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C. Wittmann · A.-P. Zeng · W.-D. Deckwer

Growth inhibition by ammonia and use of a pH-controlled feeding strategy for the effective cultivation of *Mycobacterium chlorophenolicum*

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Abstract The inhibitory effect of ammonia on the growth of the polychlorinated xenobiotic-degrading bacterium *Mycobacterium chlorophenolicum* was examined. The strain is inhibited by both the ionized and nonionized forms of ammonia. At pH 6.9 50% reduction of the growth rate was observed at 6.8 $g l^{-1}$ total ammonium. For 23 experiments performed in shake-flask culture at different pH values and ammonium concentrations a growth model based on the extended Monod kinetic fits the data with a deviation of 5.3%. To overcome growth inhibition in bioreactors a pH-controlled feeding strategy was developed for effective cultivation of *M. chlorophenolicum* at an ammonium level below 0.3 $g l^{-1}$. The ammonium addition was controlled on-line by the stoichiometric interdependence of ammonium consumption and pH decline. With this on-line control strategy a biomass concentration as high as 26.2 $g l^{-1}$ can be achieved within less than 1 week of cultivation, compared to a biomass concentration of 15.5 $g l^{-1}$ in normal batch culture after 2 weeks of cultivation. The yield is also increased from 0.32 g to 0.43 g biomass (g glucose)$^{-1}$. The strategy developed provides an effective method for the production of biomass of *M. chlorophenolicum* serving as the inoculum in remediation technologies.

Introduction

Interest in the physiology of *Mycobacterium chlorophenolicum*, formerly *Rhodococcus chlorophenolicus* (Häggblom et al. 1994), has been stimulated by the increasing research on well-performing biodegraders for bioremediation of contaminated soils. *M. chlorophenolicum* is known to degrade a variety of polychlorinated aromatic compounds (Apajalahti et al. 1986). Owing to its excellent performance in natural soils *M. chlorophenolicum* has received increasing interest as an inoculant for treating large quantities of contaminated soil (Briglia et al. 1990). *Mycobacteria* belong to the nocardioform actinomycetes that possess a widespread degradation potential towards xenobiotics. Several members of the genus have been found to degrade a variety of toxic substances, which lead to different applications in environmental biotechnology (Häggblom 1992; Hartmans and de Bont 1992; Nohynek et al. 1993). However, the majority of indigenous soil bacteria, including members from the actinomycetes group, are difficult to cultivate under laboratory conditions (van Elsas and van Overbeek 1993). In general, *Mycobacteria* have a much longer generation time than many other bacteria, which is claimed to be one of the major problems in their cultivation and application (Ratledge 1982). *M. chlorophenolicum* shows a generation time of about 13 h and 30 h when cultivated on sorbitol and glucose respectively (Wittmann et al. 1994, 1995). Ammonium can be used as a source of nitrogen by most species of *Mycobacterium* and was reported to be detrimental to growth (Ratledge 1982). However, no quantitative information is available.

This article reports on the influence of ammonium/ammonia on the growth of the pentachlorophenol degrader *M. chlorophenolicum* and introduces a fed-batch strategy for the cultivation of this strain. The aim of the study was the development of cultivation strategies to supply sufficient biomass for contaminated soil remediation. The efficiency of inoculum production (e.g. high biomass yield and concentration) plays an important role in the field of bioremediation, since inoculum size and remediation effect are closely related (Ramadan et al. 1990).

C. Wittmann · A.-P. Zeng (✉) · W.-D. Deckwer
GBF-Gesellschaft für Biotechnologische Forschung mbH,
Biochemical Engineering Division, Mascheroder Weg 1,
D-38124 Braunschweig, Germany.
Fax: 49 531 6181 111

Material and methods

Strain and medium

Mycobacterium chlorophenolicum, formerly *Rhodococcus chlorophenolicus* (Häggblom et al. 1994), was obtained as the type strain *Mycobacterium chlorophenolicum* PCP-1^T (DSM 43826) from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were grown on a mineral medium described previously (Wittmann et al. 1994), which has been modified and optimized for high biomass production (unpublished results). The concentration of glucose that was chosen as sole carbon and energy source was 10 g l^{-1} in shake-flask cultures. In batch and fed-batch cultivations the initial glucose concentration was adjusted in the range 50–75 g l^{-1}. The medium contained (g glucose^{-1}) 60 mg Na_2HPO_4, 30 mg KH_2PO_4, 50 mg yeast extract (Bacto yeast extract, Difco Laboratories), 10 mg $MgSO_4 \cdot 7H_2O$, 700 µg $CaCl_2 \cdot 2H_2O$, 500 µg $FeSO_4 \cdot 7H_2O$, 1 ml trace element solution according to Widdel and Pfennig (1977), 10 mg citrate monohydrate and 20 µg thiamine hydrochloride. For the preparation of the agar plate culture, complex DSM-65 medium (Claus et al. 1983) was used.

