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Physiological characterization and cultivation strategies of the pentachlorophenol-degrading bacteria *Sphingomonas chlorophenolica* RA2 and *Mycobacterium chlorophenolicum* PCP-1

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The physiological characteristics of growth and pentachlorophenol degradation of the bacteria *Sphingomonas chlorophenolica* RA2 and *Mycobacterium chlorophenolicum* PCP-1 were studied quantitatively in liquid culture under various conditions of pH, temperature, pO_2, pCO_2 and PCP concentration. Concerning their metabolic properties, RA2 and PCP-1 can be regarded as r-strategist and K-strategist, respectively. RA2 showed a higher activity concerning growth and PCP degradation than PCP-1 under optimum conditions. However, PCP-1 performed better under extreme conditions. Maximum growth rates or RA2 and PCP-1 on glucose were 0.21 h^{-1} and 0.024 h^{-1} and maximum PCP degradation rates 315 and 40 μmol (g of dry cells)$^{-1}$ h^{-1}, respectively. Optimized cultivation for RA2 on a technical scale led to the production of 40 g L^{-1} of cell dry mass within 55 h. The cultivation strategy including pH-controlled ammonium feeding can be used to effectively produce sufficient biomass of both strains for both research and application as inoculants in soil clean-up.

Keywords: pentachlorophenol; biodegradation; growth physiology; cultivation; *Mycobacterium chlorophenolicum* PCP-1; *Sphingomonas chlorophenolica* RA2

Introduction

The extensive use of pentachlorophenol (PCP) in the past has caused serious pollution of the environment and initiated intensive research on PCP-degrading microorganisms. During recent years, a number of bacteria have been isolated from the environment for their ability to degrade PCP [2,8,19,21–23,24]. Recent taxonomic studies revealed that bacterial PCP degraders mainly belong to the genera *Mycobacterium* [13] and *Sphingomonas* [7,17]. The degradation biochemistry of PCP-mineralizing bacteria has been studied in great detail and was reviewed recently by Fetzner and Lingens [10] and Orser and Lange [18]. In addition, a number of studies were carried out concerning the application of degrader strains to soil remediation as reviewed by Häggblom and Valo [13] and McAllister *et al* [16]. However, present knowledge of the physiological characteristics and optimum growth conditions for *S. chlorophenolica* RA2 and especially *M. chlorophenolicum* PCP-1 is rather limited, despite the importance of producing their biomass in large quantity, one of the key parameters for effective application of degrader strains in large-scale bioremediation. Moreover, understanding the impact of environmental conditions on growth and PCP degradation of RA2 and PCP-1 may contribute to their successful use for remediation of contaminated waters and soils.

In the present work, the growth and pentachlorophenol degradation of *S. chlorophenolica* RA2 and *M. chlorophenolicum* PCP-1 were characterized in liquid culture and compared under various cultivation conditions. Optimum conditions for growth and PCP degradation were identified and used for effective cultivation of the strains.

Materials and methods

Strains

Mycobacterium chlorophenolicum PCP-1^T (DSM 43826) and *Sphingomonas chlorophenolica* RA2 (DSM 8671) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Media and substrates

S. chlorophenolica RA2 was grown on a complex medium (TSB medium) containing 15 g L^{-1} of tryptic soy broth (Difco Laboratories, Detroit, USA), a mineral salt medium (RA2 medium) as reported [19] and an enriched RA2 medium containing per g of carbon source: 300 mg of $(NH_4)_2SO_4$, 60 mg of Na_2HPO_4, 30 mg of KH_2PO_4, 10 mg of $MgSO_4 \cdot 7H_2O$, 700 μg of $CaCl_2 \cdot 2H_2O$, 500 μg of $FeSO_4 \cdot 7H_2O$, 1 ml of trace element solution according to Widdel and Pfennig [25] and 10 mg of citrate monohydrate. *M. chlorophenolicum* PCP-1 was grown as described earlier [26]. Glucose was used as carbon and energy source in all experiments, unless otherwise stated. In shake flask cultures, the pH was established with 65 mM phosphate buffer.

