Thermochimica Acta 471 (2008) 86–89

Contents lists available at ScienceDirect

Thermochimica Acta

journal homepage: www.elsevier.com/locate/tca

Calorimetric investigation of *m*-methoxyphenol effect on *Chromobacterium violaceum* activity in soil

Denise A. Oliveira[a], Nieves Barros[b], Claudio Airoldi[a,*]

[a] *Instituto de Química, Universidade Estadual de Campinas, Caixa Postal 6154, 13084-971 Campinas, São Paulo, Brazil*
[b] *Department of Applied Physics, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain*

ARTICLE INFO

Article history:
Received 6 November 2007
Received in revised form 6 March 2008
Accepted 10 March 2008
Available online 18 March 2008

Keywords:
Microcalorimetry
Soil microorganisms
Chromobacterium violaceum
m-Alkoxyphenol

ABSTRACT

The microbial activity of *Chromobacterium violaceum* inoculated in sterile and natural red latosol soil samples was monitored by calorimetry to investigate metabolism of the native organic matter, easily degradable substrates (glucose) and the bacterial inhibitor *m*-alkoxyphenol. The results show that *C. violaceum* in sterile soil grows for a few hours, or, if easily degradable nutrients are available in soil, for 80 h. Inoculation of *C. violaceum* in unsterilized soil affected the metabolism of the native microflora in the presence of *m*-methoxyphenol with increases in the dissipation of heat per unit of growth.

1. Introduction

Chromobacterium violaceum, a Gram-negative rod-shaped bacterium, is a saprophyte of soil and water in tropical and subtropical areas and although it is generally considered to be non-pathogenic [1], some cases of fatal septicemia caused by this bacterium have been reported [2,3]. High numbers of this bacterium can be found in water and in soil on the banks of the Negro river [4], one of the largest tributaries of the Amazon river in Brazil. The metabolites synthesized by *C. violaceum* have potential application in several biotechnological and pharmacological research areas, and for this reason its genome sequencing has been promoted by a Brazilian Laboratories Consortium [5–7]. It can hydrolyze plastic films, producing cyanide, and this process could be useful in the extraction of gold, avoiding the use of mercury and the consequent environmental contamination [2,5].

There are no reports on the metabolism of *C. violaceum* in soil. The activity of this bacteria was measured previously by calorimetry in pure cultures to determine the effects of anionic surfactants [8,9] and uncouplers of oxidative phosphorylation [10,11]. Calorimetry can be applied to monitor the activity of *C. violaceum* in soil, and addition of nutrients can be used to stimulate microbial activity to obtain information on microbial degradation of soil substrates [12–17]. The present investigation measured the thermal effects of *C. violaceum* metabolism in soil to obtain information on bacterial production of CO_2 from soil organic carbon. A second objective of this study is to explore the toxicity of *m*-methoxyphenol on *C. violaceum* in soil and on soil microflora. *m*-Alkoxyphenol is commonly employed in biotransformation reactions [18] due to the high lipophilic activity with irreversible damage to cell walls and membranes [11]. The effect of these compounds on microbial respiration by flow-through calorimetry [10,18] has previously been used as an indicator of their toxicity. A second objective of this study is to explore the toxicity of *m*-methoxyphenol on *C. violaceum* in soil and on soil microflora.

2. Experimental

2.1. Maintenance and storage

A *C. violaceum* suspension (1 ml) was inoculated into a 1500 ml reactor flask (B. Braun Biotech, Biostat B2) containing the sterilized culture medium whose composition ($g\,l^{-1}$) was: 3.0 of yeast extract, 7.5 of glucose and 7.5 bacteriologic peptone in distilled water. This culture medium was maintained at 298 K under shaking (200 rpm) with an air flow of $2.0\,l\,min^{-1}$, for 14 h.

The cells were separated from the culture medium by centrifugation at 4000 rpm for 20 min, washed three times and suspended in sterilized phosphate buffered solution (PBS), whose composition ($g\,l^{-1}$) was: 8.0 NaCl, 0.20 KCl, 1.15 Na_2HPO_4 and 0.20 KH_2PO_4; then the mixture was centrifuged and the cells suspended again in 100 ml sterile PBS solution containing 10% of dimethylsulfoxide.

