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Calorimetric study of activity of the *m*-alkoxyphenols on the metabolism of *Chromobacterium violaceum*: Simultaneous measurements of dissolved oxygen tension

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Abstract

Calorimetric power versus time curves together with simultaneous measurements of dissolved oxygen for metabolism of *Chromobacterium violaceum* at 25 °C are reported in order to study the effect of a homologous series of *m*-alkoxyphenols. The inhibitory action of the compounds followed the order *m*-penthoxyphenol > *m*-buthoxyphenol > *m*-propoxyphenol > *m*-ethoxyphenol > *m*-methoxyphenol. The hydrophobicity of these compounds is inversely proportional to respective rates of oxygen consumption or heat production.
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Keywords: Flow microcalorimetry; *m*-Alkoxyphenols; Metabolism; Uncouplers; *Chromobacterium violaceum*

1. Introduction

Chromobacterium violaceum is a Gram-negative rod-shaped bacterium. It is a common saprophyte found in water and soil from tropical and subtropical regions of the world and is generally considered to be non-pathogenic [1], despite a few cases reported of fatal septicemia [2,3]. In Brazil, large amounts of *C. violaceum* are found in the water and in the soil on the banks of the Negro river, one of the largest tributaries of the Amazon river [4]. Several metabolites produced by *C. violaceum* present potential application in pharmaceutics, cosmetic, textile and food areas [5–7]. But some aspects of its metabolism remain unexplored.

Evaluation of sensitivity to uncouplers can be an important contribution about the metabolism of this microorganism. Continuous flow calorimetry allows following in real time the effect of *m*-alkoxyphenols on *C. violaceum* metabolism.

The measurement of DOT as a complementary variable helps elucidate the mode of action of xenobiotics on specific metabolic routes. Such measurements are normally carried out separately or in the case of flow experiments (perfusion mode), by connecting the sensors (electrodes) directly to the calorimetric cell [8–10].

In this work, simultaneous measurements of dissolved oxygen tension (DOT) and thermal effect were carried out with the aim to study the effect of phenols on respiring cells of *C. violaceum*.

Alkoxyphenols are known for their antibacterial activity and have been employed in some biological reactions used in production of flavors [11,12]. Recent studies also showed potential applications of these phenols as cytostatic drugs in tumor chemotherapy [13]. They are active against Gram-negative and Gram-positive bacteria and their mode of action is based on the inhibition of cellular respiration through the uncoupling of oxidative phosphorylation [14]. The main cellular target of *m*-alkoxyphenols is the cytoplasm and the extension of this effect is directly related to their solubility in lipids. The *m*-alkoxyphenols with higher lipophilicity are able to cross the cell membrane or promote irreversible damage to cell walls and cell membranes [15].

The influence of *m*-alkoxyphenols on microbial metabolism has already been investigated using flow-through calorimetry [14,15]. In these studies, the medium

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composition did not allow cells to grow in number because glucose was the sole carbon source and the microbial utilization of endogenous nitrogen cannot be detected in this short period of experiment (45 min).

2. Experimental

The effect of the *m*-alkoxyphenols on the metabolic rate (MR) was determined with a Thermometrics 2277 thermal activity monitor (TAM) calorimeter. The variable MR is defined by comparing the maximum height of the thermal power versus time curve for each compound with the maximum height of control curve [16]. All the experiments were carried out at 298 K in the flow-through mode (0.6 cm^3 flow cell volume). A continuous flow between the calorimeter and an external microreactor was maintained by a peristaltic pump (LKB Bromma 2132, Microperpex) at 0.50×10^{-3} $dm^3\,s^{-1}$. The calorimeter was chemically calibrated according to the procedure described by O'Neill et al. [17]. A flow of a phosphate buffer plus glucose solution (K_2HPO_4, 8.75 g dm^{-3}; KH_2PO_4, 1.88 g dm^{-3}; glucose, 1.80 g dm^{-3}) was used in the experiments. The solution from external reactor with or without (defined as control) *m*-alkoxyphenol was pumped to the calorimeter to establish a base line. To start the experiment, a sample of *C. violaceum* culture was added to the reactor. Immediately before the inoculation, the *C. violaceum* culture (previously stored in cryogenic ampoules and maintained under liquid nitrogen) was thawed for 3 min in a water bath at 310 K. The total volume added to the microreactor was 15.0×10^{-3} dm^{-3}. The *m*-alkoxyphenols, purchased from Eastman Kodak Co. Ltd., were previously distilled under vacuum and used at 3.00 and 14.0 mmol dm^{-3}. After each measurement, the flow cell and Teflon tubing were washed for 30 min with sodium dodecyl sulphate (0.02 mol dm^{-3}), ethanol (70%) and finally sterile deionized water.