Culture conditions

The shake flask studies were carried out on a rotary shaker (120 rpm) with 10 g L^{-1} of carbon source as initial substrate concentration. To study the influences of temperature and

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pH on growth of PCP-1, a reactor system with six parallel vessels of 300-ml working volume was used (Sixfors Multiple Fermenter System, Infors, Bottmingen, Switzerland). The influence of O_2 and CO_2 was studied in a 3-L bioreactor system (SGI, Toulouse, France) at pH 7.0 and 30°C. Gas flow rate was controlled at 1.2 L min^{-1} for PCP-1 and 1.5 L min^{-1} for RA2 using thermal gas flow meters (5850 TR, Brooks Instrument, Veenendaal, The Netherlands). Gas mixtures for defined pO_2 and pCO_2 values were adjusted by a control unit (5876A-2, Brooks Instrument). Technical scale cultivation was performed using a 200-L stirred tank reactor (Type 200, B Braun and Diessel Biotech, Melsungen, Germany) and a process control system (μXL, Yokagawa, Japan) at an aeration rate of 60 L min^{-1}, pH 7.0 (controlled by 15 N NH_4OH) and 30°C. Enriched RA2 medium with 50 g L^{-1} glucose and a linear glucose feeding after the batch phase, adding 6.5 kg of glucose with a feed rate of 1.6 kg h^{-1}, were used. Cultures of PCP-1 with an optical density of 2 were preinduced for degradation studies by consecutively adding PCP in increased concentrations (10, 20 and 30 μmol L^{-1}, respectively). The cultures of RA2 were preinduced twice with 100 and 200 μmol L^{-1} starting at a cell concentration yielding 0.5 OD units. Each subsequent dose of PCP was added during the induction after about 70–90% of the previously added PCP had been degraded.

Analytical methods

The analysis of carbohydrates, organic alcohols and acids, ammonium, optical density, biomass concentration, composition of the biomass, contents of O_2 and CO_2 in the exhaust gas and dissolved oxygen, was performed as reported elsewhere [26]. PCP was determined by gas chromatography (strain PCP-1) as described by Brandt *et al* [4] or by optical density measurement at 320 nm (strain RA2). A ratio of 1.2 $\times 10^9$ cells per mg of cell dry mass was measured for RA2.

Results

Influence of nitrogen source

The growth of RA2 on TSB medium was limited, likely due to an increase of pH from 7.3 to 8.4 and the accumulation of NH_3 from 0.3 to 100 mg L^{-1} during cultivation. With the RA2 medium, a final biomass concentration of only 0.65 g L^{-1} was achieved, probably due to an insufficient supply of nutrients like nitrogen and sulphur. Therefore, RA2 was further cultivated on an enriched RA2 medium providing sufficient amounts of all nutrients. As previously found for PCP-1, ammonium plays a key role for the growth of RA2, which was significantly inhibited by ammonium. At pH 7, a concentration of 4 g L^{-1} NH_4^+ caused a 50% reduction of the growth rate. Also, PCP degradation was inhibited significantly by ammonium (data not shown). To overcome these effects, a strategy of pH-controlled ammonia feeding was used, which was previously developed for PCP-1 [26] and which allowed effective cultivation at low ammonia concentrations. Urea was not used as an alternative nitrogen source, since RA2 did not show urease activity. In ammonia-fed cultivations of RA2 at ammonium concentrations in the range of 0.2–0.3 g L^{-1}, 13.1 g L^{-1}, biomass with a composition of 0.48 (g C) g^{-1} and 0.11 (g N) g^{-1} were produced from 29.8 g L^{-1} of glucose as sole carbon and energy source, the maximal specific growth rate being 0.19 h^{-1}. The biomass yield was calculated as 0.49 g (g glucose)$^{-1}$ and the respiratory quotient (RQ) as 0.99 mol mol^{-1}. The recoveries of carbon and nitrogen in the mass balance were 96.4% and 99.4%, respectively. Pyruvate accumulated up to 6.1 mmol L^{-1} in the late exponential growth phase. Of the added ammonium 91.4% was consumed by the cells, indicating a high efficiency of the applied feeding strategy.