Shake-flask culture

Shake-flask cultures were used for the ammonium studies and for preculture of the bioreactor. The pH of the shake-flask culture was established by a 65 mM phosphate buffer. The cultures were incubated at 30° C on a rotary shaker (120 rpm). For the study of the effects of ammonium, cells were grown in 250-ml shake flasks containing 50 ml medium with 10% inoculum. During cultivation the pH and ammonium concentrations were monitored and maintained at the desired value by off-line control. Precultures for bioreactor cultivations were grown from single colonies, cultivated on agar plates, in 1000-ml flasks with 300 ml culture volume; 300 mg $(NH_4)_2SO_4$ g glucose^{-1} was added to the precultures for nitrogen supply.

Batch and fed-batch cultures

A 3.5-l stirred-tank reactor (Set 4, Setric Genie Industriel, Toulouse, France) with a working volume of 3.0 l was used, which was instrumented with a central control unit (MOD 7F, Setric Genie Industriel, Toulouse, France). pH was automatically controlled at 7.00 ± 0.05 by a pH probe (Ingold, Steinbach, Germany), and the addition of 0.33 M H_3PO_4 and 1 M NaOH in batch cultivation and 0.33 M H_3PO_4 and 1 M and 2 M NH_4OH in fed-batch cultivation. Desmophen 3600 (Bayer, Leverkusen, Germany) was used as an antifoam agent at a concentration of 0.1% (v/v). The cultivation temperature was 30.0° C. Dissolved oxygen was measured by an oxygen probe and maintained above 30% by variation of the stirrer speed in the range 500–750 rpm. O_2 consumption and CO_2 production were measured by a paramagnetic oxygen analyzer (Oxygor 6N, Maihak, Hamburg, Germany) and an infrared CO_2 analyzer (Defor, Maihak, Hamburg, Germany) respectively. The gas flow rate was controlled at 600 ml min^{-1} for cultivations up to 50 g l^{-1} glucose and at 1200 ml min^{-1} for cultivations with initial glucose concentrations higher than 50 g l^{-1}, using a thermal gas flow meter (5850 TR, Brooks Instrument, Veenendaal, Netherlands) and a control unit (5876A-2, Brooks Instument, Veenendaal, Netherlands) to achieve sufficient oxygen supply. The bioreactor was inoculated with 300 ml cells (10% of the volume) in the exponential growth phase.

Analytical methods

Glucose and ammonium concentrations were measured with an enzymatic glucose analyser (Yellow Springs Instruments, Ohio, USA) and an ammonia probe (Orion, Cambridge, USA) respectively. For the determination of sugars, organic alcohols and acids an HPLC system, based on ion-exclusion chromatography, was used (HPLC Autosampler 360, Pump system 322, ORH-801 ion-exclusion column, IonGuard GC 801 guard column; Kontron Instruments, Neufarn, Germany). The analysis conditions were 35° C, 5 mM sulfuric acid as eluent at a flow rate of 0.50 ml min^{-1} and refractive index detection (detector ERC-7512, ERMA, Tokyo, Japan). The samples were filtered through a 0.45-µm membrane before injection. Growth was measured by absorbance with a photometer (Ultrospec IIE, Pharmacia, Freiburg, Germany) at 650 nm. Cell dry mass was determined by drying up to constant weight at 40 °C under vacuum. Cells were collected after 30 min centrifugation (Megafuge 1.0R, Heraeus Sepatech, Hanau, Germany) at 5500 rpm twice before drying. After the first centrifugation the pellet was washed with deionized water. The elementary composition (C, H, N) of the cell dry mass was measured with a thermoanalytical analyzer (PE2400 series II CHNS/O analyzer, Perkin Elmer, Norwalk, USA). The average values determined were 0.4760 g C (g biomass)$^{-1}$, 0.0678 g H (g biomass)$^{-1}$ and 0.0729 g N (g biomass)$^{-1}$.