The effect of *m*-alkoxyphenols on the medium dissolved oxygen tension was evaluated by simultaneously monitoring DOT and thermal effect. The apparatus of DOT measurement consisted of an oxymeter (Orion, 810A Plus), containing a polarographic oxygen sensor (Orion, 081010F). A glass cell constructed for the oxygen electrode was connected to the flow line of calorimeter. After preliminary experiments, this apparatus was used to study the effect of 3.00 mmol dm^{-3} *m*-alkoxyphenols on the metabolism of *C. violaceum*. DOT values were collected periodically and plotted versus time. Thermal power and DOT values obtained for each compound were compared by their respective curves in relation to time. Moreover, the rate of oxygen consumption was calculated through linear regression of DOT versus time curves.

Due to the low solubility of *m*-alkoxyphenols with longer side chains, the solutions of these compounds were prepared with acetone as solvent. Some control experiments were done with buffer solution plus glucose and a known percentage of acetone to evaluate the effect of this solvent on bacterium metabolism. The measurements revealed that 5.00% of acetone is the maximum percentage that could be used without interference. The experiments were carried out with 3.00 and 14.0 mmol dm^{-3} *m*-alkoxyphenols, using 5.0 and 1.8% acetone, respectively.

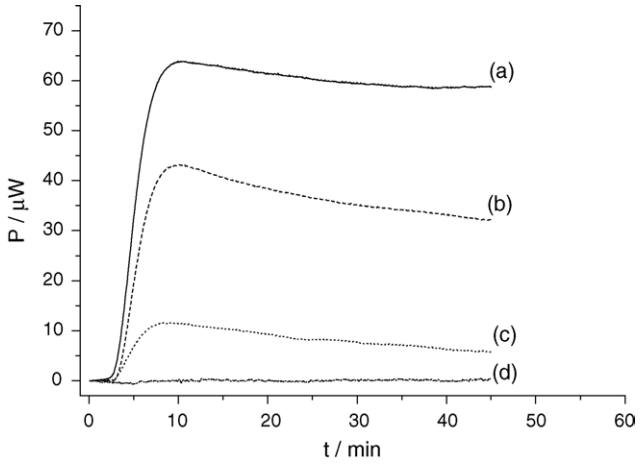

Fig. 1. Thermal power vs. time curves for metabolism of *C. violaceum*. Phosphate buffer solution plus glucose, 14.0 mmol dm^{-3} *m*-alkoxyphenol and 1.80% acetone. The medium without 14.0 mmol dm^{-3} *m*-alkoxyphenol was the control: (a) control; (b) *m*-methoxyphenol; (c) *m*-ethoxyphenol; (d) *m*-propoxyphenol.

3. Results

The results presented in Fig. 1 correspond to calorimetric experiments with the compounds at 14.0 mmol dm^{-3}. The curves show clearly that the uncoupler *m*-propoxyphenol was the most toxic, drastically reducing the thermal effect, compared to other compounds and the control. Because of the toxicity, it was not possible to study the thermal effect of 14.0 mmol dm^{-3} of *m*-butoxyphenol and *m*-pentoxyphenol.

Because one of the objectives was to investigate the toxicity of this homologous series, the concentration of 3 mmol dm^{-3} was chosen to continue this work. This concentration produced a significant effect for all the compounds of the series and the results are indicated in Fig. 2. As expected, *m*-pentoxyphenol and *m*-butoxyphenol were significantly more toxic. Bacterial metabolism showed a thermal effect near zero when tested in 3.00 mmol dm^{-3} *m*-pentoxyphenol. Methoxyphenol presented a metabolic rate similar to the control. Ethoxyphenol was only slightly toxic.