Influence of PCP

To evaluate the effect of PCP concentration on growth and degradation, experiments were conducted with preinduced cells by varying the initial concentration of PCP. Growth of and PCP degradation by *S. chlorophenolica* RA2 started immediately after the inoculation without a lag phase. Strain RA2 was capable of degrading PCP up to 850 μmol L^{-1} showing a maximum q_{pcp} at 200 μmol L^{-1} PCP (Figure 1). The q_{PCP} decreased towards higher PCP concentrations; also μ decreased linearly with increasing PCP concentrations. In comparison, the rates of growth and PCP degradation of *M. chlorophenolicum* PCP-1 were much lower and more sensitive to PCP than those of RA2 (Figure 2). The maximum q_{pcp} (about 40 μmol g^{-1} h^{-1}) of PCP-1 was only one eighth of that of RA2. Significant inhibitions of growth and PCP degradation were observed at concentrations as low as 30 μmol of PCP L^{-1}. The inhibition of growth was more profound than the inhibition of PCP degradation. Whereas PCP-1 could still degrade PCP at the highest concentration studied (120 μmol L^{-1}), very limited growth was observed under these conditions. From the experimental data the concentration of PCP causing 50% reduction of μ_{max} was estimated as 30 μmol L^{-1} for PCP-1, compared to 800 μmol L^{-1} for RA2. Inhibition models of Haldane [3], Yano [28] and Aiba [1] were tested for describing the inhibition of PCP degradation activity of both organisms. A modified form of the Yano model (Eqn 1) described the data well for both organisms, while the other models resulted in unrealistic values for the maximum

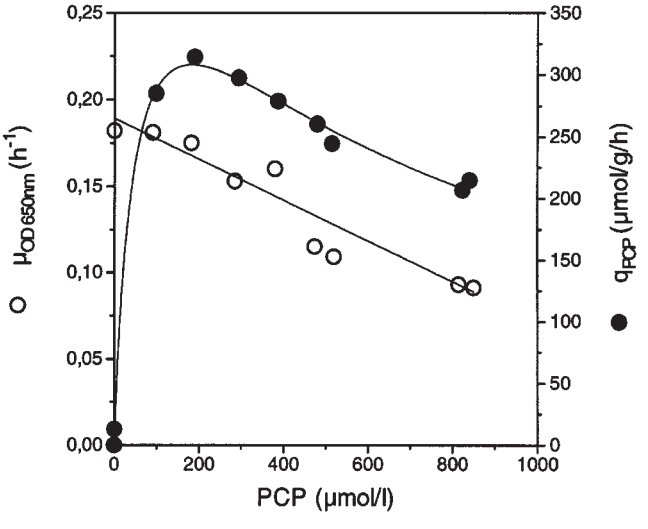

Figure 1 Effect of PCP on growth and specific PCP degradation rate of *S. chlorophenolica* RA2.

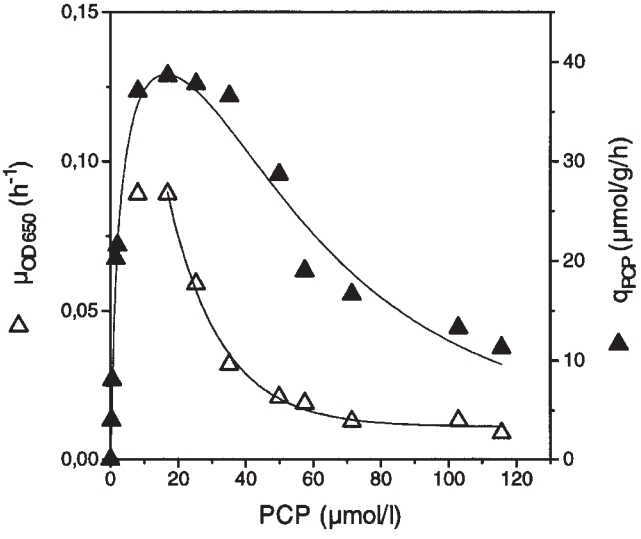

Figure 2 Effect of PCP on growth and specific PCP degradation rate of *M. chlorophenolicum* PCP-1.

degradation rate (q_{max}) (up to about 200% of deviation from experimental values).

$$q_{PCP} = \frac{q_{max} \cdot [PCP]}{K_s + [PCP] + \frac{[PCP]^3}{K_I^2}} \quad (1)$$

The estimated parameters K_s, K_I and q_{max} are summarized in Table 1. The PCP tolerance (K_I) and maximum degradation activity (q_{max}) of RA2 were much higher compared to those of PCP-1. On the other hand, strain PCP-1 showed a higher substrate affinity (K_s).