* Corresponding author.
E-mail address: airoldi@iqm.unicamp.br (C. Airoldi).

0040-6031/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.tca.2008.03.002

Then, 1 ml of this suspension was put into polypropylene ampoules (Corning).

The ampoules were inserted into a thin perforated Styrofoam plate which was placed 8 cm above the liquid nitrogen level in a Dewar Flask. When the temperature in the control ampoule reached 200 K, measured with an alcohol thermometer, the ampoules were immersed in liquid nitrogen and stored in a cryogenic cylinder [16].

Bacterial recovery after defrosting was evaluated by the number of viable cells estimated by serial dilution [19]. An aliquot (0.10 ml) was inoculated on Petri plates containing sterile, solid culture medium at 303 K for 24 h with three replicates. The number of bacterial colonies was determined by visual counting. The defrosted cultures contained 1.3×10^{10} viable cells ml^{-1}.

A 0.10 ml aliquot of the cell suspension was inoculated into the soil immediately after defrosting the ampoule for 3 min in a water bath at 310 K followed by manual shaking for 20 s.

2.2. Soil

Red Latosol soil was collected from the campus of the State University of Campinas [8]. After removing the top 5 cm, soil was collected from a depth of 5–10 cm, air dried for 1 week, and sieved (mesh size 600 μm × 600 μm) to remove roots, stones and small insects. Soil was stored in polyethylene bags at 277 ± 5 K for 2 months before calorimetric measurements. Sterile soil samples were obtained by autoclaving soil at 393 K.

2.3. Calorimetric measurements

An LKB 2277 calorimeter was used for all measurements [10–13]. Power–time curves were recorded with 1 g of soil in 5 ml stainless steel ampoules. The soil was amended with solutions as given in Table 1.

The total thermal effect, Q_T, for each experiment was calculated by integrating the area of the power–time curve with exothermic heat rate. Integration was done from the time the sample was inserted into the calorimeter until data collection was ended as shown in Figs. 1–4. The apparent microbial growth rate constant, μ, was calculated from the exponential growth portion of the curve as the slope of the line obtained by plotting the logarithm of the heat rate against time [20–22]. The bacterial biomass activated by the addition of nutrients, X_0, was determined by Sparling method [19]. The values of μ and X_0 were used to evaluate the increment in biomass, ΔX, by the equation for exponential microbial growth, $N = N_0 e^{\mu t}$. The heat yield, $Y_{Q/X} = Q_T/\Delta X$, heat dissipated per unit of biomass formed, gives information on the carbon conversion efficiency of microbial metabolism [23,24].

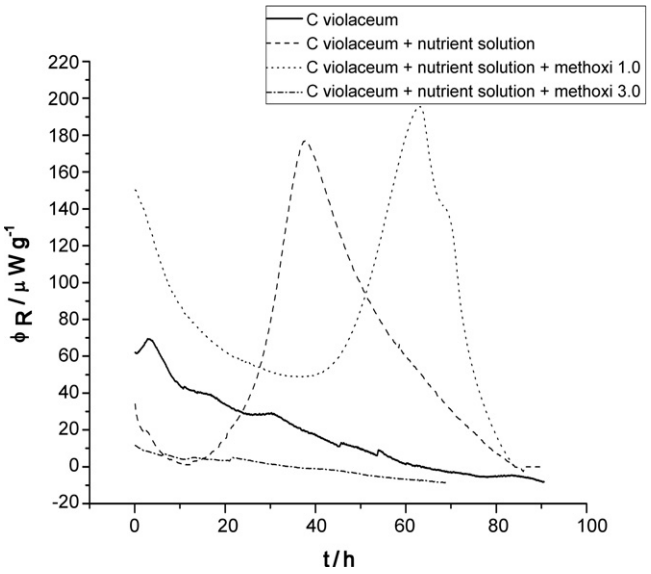

Fig. 1. Power–time curves for sterile soil inoculated with: (i) *C. violaceum*; (ii) *C. violaceum* and nutrient solution; (iii) *C. violaceum*, nutrient solution and 1.0 mM methoxyphenol; (iv) *C. violaceum*, nutrient solution and 3.0 mM methoxyphenol.