Calculations

The following calculations were used for the evaluation of experimental data. With the ammonia probe the total ammonium, including both ionized and nonionized forms, is determined. The corresponding concentrations of NH_4^+ and NH_3 were calculated by Eqs. 1 and 2. For this purpose the actual pH value and the corresponding dissociation constant in water at 30° C ($k_B = 1.820 \times 10^{-5}$ mol l^{-1}) according to Weast et al. (1990) were used. M_{NH_3} and $M_{NH_4^+}$ are the relative molecular masses of ammonia and ammonium respectively.

$$c_{NH_4^+} = \frac{k_B \cdot c_{NH_4\,measured}}{c_{OH^-} + k_B} \quad (1)$$

$$c_{NH_3} = \frac{c_{OH^-}\ c_{NH_4\,measured}}{c_{OH^-} + k_B} \frac{M_{NH_3}}{M_{NH_4^+}} \quad (2)$$

The oxygen uptake rate (QO_2) and carbon dioxide evolution rate (QCO_2) are calculated from effluent gas analysis. Calculation of the respiratory quotient was based on QO_2 and QCO_2. For mass balances the dilution of culture volume by pH control and its decrease by sampling were considered. The carbon mass balance was based on the consumption of the substrate (glucose) for the formation of biomass and CO_2. The nitrogen balance was calculated to confirm the effectivity of the ammonium feeding. It includes the addition of NH_4OH during pH control.

Results

Inhibition of *M. chlorophenolicum* by ammonium/ammonia

In shake-flask studies growth was followed in the presence of different initial ammonium concentrations in the range 0.25–7.80 g l^{-1} at pH = 6.9. Both $(NH_4)_2SO_4$ and NH_4Cl were used to test whether ammonium is the only inhibiting substance. Samples were taken periodically to measure biomass as absorbance and hence to

calculate the growth rate. The results are shown in Fig. 1. The growth rate linearly decreased with increasing amount of total ammonium present in the culture, irrespective of the ammonium salt applied, indicating that growth inhibition is due to ammonium/ammonia alone. At pH 6.9 the growth rate reduced to 0.012 h^{-1} (50% μ_{max}), if the total ammonium concentration was about 6.8 $g l^{-1}$.

In order to verify further that the inhibition is merely due to NH_4^+ or NH_3 or both of them, experiments were carried out at different pH values. To this end, shake-flask cultures with four different initial concentrations of $(NH_4)_2SO_4$ at pH values 6.0, 6.9 and 7.4 were studied. The resulting growth rates at different cultivation conditions are shown in Fig. 2. Neutral pH appears to be optimal for growth. However, with increasing ammonium concentration the growth rate decreased for all pH values. At pH 6.0 *M. chlorophenolicum* grows better than at alkaline pH 7.4. These results agree well with our previous work with this strain that showed a pH optimum at 7.0 and especially a good tolerance of lower pH values for growth on sorbitol (Wittmann et al. 1995). The acid-fast characteristic has been shown to be a typical feature of the genus *Mycobacterium* (Wayne and Kubica 1986). The decrease of specific growth rate (μ) with increasing concentration of total ammonium seems to be more profound at pH 7.4 than at other pH values.

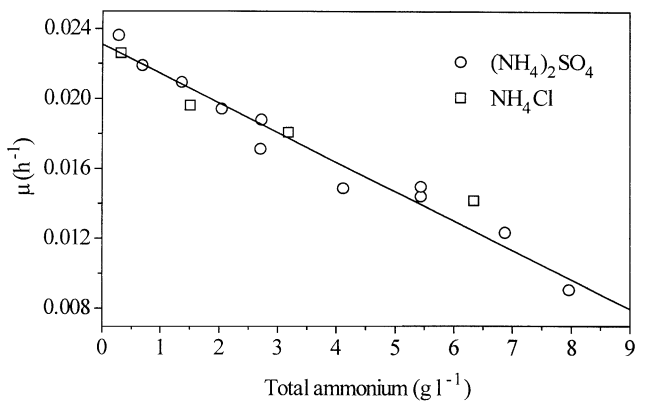

Fig. 1 Influence of total ammonium concentration on growth of *M. chlorophenolicum* at pH 6.9 in shake-flask culture. μ specific growth rate

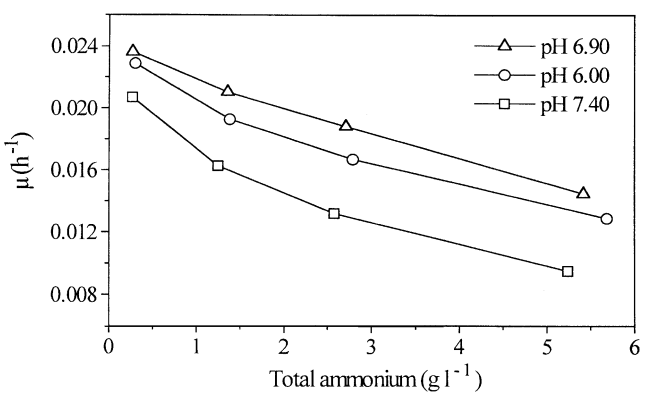

Fig. 2 Influence of pH on ammonium inhibition of *M. chlorophenolicum*

Modelling of ammonium/ammonia inhibition

In the literature the dependence of the growth rate on inhibitory substrates ($c_S = c_I$) is often described by the following extended Monod kinetics (Aiba and Shoda 1969):