Influence of temperature and pH

Both strains are mesophilic and exhibited optimum growth temperatures of 33°C for RA2 (μ = 0.21 h^{-1} on glucose) and 30°C for PCP-1 (μ = 0.07 h^{-1} on sorbitol) (Figure 3a and b). Whereas PCP-1 grew up to 45°C, complete growth inhibition of RA2 occurred at 36°C. The PCP degradation by RA2 was similarly influenced by temperature. A maximal rate of PCP degradation, ie 315 μmol g^{-1} h^{-1}, was obtained at about 30°C and a rapid loss of activity occurred at higher temperatures. In contrast, PCP-1 was able to degrade PCP over the whole temperature range tested. An increase of temperature led to an exponential increase of the degradation rate. The highest activity (67 μmol g^{-1} h^{-1}) was found at 41°C, 10°C above the optimum temperature for growth. The pH optimum curves for RA2 and PCP-1 exhibited maxima at pH 7.0 (Figure 4a and b). For RA2,

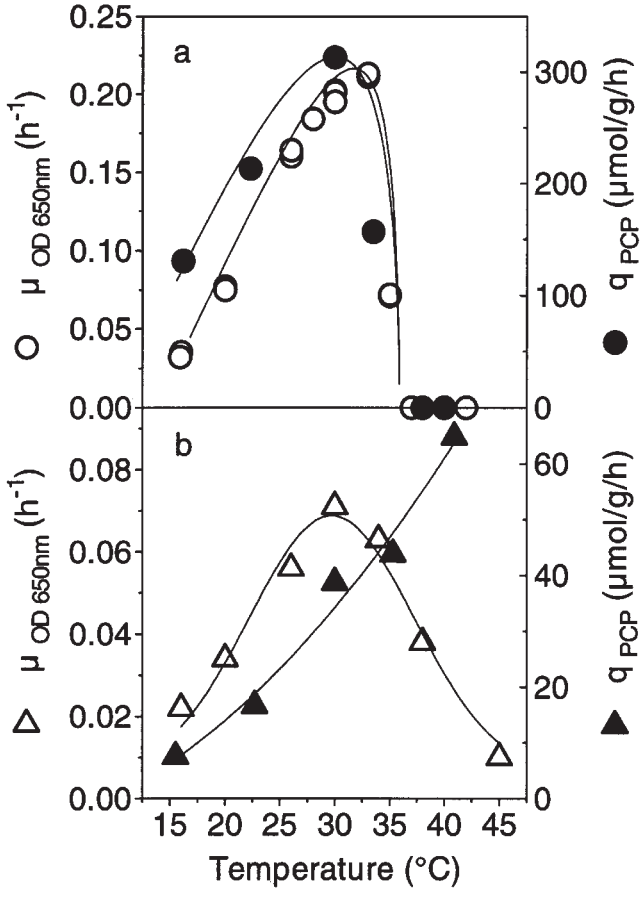

Figure 3 Effect of temperature on growth and PCP degradation activity of *S. chlorophenolica* RA2 (a) and *M. chlorophenolicum* PCP-1 (b).

a reduction of μ to about 30% of the maximum rate was observed at pH 5.0 and no growth occurred at pH 4.0. In contrast, PCP-1 showed better tolerance for acidic pH. The specific growth rate of this strain remained nearly constant down to pH 5.5 and slow growth of PCP-1 was observed at pH 4.5. At alkaline pH values, μ decreased steeply in the case of PCP-1, whereas RA2 grew well. The pH optima for PCP degradation by RA2 and PCP-1 were at a pH more alkaline than the pH optimum for growth (Figure 4a, b). Neither strain degraded PCP at pH 5. Additionally, no growth was observed under these conditions in the degradation experiments.

Influence of oxygen and carbon dioxide

Defined step changes of oxygen and carbon dioxide supply were performed during batch cultivation and the responding maximum growth rate (μ_{max}) was measured to assess the

Table 1 Parameters of inhibition kinetics of *S. chlorophenolica* RA2 and *M. chlorophenolicum* PCP-1 by PCP estimated by the modified Yano model (Eqn 1)

Organism	q_{max} (μmol g^{-1} h^{-1})	K_s (μmol L^{-1})	K_i (μmol L^{-1})	Dev (%)
S. chlorophenolica RA2	348	27	1011	3.4
M. chlorophenolicum PCP-1	48	2.6	58	7.4