3. Results

Power–time curves of *C. violaceum* activity in sterile soil, with and without additional nutrient solution containing glucose and ammonium sulfate and *m*-methoxyphenol are shown in Fig. 1. The curve obtained when the bacteria is inoculated in sterile soil without any amendments shows it is not able to use the soil organic matter as a food and energy source, after a short period of low activity (about 10 h), the curve declines to a null value. The sample treated with nutrient solution showed an increase of microbial activity after a lag phase of about 20 h. Bacterial activity is totally inhibited by 3.0 mM methoxyphenol. This inhibition was not observed at 1.0 mM, although the increase of the heat rate occurs after a lag phase of almost 40 h, double that inoculated with the nutrient solution only.

The duration of the exponential region of the growth curve (PT), the apparent microbial growth rate constant (μ), and the heat yield ($Y_{Q/X}$) of the sterile samples amended with the nutrient solution are similar to those of the sterile samples amended with nutrient solution and 1.0 mM methoxyphenol (Table 1). Methoxyphenol at 1.0 mM increased the initial bacterial biomass (X_0), 540 μg g^{-1}, compared with 185 μg g^{-1} in samples with no methoxyphenol. Methoxyphenol also increased the total heat dissipated, Q_T.

Table 1
Summary of experimental conditions and thermochemical data obtained from all experiments

Experiment	Q_T (J g^{-1})	PT (h)	μ (h^{-1})	X_0 (mg g^{-1})	ΔX (mg g^{-1})	$Y_{Q/X}$ (kJ $g^{-1}X$)
st soil + Cv	–	–	–	–	–	–
st soil + Cv + nut	17.8	19.8	0.127	0.19	2.3	7.8
st soil + Cv + nut + mtx 1	32.2	18.6	0.116	0.54	4.7	6.9
st soil + Cv + nut + mtx 3	–	–	–	–	–	–
unst soil + nut	25.2	14.4	0.268	0.54	25	1.0
unst soil + mtx 1	5.5	5.4	–	–	–	–
unst soil + mtx 3	16.3	9.8	0.208	0.21	1.6	10.4
unst soil + nut + mtx 1	49.4	8.7	0.321	0.29	4.6	10.7
unst soil + nut + mtx 3	40.9	13.0	0.297	0.16	7.5	5.5
unst soil + Cv + nut + mtx 1	35.2	10.9	0.095	13.4	3.8	9.4
unst soil + Cv + nut + mtx 3	44.5	13.5	0.084	0.81	2.5	17.8

St, Sterile; unst, unsterilized; Cv, 0.10 ml of *C. violaceum* culture; nut, 0.30 ml of solution containing 3.0 mg glucose and 3.0 mg ammonium sulfate; mtx 1, 0.30 ml of 1.0 mM *m*-methoxyphenol; mtx 3, 0.30 ml of 3.0 mM *m*-methoxyphenol.

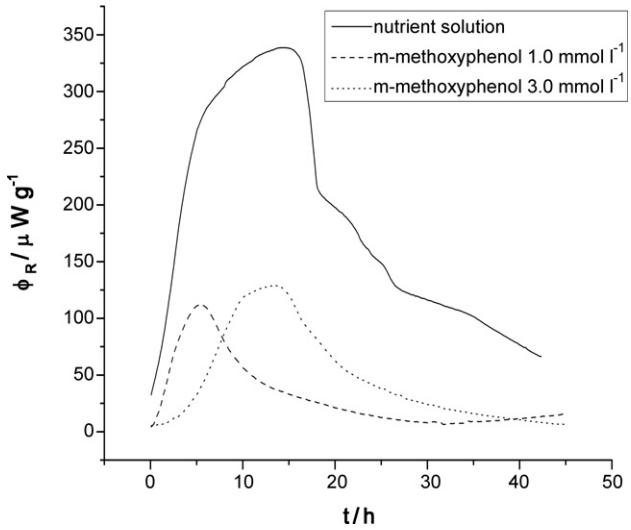

Fig. 2. Power–time curves of unsterilized soil amended with the nutrient solution and with 1.0 and 3.0 mM methoxyphenol.