4. Discussion

The inhibition of the respiratory process (100, MR) varied considerably as a function of length of the side chain of the *m*-alkoxyphenol. The influence of the uncouplers on the *C. violaceum* metabolism followed, as expected, the toxicity order: *m*-pentoxyphenol > *m*-butoxyphenol > *m*-

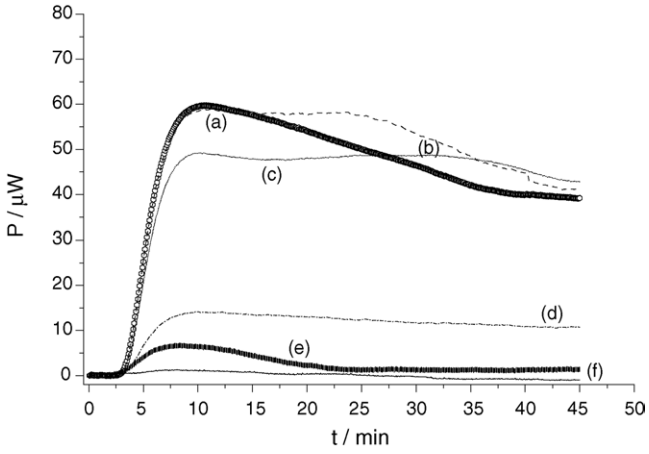

Fig. 2. Thermal power vs. time curves for metabolism of *C. violaceum*. Phosphate buffer solution plus glucose, 3.00 mmol dm^{-3} *m*-alkoxyphenol and 5.00% acetone. The medium without the 3.00 mmol dm^{-3} *m*-alkoxyphenol was the control: (a) control; (b) *m*-methoxyphenol; (c) *m*-ethoxyphenol; (d) *m*-propoxyphenol; (e) *m*-butoxyphenol; (f) *m*-pentoxyphenol.

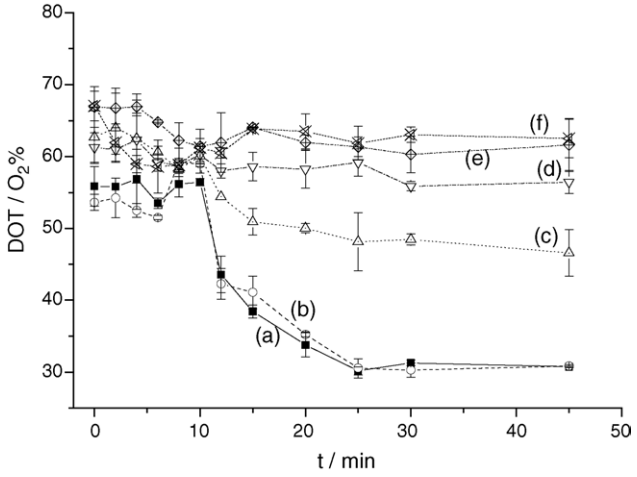

Fig. 3. DOT vs. time curves relative to the effect of *m*-alkoxyphenols on the metabolism of *C. violaceum*: (a) control; (b) *m*-methoxyphenol; (c) *m*-ethoxyphenol; (d) *m*-propoxyphenol; (e) *m*-butoxyphenol; (f) *m*-pentoxyphenol.

propoxyphenol > *m*-ethoxyphenol > *m*-methoxyphenol. The greater the membrane permeation of *m*-alkoxyphenol (due to increasing hydrophobic side chain), the higher will be the toxic effect on the microorganism [15].

Previous investigations of the effect of these compounds on metabolism of *Escherichia coli* [15] and *Saccharomyces cerevisiae* [14] showed the same behavior. Compared with *E. coli*, *C. violaceum* was almost three times more sensitive when exposed to the same conditions.

The rates of oxygen consumption of *C. violaceum* in the presence of *m*-alkoxyphenols are listed in Table 1. The hydrophobicity of uncouplers tested is inversely proportional to respective rates of oxygen consumption. This shows that the decrease in DOT is a consequence of a pronounced loss in cellular viability under the effects of *m*-alkoxyphenols with longer hydrophobic side chains.

There is no statistical difference among the rates of oxygen consumption with *m*-propoxyphenol, *m*-butoxyphenol and *m*-pentoxyphenol. This can be observed more clearly in Fig. 3. The loss of cellular viability due to the presence of a xenobiotic causes a reduction in DOT. These results are in agreement with the calorimetric data.

Fig. 4 presents the results of simultaneous measurements of thermal effect and DOT relative to exposure of *C. violaceum* to uncouplers. The control and *m*-methoxyphenol curves showed a rapid depletion of O_2 supply as a result of high *C. violaceum* activity. The flat curves indicate that exposure to *m*-alkoxyphenols of longer side chains promoted a low O_2 consumption rate as a consequence of no respiration or cellular death. A small displacement of DOT versus time curve in relation to thermal power versus time curve is probably due to the impossibility of measuring DOT inside the flow cell.