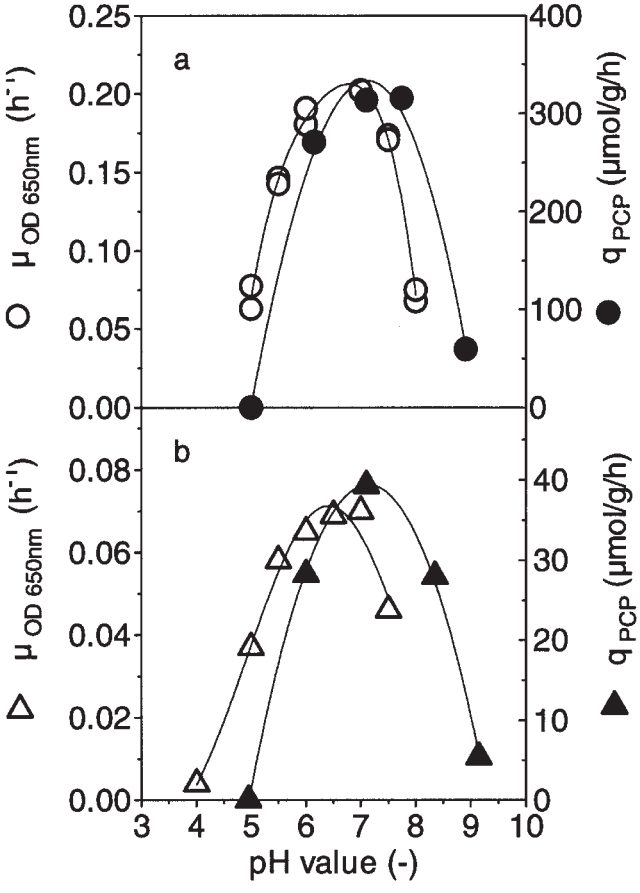

Figure 4 Effect of pH on growth and PCP degradation activity of *S. chlorophenolica* RA2 (a) and *M. chlorophenolicum* PCP-1 (b).

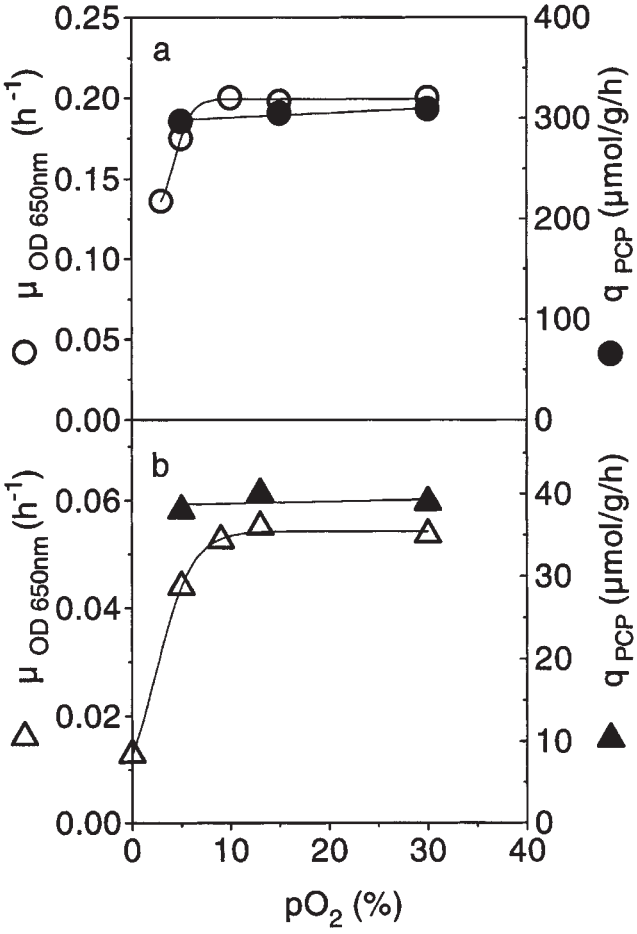

Figure 5 Influence of pO_2 on growth and PCP degradation activity of *S. chlorophenolica* RA2 (a) and *M. chlorophenolicum* PCP-1 (b).

