreactor
Batch t_0: 25 mM	0.11 U g^{-1}	n.d.
Fed-batch 1 t_0: 10 mM Feed: $t_{71,\ 93,\ 115,\ 123,\ 139,\ 163}$: 25 mM	0.13 U g^{-1}	n.d.
Fed-batch 2 t_0: 20 mM Feed: $t_{55,\ 72}$: 30 mM; t_{79}: 40 mM	0.19 U g^{-1}	n.d.
Fed-batch 3 t_0: 10 mM Feed: $t_{24,48}$, 25 mM; $t_{32,56}$, 38.5 mM; t_{72}, 55.7 mM; $t_{80,96}$, 64.3 mM	n.d.	0.30 U g^{-1}
Fed-batch 4 t_0: 10 mM Feed: t_{31}, 11.3 mM; t_{54}, 34 mM; t_{66}, 30 mM; t_{79}, 29.6 mM; t_{90}, 29.1 mM	n.d.	0.36 U g^{-1}
Fed-batch 5 t_0: 10 mM Feed: automatic	n.d.	0.31 U g^{-1}

dlFalDH yield at the end of the culture expressed in unit g^{-1} biomass (wet weight). The time course of feeding and quantity of MA-HCl supplemented to the culture are given for each culture conditions.

presence of inhibitory agents. Growth is often somewhat better on methanol, but this substrate does not provide a suitable C1-source for the production of dlFalDH. The enzyme is exclusively produced when the bacteria are grown on methylamine hydrochloride (or methylamine), as previously described by Kohler and Schwartz (1982).

In this contribution, it is shown that, via a suitable fed batch strategy, it is possible to improve the biomass yield of up to 3.5-fold and the specific enzyme production from 0.11 to 0.30 units g^{-1} biomass. A first analysis of shake flask data showed that, via a suitable fed batch strategy, it is possible to double the biomass yield. Independently of the applied feeding strategy, the growth of *H. zavarzinii ZV 580* can be divided in three growth phases (early, middle, and late). The specific growth rate is highest in the early and lowest in the late phase. The reason for the observed decrease in the specific growth rate in the later stages of the cultivation is not straightforward. Sufficient nutrients to sustain growth beyond the observed level were still present. One aspect may be an increase in the salt concentration after several rounds of MA-HCl feeding and pH correction with NaOH. Such a salt sensitivity may even become a critical bottleneck, as it may prevent implementing an exponential feeding strategy in the bioreactor. While some salt accumulation in the fermentation broth is unavoidable in a fed-batch culture, we nevertheless found that using methylamine (MA) instead of NaOH for pH correction had a beneficial effect on biomass production. This approach has the advantage that MA can also be used as C1-source by the *H. zavarzinii ZV 580*, thereby lowering the amount of MA-HCl to be added during feeding concomitant to the pH control.

A second prerequisite for transfer of the dlFalDH production to the bioreactor is the addition of a suitable antifoam agent. As shown in Table 2, between the two agents tested, the cells were very sensitive to agent Sigma-A204, where the addition of 0.5% (*v*/*v*) to the growth medium caused a 47% decrease in the final biomass yield. Such a concentration of the antifoam agent would, on the other hand, most likely not even be sufficient to prevent foaming in the bioreactor, especially towards the end of the cultivation. The reason for this sensitivity of *H. zavarzinii ZV 580* to Sigma-A204 can only be speculated upon. This particular bacterial strain needs to form rosettes to progress along its cell cycle (Zavarzin 1961). Antifoam Sigma-A204 is a surfactant and might disrupt or interfere with this interaction leading to a blockage of the cell cycle and, consequently, to proliferation inhibition. A previous study had shown that Tween 80 prevents rosettes formation (Moore and Marshall 1981). Unfortunately, that study did not provide any data on the effect of Tween 80 on bacterial growth. The bacteria much better tolerated the antifoam agent Sigma-A. However, while Sigma-A can be added to the culture medium, it unfortunately cannot be pumped into the bioreactor on an 'on demand' basis (the best protection against excessive foaming in the bioreactor) because it is not water-soluble.