$$\frac{\mu}{\mu_{max}} = \left(\frac{c_S}{K_S + c_S}\right)\left(\frac{K_I}{K_I + c_I}\right) \tag{3}$$

where c_S is the substrate concentration, c_I the inhibitor concentration, K_S the saturation constant and K_I an empirical constant having the value of the inhibitor concentration at 50% inhibition. μ/μ_{max} is the relative specific growth rate with $\mu/\mu_{max} = 1$ under conditions of $K_S \ll c_S \ll K_I$ (for $c_S = c_I$). μ_{max} is a function of the pH, which was determined by extrapolating μ to zero ammonium concentration. For microbial assimilation of ammonium the K_S value is about 0.1 mmol l^{-1} (Riesenberg 1988). In all experiments performed the total ammonium concentration was higher than 20 mmol l^{-1}. Hence, the Monod term $c_S/(K_S + c_S)$ can be neglected. This leads to the following inhibition models for ammonium (Eq. 4) and ammonia (Eq. 5):

$$\frac{\mu}{\mu_{max}} = \frac{K_{NH_4^+}}{K_{NH_4^+} + c_{NH_4^+}} \tag{4}$$

$$\frac{\mu}{\mu_{max}} = \frac{K_{NH_3}}{K_{NH_3} + c_{NH_3}} \tag{5}$$

Assuming that growth inhibitions of ammonium and ammonia are noncompetitive, a growth model that considers both NH_4^+ and NH_3 as inhibitors can be written as follows (Eq. 6):

$$\frac{\mu}{\mu_{max}} = \left(\frac{K_{NH_4^+}}{K_{NH_4^+} + c_{NH_4^+}}\right)\left(\frac{K_{NH_3}}{K_{NH_3} + c_{NH_3}}\right) \tag{6}$$

To evaluate the inhibition effects of ammonium and ammonia, experimental data at different pH values and concentrations of both ammonium and ammonia were used. A Nelder-Mead optimization algorithm included in the software Microsoft Excel 4.0 was applied to estimate the parameters of Eqs. 4–6. The results are given in Table 1. For the description of all data the consideration of only a single component as the inhibitory substance shows deviations of 7.4% and 10.8%, respectively. Figure 3 shows a comparison between the calculated and experimental values for μ/μ_{max} according to Eq. 6. The dual-inhibition growth model fits the

Table 1 Inhibition kinetics of *M. chlorophenolicum* by ammonium and ammonia using different models

Inhibition model	$K_{NH_4^+}$ ($g l^{-1}$)	K_{NH_3} ($g l^{-1}$)	Deviation (%)
Eq. 4	6.67	—	7.4
Eq. 5	—	0.03	10.8
Eq. 6	10.00	0.15	5.3

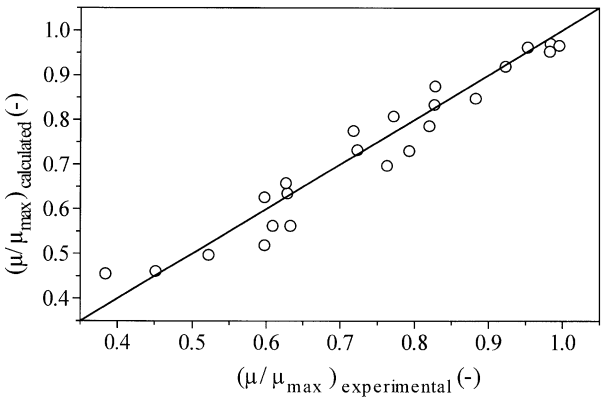

Fig. 3 Comparison of experimental and calculated relative growth rates μ/μ_{max} for *M. chlorophenolicum* at different pH values and concentrations of ammonium/ammonia

experimental data better, with an average deviation of 5.3%. At low pH values with $c_{NH_3} \ll K_{NH_3}$ the ammonia term can be neglected, which simplifies the inhibition kinetics to Eq. 4. At high pH values ammonia inhibition becomes more significant and needs to be considered.