influence of these gases on the growth of both strains, which showed similar characteristics towards oxygen supply (Figure 5a and b). Unlimited growth of both organisms occurred down to pO_2 values of about 10%. At very low O_2 concentrations in the aeration gas, pO_2 in the culture approached zero (O_2-limitation). Under these conditions, growth of PCP-1 was reduced, and the cells did not produce such metabolites as organic acids or alcohols, which are often found for microorganisms under oxygen limitation. Pyruvate accumulated in the medium of RA2 especially at low pO_2 values. The experiments performed under different oxygen supplies revealed no significant influence of pO_2 on the degradation activity of either organism in the range of 5–30% of oxygen saturation. Whereas the specific activity of RA2 was constant at about 300 μmol g^{-1} h^{-1}, the rate of PCP removal by PCP-1 was 40 μmol g^{-1} h^{-1}. The influence of carbon dioxide on growth was investigated in the range of 0.03 to about 8.0% of CO_2 in the gas phase (Figure 6a, b). Up to 2.5% CO_2, the growth of PCP-1 was enhanced, whereas pCO_2 values higher than 3.0% inhibited growth. The observed biomass yield of PCP-1 also increased from $Y_{x/s}$ = 0.43 g g^{-1} at low CO_2 concentrations to $Y_{x/s}$ = 0.51 g g^{-1} at a pCO_2 of 4%, indicating fixation of carbon dioxide. Additional studies revealed that PCP-1 is able to grow under autotrophic conditions (data not shown). Similar properties were not found for RA2.

Cultivation in technical scale

Based on the optimum growth conditions and the developed synthetic medium, the cultivation process was demonstrated for RA2 at a technical scale. The pH-controlled ammonium feeding strategy was applied. A slightly increased ammonium level of 1.0 g L^{-1} was used to overcome possible delays of the pH control system. To obtain higher biomass concentration, linear feeding with glucose was started at the end of the batch phase. The results are shown in Figure 7 a–c. The cells grew on the fully synthetic medium after a lag phase of about 10 h with a growth rate of 0.17 h^{-1}. The growth rate was lower than that observed in small-scale cultivations and might be due to an insufficient control of pH, which varied between 6.9 and 7.8 (data not shown). The pO_2 decreased down to 20% within 35 h of cultivation and was then kept constant at this value by control of the stirrer speed. After the lag phase, the specific respiration rates increased and stayed constant during the exponential growth phase. Especially at the end of the batch phase pyruvate accumulated up to 0.45 g L^{-1}, but was immediately consumed after depletion of the glucose at 45 h. During the appended feeding phase the RQ value was constant at 1.00 ± 0.03 mol mol^{-1} and with a continuously decreasing growth rate the biomass concentration doubled. Overall, 40 g cell dry mass per litre were achieved within

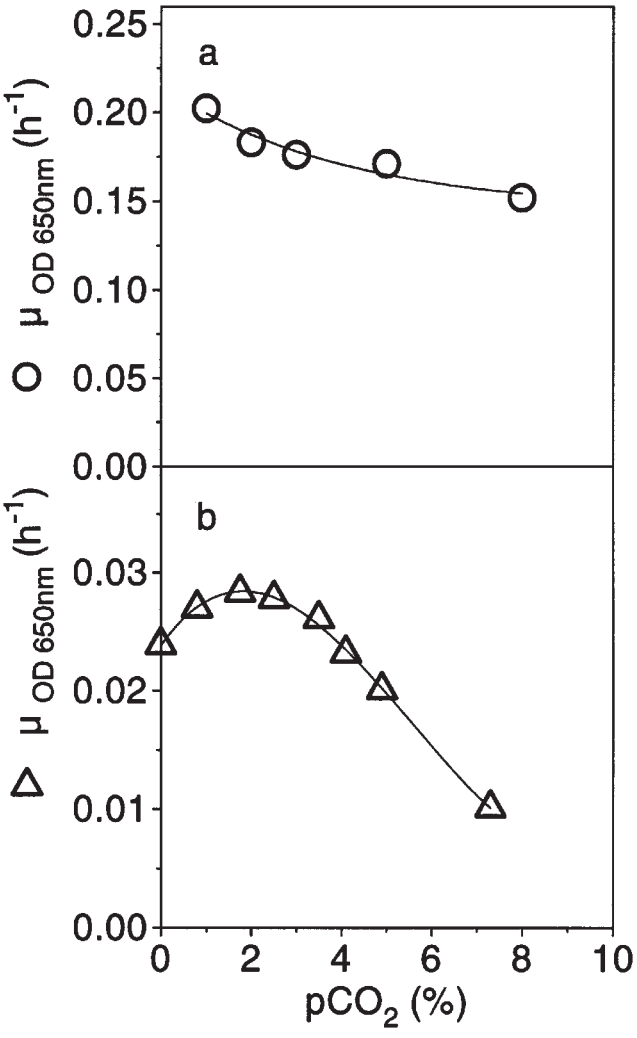

Figure 6 Influence of pO_2 on growth of *S. chlorophenolica* RA2 (a) and *M. chlorophenolicum* PCP-1 (b).