The unsterilized soil amended with nutrients showed a significant thermal effect due to activity of the native soil microflora (Fig. 2). The shape of the curves and the values listed in Table 1 indicate differences between the response of soil microflora and the response of *C. violaceum* in sterile soil. The values of Q_T, μ, X_0, and ΔX of soil with native microflora are higher than those soils with only *C. violaceum*. The opposite was observed for $Y_{Q/X}$ values. The native microflora degrades the glucose and grows more efficiently than *C. violaceum* in the sterile soil. The effect of 1.0 and 3.0 mM methoxyphenol in the unsterilized soil also differs from that of the sterile sample. The lag phase of the sterile soil amended with 1.0 mM methoxyphenol is longer than in the unsterilized soil in the same experimental conditions. Methoxyphenol at 3.0 mM inhibits the *C. violaceum* activity in the sterile sample. The unsterilized soil treated with 3.0 mM methoxyphenol shows the typical exponential increase in heat rate caused by microbial growth [21,22,25]. The $Y_{Q/X}$ values indicate the soil microflora degrades glucose more efficiently than methoxyphenol.

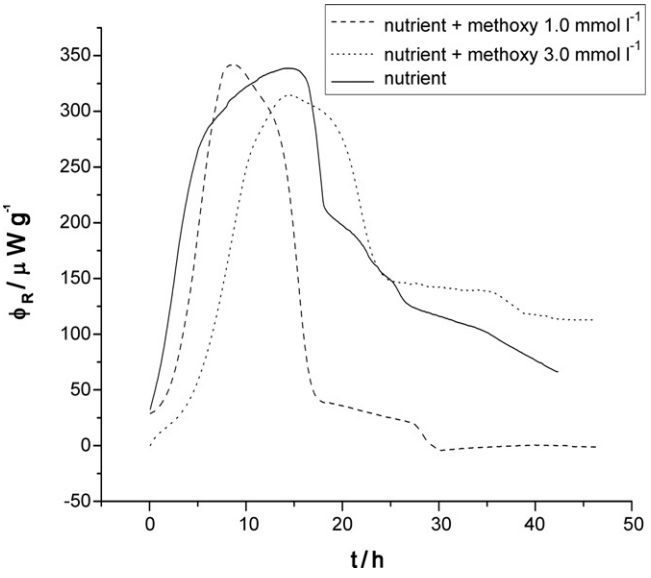

Fig. 3. Power–time curves of unsterilized soil treated with nutrients and with nutrients together with 1.0 and 3.0 mM methoxyphenol.

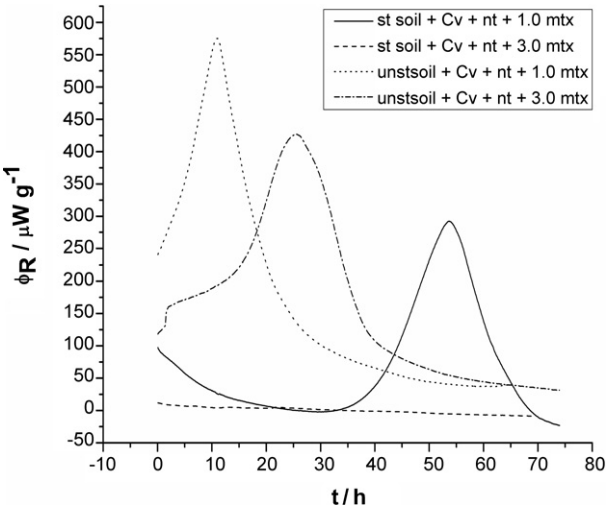

Fig. 4. Thermal effects in sterile soil inoculated with *C. violaceum* and treated with nutrients and methoxyphenol at 1.0 and 3.0 mM, and unsterililized soil inoculated with *C. violaceum* and amended with nutrients and 1.0 and 3.0 mM methoxyphenol.