Cultivation of *M. chlorophenolicum* in bioreactor

Conventional batch cultivation

Batch cultivations were carried out in a 3-l stirred bioreactor to obtain further information about the influence of ammonium/ammonia on growth of *M. chlorophenolicum*. $(NH_4)_2SO_4$ was used as ammonium source and the ammonium/glucose ratio was $0.13\ g\,g^{-1}$. Figure 4 shows the results of a batch culture with an initial glucose concentration of $50.8\ g l^{-1}$, an initial concentration of total ammonium of $6.75\ g l^{-1}$ and a pH of 7.0. Cells grow with a constant growth rate of $0.012\ h^{-1}$ until the substrate is completely consumed. Significant growth inhibition is observed, which leads to a 50% reduction of the growth rate during the whole process, as compared to the value of $0.024\ h^{-1}$ determined for low ammonia concentrations at the same pH. The observed inhibition agrees well with shake-flask experiments in which 50% growth rate reduction was found at about $6.8\ g l^{-1}$ ammonium. After 280 h of cultivation, an absorbance of 37.5 (corresponding to $15.5\ g l^{-1}$ cell dry mass) was reached.

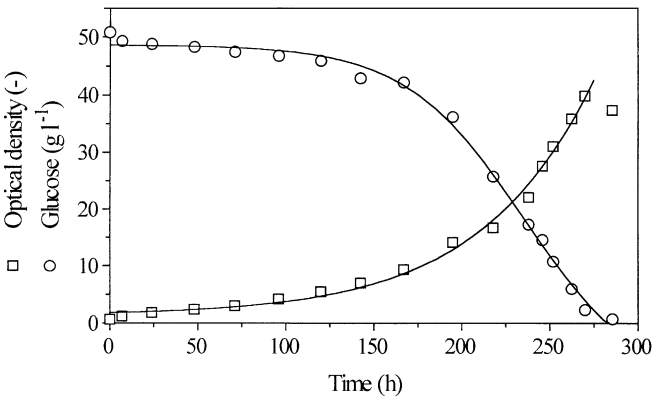

Fig. 4 Growth and substrate consumption of a conventional batch cultivation of *M. chlorophenolicum* with initial concentrations of $50\ g l^{-1}$ glucose and $6.75\ g l^{-1}$ ammonium

During the batch cultivation an alkaline solution was added to maintain the pH at 7.0. In general, the decrease of pH during microbial growth may be caused by the production of acid metabolites, the consumption of basic nutrients or the production of CO_2. The pH will also fall mainly as a result of the utilization of ammonium (Pirt 1975). Concerning the cultivation of *M. chlorophenolicum* the analysis of the culture broth by HPLC shows no organic products. This is also confirmed by the nearly complete carbon mass balance (more than 95%). The addition of hydroxyl ions by the pH control system (169 mmol OH^-) and the consumption of ammonium calculated from the measured cell dry mass and the determined nitrogen content (182 mmol NH_4^+) also shows reasonable correlation in view of the possible data scattering.

Batch cultivation with pH-controlled feeding of ammonium

Obviously the uptake of ammonium/ammonia into the cells is directly linked to the pH decline of the system. This fact was used as a basis for the development of a feeding strategy that supplies sufficient nitrogen for cell growth and at the same time keeps the ammonium concentration at a low level to avoid inhibition. In principle, every proton released during the ammonium uptake acts as an on-line signal for the pH control to add an alkaline solution. By using NH_4OH as the alkaline solution, two functions can be fulfilled simultaneously: the addition of one hydroxyl ion to neutralize the released proton for pH control and the addition of one molecule ammonium to replace the consumed one. The ammonium feeding can be automatically controlled by the pH probe.

The feeding strategy was demonstrated in batch cultivations with relatively high initial glucose concentrations. Results of a culture with $74.5\ g l^{-1}$ glucose are presented in Figs. 5 and 6. In this culture, the initial

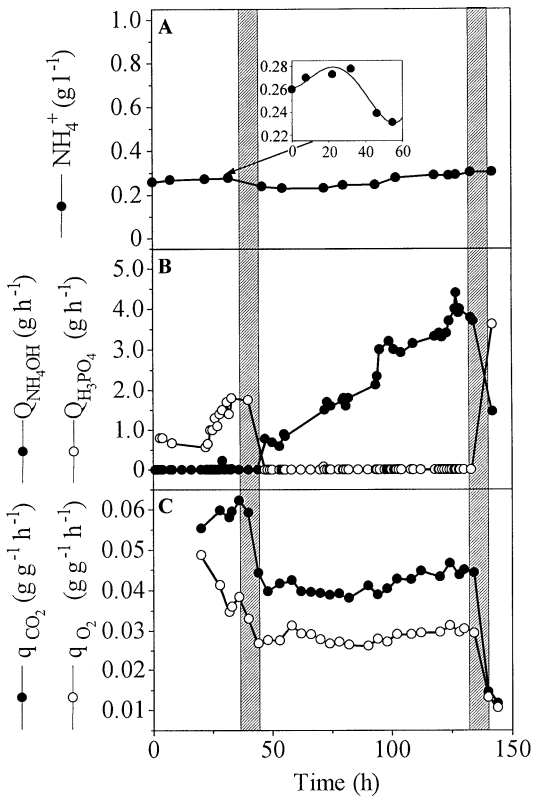

Fig. 5A–C Physiological characteristics of a batch cultivation of *M. chlorophenolicum* with on-line controlled ammonium feeding. **A** Ammonium concentration in culture; **B** acid and alkaline consumption rates; **C** Specific consumption rate of O_2 and specific production rate of CO_2