The three growth phases were also observed in the bioreactor. While the specific growth rates in the three growth phases were similar to those calculated for the fed-batch shaker flask cultures, the final biomass was somewhat lower. The reason for this reduced final biomass in the bioreactor is at present unclear. An experimental error cannot be excluded, although the effect was observed reproducibly. It is also possible that the fermentation parameters and/or feeding strategy were somehow less successful in the bioreactor than in the shake flask or that the controlled environment in the bioreactor led to an improved C1-source uptake/metabolism and thus to an earlier starvation of the bacteria inducing a lag phase (observable as slight reduction in biomass in the bioreactor after 55 h of cultivation, Fig. 4). To avoid the occurrence of such starvation periods, an automatic 'on demand' feeding strategy was developed. While this rendered bioproduction of dlFalDH more convenient, it did, at first glance, not improve the biomass yield of the process, which dropped to 1.5 g l^{-1} (Fig. 5).

Independently of the applied feeding strategy, the growth of *H. zavarzinii ZV 580* seemed to correlate with the methylamine concentration in the culture medium. In this context, the specific growth rates of the early and late phases were not influenced either by the culture vessel (shake flask vs bioreactor) or by the process parameters (manual vs automatic feeding and pH control). However, the 'on-demand' feeding strategy led to a more than twofold increase of $\mu_{\text{middle phase}}$. An explanation for this observation can only be speculated upon. Hyphal bacteria are normally present in culture as heterogeneous population of cells (swarmer, hyphal/budding stages, and rosette-like aggregates); each population might have a different sensitivity to the physico-chemical parameters of the fermentation medium. However, as this genus utilizes C1 carbon compounds as the sole source of carbon and energy, it is questionable if this consumption varied according to the life cycle stages (Hirsch and Conti 1964a,b; Moore 1981). Interestingly, the highest specific growth rate (0.12 h^{-1}) was achieved at low methylamine concentration. Two mechanisms may contribute to this observation. It is possible that, in general, too high a concentration of nutrients results in growth inhibition for this oligotrophic strain, which in nature is used to live at low nutrient levels (Hirsch 1986). On the other hand, the data presented in Fig. 5 demonstrate that the diminution of the growth in the late phase is not related to a C1-source limitation. It might be linked to the depletion of additional limiting nutrients or may be caused by the accumulation of inhibitory metabolites in the

fermentation medium. However, as an increasing salt concentration in the fermentation medium also inhibits cellular growth, an increasing osmomolarity of the medium caused by increased methylamine concentration may also negatively influence the growth rate. This assumption is also supported by the fact that pH correction with methylamine led to a significant increase of the biomass yield.

Data analyzing the kinetics of dlFalDH expression during the fermentation (Fig. 5) suggest again a correlation between the cell growth, the methylamine consumption rate, and the quantity of enzyme produced. In particular, a lower enzyme production rate was observed in the late phase of the cultivation. This suggests a down-regulation of the dlFalDH production in correlation with the absence of MA-HCl consumption. Such a mechanism might reflect the physiological significance of dlFalDH in the formaldehyde metabolism in *H. zavarzinii ZV 580* cultured on C1 compounds. A supplementary set of analysis of the enzyme production at the RNA level with for example RT-PCR or quantitative RT-PCR should clarify this point and allow us to further improve the enzyme production. Based on the results achieved, we conclude that there are two major factors that seem to positively affect dlFalDH productivity: culture in the fed-batch mode in the bioreactor and active methylamine consumption.

Acknowledgements This work was supported by the German Research Foundation (DFG, project number Fr 830/10–1). Thanks to Nancy Nixdorf for the GC analysis. Strain *Hyphomicrobium zavarzinii ZV 580* (Institute für Allgemeine Mikrobiologie, University of Kiel, Germany, accession number IFAM ZV-580, EMBL accession number Y14306) was a generous gift from Dr. Vorholt (INRA/CNRS, Castanet-Tolosan, France).

