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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*

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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).

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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].

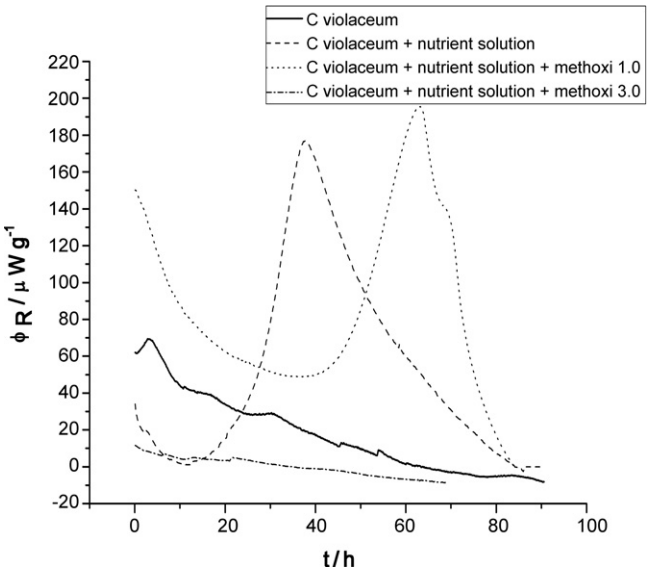

Fig. 1. Power–time curves for sterile soil inoculated with: (i) *C. violaceum*; (ii) *C. violaceum* and nutrient solution; (iii) *C. violaceum*, nutrient solution and 1.0 mM methoxyphenol; (iv) *C. violaceum*, nutrient solution and 3.0 mM methoxyphenol.

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.