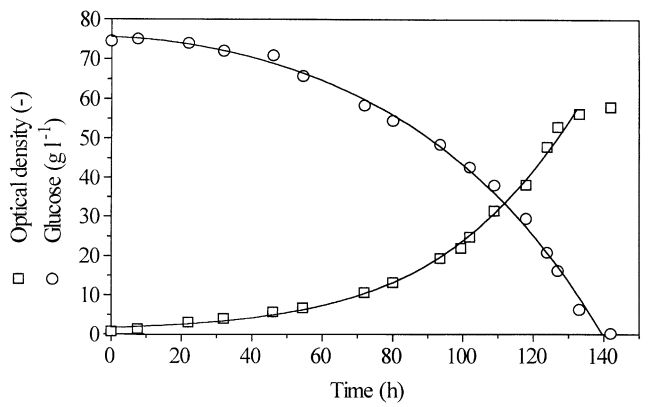

Fig. 6 Growth and substrate consumption of a batch cultivation of *M. chlorophenolicum* with on-line controlled ammonium feeding and initial concentrations of 75 g l^{-1} glucose and 0.26 g l^{-1} ammonium

ammonium concentration was about 0.26 g l^{-1}, which resulted from non-consumed ammonium of the preculture. The whole cultivation can be divided into three phases (see Fig. 5). At the beginning the added yeast extract is consumed, which is underlined by an increase in ammonium concentration due to deamination of amino acids contained in the yeast extract. In this phase acid is consumed to buffer the ammonium increase (phase one). The specific respiration activity of the cells, expressed as qCO_2 and qO_2 is high, because the existing building blocks allow fast biomass formation. The ammonium concentration reaches a temporary maximum value of 0.28 g l^{-1} after about 40 h of cultivation, indicating that the yeast extract is consumed. The consumption of acid stops and the metabolism changes to using only NH_4^+ as N source. At this point the release of H^+ triggers the ammonium feeding (phase two). A slight increase of ammonium up to a final concentration of 0.30 g l^{-1} was observed. It should be mentioned that the net increase of ammonium was only 0.04 g l^{-1}. In other experiments with low starting values of ammonium an ammonium level as low as 0.1 g l^{-1} was obtained.

Table 2 Experimental results of conventional and ammonium-fed batch cultivation of *M. chlorophenolicum*. $Y_{x/Glc}$ biomass yield on glucose, X amount of biomass, μ specific growth rate

Cultivation	Conventional batch	Ammonium-fed batch
c^i_{Glc} (g l^{-1})	50.8	50.3
$c^i_{NH_4^+}$ (g l^{-1})	6.80	0.30
$C_{recovery}$ (%)	95.1	95.6
$N_{recovery}$ (%)	—	101.0
μ (h^{-1})	0.012	0.024
$Y_{x/Glc}$ (g g^{-1})	0.32	0.43
NH_4^+X (%)	—	94.4
X (g l^{-1})	15.51	18.38
Productivity (g l^{-1} h^{-1})	0.054	0.129

The specific respiration rates qCO_2 and qO_2 remain relatively constant untill the total glucose is consumed at 140 h of cultivation time (Fig. 5). The calculated average respiratory quotient in the exponential growth phase, 1.06 mol mol^{-1}, agrees well with the theoretical value of 1.07–1.08 mol mol^{-1} for the purely oxidative metabolization of glucose in yeasts (Sonnleitner and Käppeli 1986). After the substrate is completely consumed, cells change to endogenous metabolism and start to lyse (phase three). Respiration activity sharply decreases, while the respiration quotient changes to 0.8, which is expected for the degradation of proteins. This degradation is linked to the release of ammonium and acid consumption. Cells grew with a specific growth rate between 0.024–0.027 h^{-1} over the whole process indicating no significant inhibition by ammonium. After 140 h of cultivation, an absorbance of 58.0 (corresponding to 26.2 g l^{-1} cell dry mass) was reached (Fig. 6).