References

Borodina E, Kelly DP, Rainey FA, Ward-Rainey NL, Wood AP (2000) Dimethylsulfone as a growth substrate for novel methylotrophic species of *Hyphomicrobium* and *Arthrobacter*. Arch Microbiol 173(5–6):425–437

Conti SF, Hirsch P (1965) Biology of budding bacteria.3. Fine structure of *Rhodomicrobium* and *Hyphomicrobium* spp. J Bacteriol 89(2):503–512

Gallagher SR (1995) One-dimensional electrophoresis using non-denaturing conditions. Current Protocols in Protein Science 2:10.3.1–10.3.11

Hirsch P (1986) Microbial life at extremely low nutrient levels. Adv Space Res 6(12):287–298

Hirsch P, Conti SF (1964a) Biology of budding bacteria.1. Enrichment isolation + morphology of *Hyphomicrobium* spp. Archiv Fur Mikrobiologie 48(4):339–357

Hirsch P, Conti SF (1964b) Biology of budding bacteria.2. Growth + nutrition of *Hyphomicrobium* spp. Archiv Fur Mikrobiologie 48 (4):358–367

Jérôme V, Hermann M, Hilbrig F, Freitag R (2007) Fast method for quantitative analysis of methylamine in fermentation broth by gas chromatography. in J Chrom A (in press)

Klein CR, Kesseler FP, Perrei C, Frank J, Duine JA, Schwartz AC (1994) A novel dye-linked formaldehyde dehydrogenase with some properties indicating the presence of a protein-bound redox-active quinone cofactor. Biochem J 301(Pt 1):289–295

Kohler J, Schwartz AC (1982) Oxidation of aromatic-aldehydes and aliphatic secondary alcohols by *Hyphomicrobium* spp. Can J Microbiol 28(1):65–72

Koszelak Rosenblum ME (2002) Cofactor activities of factor VIIIa and A2 subunit following cleavage of A1 subunit at Arg336. J Biol Chem 277:11664–11669

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Matzen N, Hirsch P (1982) Continuous culture and synchronization of *Hyphomicrobium* sp. B-522. Arch Microbiol V132(1):96–99

Moore RL (1981) The biology of *Hyphomicrobium* and other prosthecate, budding bacteria. Ann Rev Microbiol 35:567–594

Moore RL, Hirsch P (1973a) First generation synchrony of isolated *Hyphomicrobium* swarmer populations. J Bacteriol 116(1):418–423

Moore RL, Hirsch P (1973b) Nuclear apparatus of *Hyphomicrobium*. J Bacteriol 116(3):1447–1455

Moore RL, Duxbury T (1981) A microcultural study of the effect of mitomycin-C on *Hyphomicrobium vulgare*. Fems Microbiology Letters 11(2–3):107–109

Moore RL, Marshall KC (1981) Attachment and Rosette formation by hyphomicrobia. Appl Environ Microbiol 42(5):751–757

Paz MA, Fluckiger R, Boak A, Kagan HM, Gallop PM (1991) Specific detection of quinoproteins by redox-cycling staining. J Biol Chem 266:689–692

Poindexter JS (1964) Biological properties and classification of caulobacter group. Bacteriol Rev 28(3):231–295

Rothe GM (1994) Chemistry of enzyme visualization. In: Rothe GM (ed) Electrophoresis of enzymes: laboratory methods. Springer, Berlin, pp 141–179

Steyn-Parv EP, Beinert H (1958) On the mechanism of dehydrogenation of fatty acyl derivatives of coenzyme A. J Biol Chem 233 (4):843–852

Zahn JA, Bergmann DJ, Boyd JM, Kunz RC, DiSpirito AA (2001) Membrane-associated quinoprotein formaldehyde dehydrogenase from *Methylococcus capsulatus* bath. J Bacteriol 183(23):6832–6840

Zavarzin GA (1955) Bacteriology of the surface stratum in natural water supply of the Volga Delta. Tr Latv Padomju Soc Repub Zinat Akad Mikrobiol Inst 4:196–201

Zavarzin GA (1960) The developmental cycle and the nuclear apparatus of *Hyphomicrobium vulgare* Stutz. et Hartleb. Mikrobiologiia 29:38–42

Zavarzin GA (1961) Budding bacteria. Mikrobiologiia 30:952–975