Fig. 3 shows the power–time curves obtained when methoxyphenol is added to the unsterilized soil together with the nutrient solution and the curve of the unsterilized soil amended with nutrient solution only. The values of Q_T of the unsterilized soil treated with nutrients and methoxyphenol at 1.0 and 3.0 mM are higher than Q_T observed with soil treated only with nutrients. $Y_{Q/X}$ was larger for soil treated with nutrients and methoxyphenol.

The effects of inoculation of *C. violaceum* in unsterilized soil with nutrients and 1.0 and 3.0 methoxyphenol are shown in Fig. 4 and compared to the plots obtained from the sterile soil. The curves show differences in the duration of their lag phases which are shorter in the unsterilized samples. $Y_{Q/X}$ increases very much if the bacterium is inoculated in unsterilized soil treated with glucose and methoxyphenol at the high concentration. Table 1 shows that μ also decreases remarkably when *C. violaceum* is inoculated in the unsterilized soil. This value changes from about 0.3 h^{-1}, in the unsterilized soil to about 0.09 h^{-1}.

4. Discussion

Addition of easily degradable C sources to soil stimulate microbial growth characterized by an exponential increase of the heat rate [11,21,22,26]. The power–time curves in this work show the typical pattern of microbial growth with the exception of the sterile sample inoculated with *C. violaceum* that was unable to grow using the organic matter in the red latosol soil. *C. violaceum* needs an easily degradable C source to be able to grow. Other studies of survival and growth of bacteria introduced in soil show increase in number upon addition of 0.1% or 1.0% glucose and show a pattern of growth similar to that reported here [27]. The 5 ml calorimeter ampoules contain about 4 ml of air with about 33 μmol of O_2. From Thornton's rule (−455 kJ mol^{-1} O_2), this amount of O_2 could produce a maximum of only 15 J, significantly less than many of the Q_T values in Table 1. Another electron acceptor must be being used by the bacteria, probably NO_3^- (−497 kJ mol^{-1}/NO_3^- for reduction to NH_3 or −505 kJ mol^{-1} for reduction to N_2) for oxidation of organic compounds.

C. violaceum grows at a lower rate and develops a more dissipative or less efficient metabolism than the native soil microflora as shown by $Y_{Q/X}$ [22,28]. Variations in $Y_{Q/X}$ values can be attributed to adaptations of the microflora to the environment [29], microbial competition for the substrate added [30] or to changes in the

soil biomass structure [31]. In all cases, inoculation of *C. violaceum* altered the microflora metabolism.

Methoxyphenol is degraded and assimilated by both *C. violaceum* and the native soil microflora. The observed effect depends on the quantity of methoxyphenol added to the soil, 3.0 mM depletes the initial active native microflora, X_0, and causes a more dissipative metabolism. Both factors, active biomass depletion and higher dissipative metabolism, can be considered as a toxic effect [32,33]. In a previous paper, methoxyphenol appears to not affect respiration rates of *C. violaceum* [34], while results in this paper suggest a quite clear toxic effect on *C. violaceum* and on native microbial metabolism. Methoxyphenol also altered the microbial glucose degradation since unsterilized soil amended with both glucose and methoxyphenol show a decay of X_0 and higher $Y_{Q/X}$ values than those of the unsterilized soil amended only with glucose. As higher dissipation of heat is associated to higher levels of CO_2 respired in soils [12,19,35] the results may be considered as an undesirable effect, since the Kyoto protocol requests control of CO_2 released by soil carbon utilization. The loss of microbial efficiency in soil could thus contribute to global warming [36]. The metabolic efficiency could be a more sensitive indicator for diagnosing toxic effects than measurements of respiration or heat rates.

Acknowledgements

The authors are indebted to FAPESP and CNPq for fellowships and financial support. Also N. Barros thanks to the Ministerio de Educación y Ciencia in Spain and to the Consejería de Educación y Ciencia of Asturias for financial support, research projects MAT 2006-01997 and PC 06-020. All the authors are especially thankful to Dr. Lee Hansen for his important contribution to this article.