Comparison of conventional and ammonium-fed batch cultures

Table 2 shows stoichiometric data of a conventional batch and a batch culture with ammonium feeding with

M. chlorophenolicum at an initial glucose concentration of about 50 $g l^{-1}$. The C and N recoveries have values between 95.1% and 101.0% thus confirming the consistency of the experimental data. In conventional batch mode, bacteria grew only with 50% of the growth rate achieved with on-line feeding of ammonium. Owing to this growth inhibition the biomass yield on glucose was also reduced in the presence of a high ammonium concentration, the observed value of 0.32 $g g^{-1}$ being only 75% of that achieved with ammonium feeding. Cells have to spend energy to overcome the toxic conditions. In the culture with ammonium feeding 94.4% of the added ammonium was consumed for biomass formation. The remaining 5.6% of the added ammonium resulted partly from acidification caused by the increase of carbon dioxide concentration in the gas phase during cultivation. Quantitative estimation of this effect can be made by considering the absorption and subsequent dissociation of CO_2 to carbonate and bicarbonate (Zeng 1995). In this way about 65% of the observed increase in ammonium concentration can be accounted for. With the developed feeding strategy the productivity of the process could be increased by 240% compared to the batch mode, giving higher cell dry mass in half the time. This increase of productivity will be more significant at a higher initial glucose concentration. The final biomass concentration will, of course, increase further as well. For example, in the cultivation with an initial glucose concentration of 74.5 $g l^{-1}$, including ammonium feeding, a productivity of 0.184 $g l^{-1} h^{-1}$ was achieved (Fig. 6).

Discussion

The growth of *M. chlorophenolicum* was shown to be strongly inhibited by ammonium. At high pH the inhibition of ammonia was also significant. These inhibition effects can be described by an extended Monod kinetics. The calculated values of $K_{NH_3} = 0.15\ g l^{-1}$ and $K_{NH_4^+} = 10.0\ g l^{-1}$ indicate that ammonia is more toxic than ammonium, which is known for a number of microorganisms (Bajpai and Ianotti 1988). Growth inhibition by ammonium/ammonia has been reported for a number of microorganisms and mammalian cells (Ratledge 1982; Bajpai and Ianotti 1988; Newland et al. 1994). Several mechanisms for ammonium inhibition have been proposed such as the change of intracellular pH value, the increase of maintenance energy requirement, or the inhibition of specific enzymatic reactions. For example, Prasada-Reddy and Venkitasubramanian (1975) observed an inhibitory effect of ammonium on the activity of enzymes of the citric acid cycle in several *Mycobacteria* strains. It remains to be answered which mechanism is mainly responsible for the observed growth inhibition of *M. chlorophenolicum*. Whatever the mechanism is, for the development of an effective cultivation strategy it appears to be essential to keep the ammonium level low. From the viewpoint of bioremediation this ammonium sensitivity should also be considered when using ammonium-containing fertilizers for the enhancement of degradation activity. To overcome the problem of ammonium inhibition during cultivation of microorganisms several strategies are possible such as the use of alternative nitrogen sources and feeding cultivations. *M. chlorophenolicum* is able to assimilate ammonium and nitrate as nitrogen sources. In the literature, media for the cultivation of this strain are described that are based on these compounds (Sundman 1964; Wittmann et al. 1994). Ammonium is directly integrated into the biomass without any preceding modifications. In comparison, the assimilation of nitrate is linked to the consumption of reduction power, which leads to lower biomass yield compared to ammonium (Babel 1988). Growth of *M. chlorophenolicum* in the presence of urea as the sole source of nitrogen is linked to an increase in ammonium concentration and in pH as a result of the enzymatic cleavage of urea during its microbial consumption. In this work a strategy of pH-controlled feeding of ammonium was developed, which is based on the stoichiometry of ammonium consumption and release of protons in the culture. Using this strategy the ammonium level can be kept in the range of 0.1–0.3 $g l^{-1}$. This results in significantly higher growth rate, higher biomass yield and higher biomass concentration up to 30 $g l^{-1}$, compared to conventional batch cultivation. The strategy developed provides an effective method for the production of large amounts of biomass of *M. chlorophenolicum* for soil remediation. It should be possible to perform continuous cultivation of the strain with ammonium feeding in the form of an auxostat maintaining a constant ammonium level.