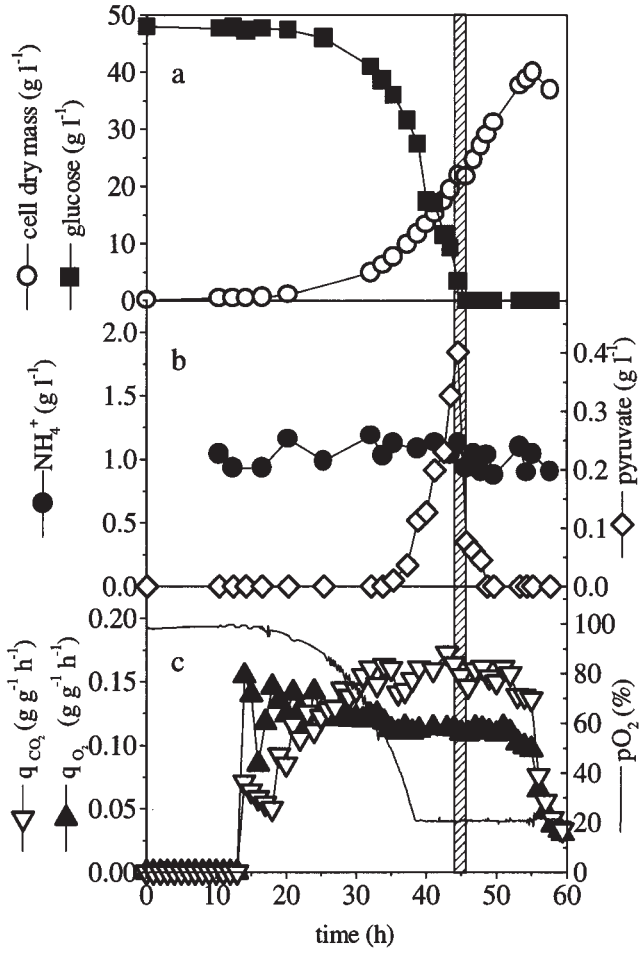

Figure 7 Physiological characteristics of technical scale cultivation of *S. chlorophenolica* RA2 with on-line controlled ammonia feeding and linear glucose feeding after 45 h: glucose and cell dry mass concentration (a); ammonium and pyruvate concentration (b); specific rates of CO_2 production (q_{co2}) and O_2 consumption (q_{o2}) and dissolved O_2 concentration (pO_2) (c).

55 h of cultivation. The ammonium concentration remained constant at the initial value of 1 g L^{-1}. Thus, a constant stoichiometric ratio between ammonium consumption and pH decrease existed under carbon excess and carbon limitation. The carbon and nitrogen recovery were 96.8 and 99.5%, respectively. The volumetric productivity was 0.72 g L^{-1} h^{-1}.

Discussion

The genera *Sphingomonas* and *Mycobacterium* comprise the best studied bacteria capable of complete mineralization of PCP. The biochemistry of PCP degradation is well known for *Arthrobacter* sp ATCC 33790, *Pseudomonas* sp RA2, *Flavobacterium* sp ATCC 39723 and *Pseudomonas* sp SR3, which were all recently shown to belong to a single species, *S. chlorophenolica* [7,17] and are represented in this work by *S. chlorophenolica* RA2. *M. chlorophenolicum*, previously *Rhodococcus chlorophenolicus*, represents the Gram-positive species for which PCP degradation biochemistry has also been elucidated in great detail. However, the general biochemistry and requirements for optimal growth and PCP degradation of *S. chlorophenolica* RA2 and *M. chlorophenolicum* PCP-1 are poorly understood and were, therefore, the topic of the present paper.

Our results show distinct differences in requirements for growth and PCP degradation between the two PCP degraders. The data obtained may be of value when cultivating and applying these strains for bioremediation and in evaluating the results of such efforts.