References

[1] P.H.A. Sneath, in: N.R. Krieg, J.E. Holt (Eds.), Bergeyĭs Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, USA, 1984.
[2] S.C. Kaufman, D. Ceraso, A. Schugurensky, J. Clin. Microbiol. 23 (1986) 956.
[3] R. Ponte, S.G. Jenkins, Pediatr. Infect. Dis. J. 11 (1992) 583.
[4] L.R. Caldas, Photochem. Photobiol. 26 (1997) 1.
[5] N. Durán, C.F.M. Menck, Crit. Rev. Microb. 27 (2001) 222.
[6] T. Hoshino, T. Hayashi, T. Uchiyama, Biosci. Biotech. Biochem. 58 (1994) 1338.
[7] M. Gillis, J. De Ley, A. Ballows, The Procaryotes, Springer Verlag, New York, USA, 1992.
[8] M.M. Basheer, P.L.O. Volpe, C. Airoldi, Int. J. Pharm. 282 (2004) 163.
[9] M.M. Basheer, C.E. Perles, P.L.O. Volpe, C. Airoldi, J. Solution Chem. 35 (2006) 625.
[10] D.A. Oliveira, C.P. Lisboa, P.L.O. Volpe, Thermochim. Acta 436 (2005) 26.
[11] R.D. Pereira, A. Matheus, P.L.O. Volpe, J. Ferm. Bioeng. 85 (1998) 302.
[12] S.A.M. Critter, J.A. Simoni, C. Airoldi, Thermochim. Acta 232 (1994) 145.
[13] N. Barros, S. Feijóo, R. Balsa, Thermochim. Acta 296 (1997) 53.
[14] S.A.M. Critter, S.S. Freitas, C. Airoldi, Appl. Soil Ecol. 18 (2001) 217.
[15] S.A.M. Critter, S.S. Freitas, C. Airoldi, Thermochim. Acta 394 (2002) 133.
[16] S.A.M. Critter, S.S. Freitas, C. Airoldi, Thermochim. Acta 410 (2004) 35.
[17] S.A.M. Critter, S.S. Freitas, C. Airoldi, Thermochim. Acta 417 (2004) 275.
[18] P.L.O. Volpe, J. Braz. Chem. Soc. 8 (1997) 343.
[19] G.P. Sparling, Soil Biol. Biochem. 34 (1983) 381.
[20] N. Barros, S. Feijóo, J.A. Simoni, A.G.S. Prado, F.D. Barboza, C. Airoldi, Thermochim. Acta 328 (1999) 99.
[21] T. Kimura, K. Takahashi, J. Gen. Microbiol. 131 (1985) 3083.
[22] L. Nuñez, N. Barros, I. Barja, Thermochim. Acta 237 (1994) 73.
[23] E.H. Battley, Thermochim. Acta 309 (1998) 17.
[24] N. Barros, S. Feijóo, Biophys. Chem. 104 (2003) 561.
[25] S.A.M. Critter, S.S. Freitas, C. Airoldi, Thermochim. Acta 394 (2002) 145.
[26] I. Barja, L. Nuñez, Soil Biol. Biochem. 31 (1999) 441.
[27] M.J. Acea, C.R. Moore, M. Alexander, Soil Biol. Biochem. 20 (1988) 509.
[28] U. von Stockar, I. Marison, Thermochim. Acta 229 (1993) 157.
[29] L. Gustafsson, Thermochim. Acta 251 (1995) 69.
[30] N. Barros, C. Airoldi, J.A. Simoni, B. Ramajo, A. Espina, J.R. García, Thermochim. Acta 441 (2006) 89.
[31] R.K. Thiet, S.D. Frey, J. Six, Soil Biol. Biochem. 38 (2006) 837.
[32] J.W. Stucky, Eur. J. Biochem. 109 (1980) 269.
[33] L.D. Hansen, C. Mcfarlane, N. McKinnon, B.N. Smith, S.R. Criddle, Thermochim. Acta 422 (2004) 55.
[34] Denise A. Oliveira, Chrislane P. Lisboa, Pedro L.O. Volpe, Thermochim. Acta 436 (2005) 26–29.
[35] A. Tancho, R. Mercks, R. Schoovaerts, K. Vlassak, Thermochim. Acta 251 (1995) 21.
[36] W.H. Schlesinger, J.A. Andrews, Biogeochemistry 48 (2000) 7.