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References

Aiba S, Shoda M (1969) Reassessment of product inhibition in alcohol fermentation. J Ferment Technol 47:790–794

Apajalahti JHA, Kärpänoja P, Salkinoja-Salonen MS (1986) *Rhodococcus chlorophenolicus* sp. nov., a chlorophenol-mineralizing actinomycete. Int J Syst Bacteriol 36:246–251

Babel W (1988) Effizienz und Geschwindigkeit von Wachstum und Vermehrung. In: Hecker M, Babel W (eds) Physiologie der Mikroorganismen. Fischer, Stuttgart New York, pp 72–90

Bajpai RK, Ianotti EL (1988) Product inhibition. In: Erickson LE, Fung YC (eds) Handbook on anaerobic fermentation. Dekker, New York, pp 207–241

Briglia M, Nurmiaho E, Vallini G, Salkinoja-Salonen MS (1990) The survival of the pentachlorophenol-degrading *Rhodococcus chlorophenolicus* PCP-1 and *Flavobacterium* sp. in soil. Biodegradation 1:273–281

Claus D, Lack P, Neu B (1983) Deutsche Sammlung von Mikroorganismen. Catalogue of strains, 3rd edn. Deutsche Sammlung von Mikrrorganismen, Braunschweig, Germany

Elsas JD van, Overbeek LS van (1993) Bacterial responses to soil stimuli. In: Kjelleberg S (ed) Starvation of bacteria. Plenum, New York, pp 55–79

Häggblom MM (1992) Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol Rev 103:29–72

Häggblom MM, Nohynek LJ, Palleroni N, Kronquist K, Nurmiaho-Lassila EL, Salkinoja-Salonen MS, Klatte S, Kropenstedt RM (1994) Transfer of polychlorophenol-degrading *Rhodococcus chlorophenolicus* (Apajalahti et al. 1986) to the genus *Mycobacterium* as *Mycobacterium chlorophenolicum* comb. nov. Int J Syst Bacteriol 44:485–493

Hartmans S, Bont JAM de (1992) The genus *Mycobacterium* – nonmedical. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KM (eds) The prokaryotes, vol 2, 2nd edn. Springer, New York Berlin Heidelberg, pp 1214–1237

Newland M, Kamal MN, Greenfield PF, Nielsen LK (1994) Ammonia inhibition of hybridomas propagated in batch, fed-batch, and continuous culture. Biotechnol Bioeng 43:434–438

Nohynek LJ, Häggblom MM, Palleroni NJ, Kronquist K, Nurmiaho-Lassila EL, Salkinoja-Salonen MS (1993) Characterization of a *Mycobacterium fortuitum* strain degrading polychlorinated phenolic compounds. Syst Appl Microbiol 16:126–134

Pirt SJ (1975) Principles of microbe and cell cultivation. Blackwell, Oxford London Edinburgh

Prasada Reddy TL, Venkitatasubramanian TA (1975) Metabolism of *Mycobacteria*. J Sci Ind Res 34:319–328

Ramadan MA, El-Tayeb OM, Alexander M (1990) Inoculum size as a factor limiting success of inoculation for bioremediation. Appl Environ Microbiol 56:1392–1396

Ratledge C (1982) Nutrition, growth and metabolism. In: Rathledge C, Stanford J (eds) The biology of the mycobacteria, vol 1. Physiology, identification and classification. Academic Press, London, pp 185–271

Riesenberg D (1988) Die Regulation der Stickstoffassimilation. In: Hecker M, Babel W (eds) Physiologie der Mikroorganismen. Fischer Stuttgart New York, pp 156–166

Sonnleitner B, Käppeli O (1986) Growth of *Saccharomyces cerevisiae* is controlled by its limited respiratory capacity: verification of a hypothesis. Biotechol Bioeng 28:927–937

Sundman V (1964) A description of some lignanolytic soil bacteria and their ability to oxidize simple phenolic compounds. J Gen Microbiol 36:171–183

Wayne LG, Kubica GP (1986) Genus *Mycobacterium*. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. Williams & Wilkins, Baltimore, pp 1436–1457

Weast RC, Lide DR, Astle MJ, Beyer WH (eds) (1990) Handbook of chemistry and physics, 70th edn, CRC Press, Boca Raton, Fla, p D-165

Widdel GS, Pfennig N (1977) A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, *Desulfotomaculum acetoxidans*. Arch Microbiol 112:119–122

Wittmann C, Zeng AP, Deckwer WD (1994) Minimal nutrient requirement for growth of *Mycobacterium chlorophenolicum*. Biotechnol Lett 16:1103–1106

Wittmann C, Zeng AP, Deckwer WD (1995) Physiological and kinetic studies on growth of *Mycobacterium chlorophenolicum* for pentachlorophenol degradation. Poster paper (JEP-165), Abstract Book, Volume 4, 7th European Congress on Biotechnology, Feb. 19–23, 1995, Nice, France.

Zeng AP (1995) Effect of CO_2 absorption on the measurement of CO_2 evolution rate in aerobic and anaerobic continuous cultures. Appl Microbiol Biotechnol 42:688–691