In general, RA2 grew and degraded PCP faster than did PCP-1 under optimum conditions. Organisms with high growth rate and low substrate affinity have been described as r-strategists, while the term K-strategists has been used for organisms with low growth rate and high substrate affinity [5]. Based on the metabolic properties revealed by this work, RA2 can be regarded as an r-strategist and PCP-1 as a K-strategist. *S. chlorophenolica* RA2 was more tolerant to PCP compared to *M.chlorophenolicum* PCP-1 in both growth and PCP degradation. The inhibition constants measured in this work for RA2 are comparable to those reported for the growth of *S. chlorophenolica* ATCC 33790 (previously *Arthrobacter* sp) [9]. The sensitivity of growth towards PCP, previously shown for several strains of the

genus *Mycobacterium* [12], was also found for strain PCP-1. In addition, our results suggest that PCP degradation activity is inhibited by PCP, but is less affected than growth. The similar values of RA2 for 50% inhibition of growth (K_1 = 800 μmol L^{-1}) and PCP degradation (K_1 = 1010 μmol L^{-1}), reveal a correlation of growth and degradation. In contrast, PCP-1 revealed a very different behaviour. The inhibition constant of PCP degradation (58 μmol L^{-1}) was nearly twice as high as that of the growth (30 μmol L^{-1}), indicating that this strain effectively conserved its degradation potential under non-optimum growth conditions. Strain PCP-1 had a much higher affinity for PCP compared to RA2 as suggested by the tenfold lower value of K_s. The K_s of 27 μmol L^{-1} for RA2 fits well with the K_s value for the purified PCP-4-monooxygenase of *S. chlorophenoloca* ATCC 39723, which was determined as 30 μmol L^{-1} [27]. It is noted that Gu and Korus [11] obtained a different K_s value of 143 μmol L^{-1} for whole cells of this strain, which might be due to the choice of a different inhibition model. The differences between RA2 and PCP-1 concerning the tolerance ($K_{1,RA2} > K_{1,PCP-1}$) and affinity ($K_{S,RA2} > K_{S,PCP-1}$) towards PCP reveal that RA2 is adapted to relatively high concentrations of PCP, while PCP-1 is adapted to relatively low concentrations of PCP. The strain RA2 performed poorly at low pH and high temperature; the opposite was observed for PCP-1. It is interesting to note that whereas whole cells of RA2 did not mineralize PCP at 38°C, isolated PCP-degrading enzymes of *S. chlorophenolica* ATCC 39723 were active up to 45°C [27], implying the importance of good growth conditions for effective PCP degradation by this species. *S. chlorophenolica* RA2 tolerated alkaline conditions, whereas *M. chlorophenolicum* PCP-1 performed better at acidic pH. The inhibiting influence of low pH for PCP degradation by *S. chlorophenolica* was also observed in soil [10]. The growth limitations observed for both organisms under pO_2 values below 10% should be considered for effective cultivation. Carbon dioxide significantly influenced the metabolism of PCP-1. Mycobacterial growth is frequently enhanced by carbon dioxide [20]. In contrast, the growth of RA2 was not affected by carbon dioxide. The observed increase in biomass yield of PCP-1 at high pCO_2 levels may result from additional fixation of carbon dioxide, since PCP-1 was found to be facultative autotrophic. Growth under autotrophic conditions was previously shown for other *Mycobacterium strains* like *M. smegmatis, M. fortuitum, M. marinum* [15] or the propen-degrading *M.Py1* [6]. The ability of *M. chlorophenolicum* PCP-1 to grow under autotrophic conditions, at low pH and at high temperature may suit it to soil clean-up by composting [13]. The high PCP affinity of PCP-1 is particularly useful for PCP degradation in soil where restricted bioavailability due to adsorption may occur. pH-controlled ammonium feeding was previously developed for cultivation of *M. chlorophenolicum* PCP-1 [26]. The metabolism of *S. chlorophenolica* RA2 is significantly inhibited by ammonium and the same strategy of ammonium feeding can be used for its effective cultivation. The production process was successfully demonstrated at a technical scale. Due to the fact that no technical problems occurred at the high cell densities of 40 g L^{-1}, a further scale-up to m^3 volume should be possible without difficulties. Whereas in cultivations of PCP-1 for field studies complex media were used [14], both strains can now be cultivated effectively under defined conditions on synthetic media with glucose as a cheap carbon source. With a ratio of 1.2×10^9 cells per mg of cell dry mass, 6.4×10^{15} cells were produced by the cultivation of RA2 at a technical scale. With a usual inoculum density of 10^6–10^7 cells per g of soil, the amount of biomass produced is sufficient for 600–6000 m^3 of soil. In comparison, a number of soils contaminated with PCP and other chlorinated phenols, recently treated with microbial remediation, have a volume of several hundreds to thousands m^3 [13]. Thus, the production process developed here can be directly applied for real remediation cases. In conclusion, the results presented in this work should be helpful in designing favourable conditions for bulk cultivation of the two strains and may also shed light on exploiting their potential in degrading PCP and other related compounds for the bioremediation of soil.

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