One of the biological effects expected for an uncoupler is, at first, to accelerate the respiratory processes, quickly increasing DOT [18]. The curves of Fig. 4 do not show this initial effect. However, the microorganism suspension spends 5 min to reach the oxygen electrode. The promotion event could have occurred before reaching the oxygen electrode. An alteration of experimental arrangement, placing

Table 1
Rates of oxygen consumption obtained during metabolism of *C. violaceum* in the presence of *m*-alkoxyphenols and in the control

Experiment	Rate of oxygen consumption (O_2 % min^{-1})
Control	1.00 ± 0.13
m-Methoxyphenol	0.95 ± 0.09
m-Ethoxyphenol	0.43 ± 0.11
m-Propoxyphenol	0.12 ± 0.03
m-Butoxyphenol	0.16 ± 0.03
m-Pentoxyphenol	0.20 ± 0.08

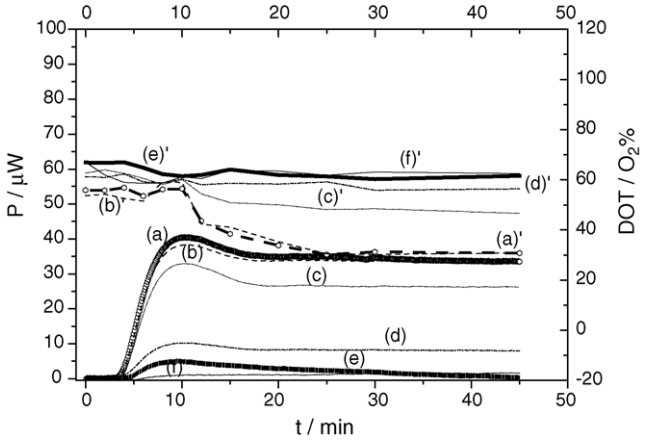

Fig. 4. DOT and thermal power vs. time curves relative to the effect of *m*-alkoxyphenols on the metabolism of *C. violaceum*: (a), (a)′ control; (b), (b)′ *m*-methoxyphenol; (c), (c)′ *m*-ethoxyphenol; (d), (d)′ *m*-propoxyphenol; (e), (e)′ *m*-butoxyphenol; (f), (f)′ *m*-pentoxyphenol.

the O_2 sensor just after the microreactor, might solve this problem.

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References

[1] P.H.A. Sneath, in: N.R. Krieg, J.G. Holt (Eds.), Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, 1984.
[2] S.C. Kaufman, D. Ceraso, A. Schugurensky, J. Clin. Microbiol. 23 (1986) 956.
[3] R. Ponte, S.G. Jenkins, Infect. Dis. 11 (1992) 583.
[4] L.R. Caldas, Photochem. Photobiol. 26 (1977) 1.
[5] M. Gillis, J. De Ley, A. Ballows (Eds.), The Procaryotes, Springer-Verlag New York Inc., NY, 1992.
[6] T. Hoshino, T. Hayashi, T. Uchiyama, Biosci. Biotech. Biochem. 58 (1994) 279.
[7] N. Durán, C.F.M. Menck, Crit. Rev. Microbiol. 27 (2001) 201.
[8] A.M. James (Ed.), The Thermal and Energetic Studies of Cellular Biological Systems, Wright, Bristol, 1987.
[9] P. Bäckman, I. Wadsö, J. Biochem. Biophys. Methods 23 (1991) 283.
[10] I. Wadsö, Chem. Soc. Rev. 26 (1997) 79.
[11] M. Doi, M. Matsui, Y. Shuto, Y. Kinoshita, Agric. Biol. Chem. 54 (1990) 2753.
[12] M. Doi, M. Ninomiya, M. Matsui, Y. Shuto, Y. Kinoshita, Agric. Biol. Chem. 53 (1989) 1051.
[13] G. Lassmann, S. Poetsch, Free Radic. Biol. Med. 19 (1995) 533.
[14] R.S. Pereira, A. Matheus, P.L.O. Volpe, J. Ferm. Bioeng. 85 (1998) 302.
[15] P.L.O. Volpe, J. Braz. Chem. Soc. 8 (1997) 343.
[16] A.E. Beezer (Ed.), Biological Calorimetry, Academic Press, London, 1980.
[17] M.A.A. O'Neill, A.E. Beezer, C. Labetoulle, L. Nicolaides, J.C. Mitchell, J. Connor, J. Orchard, R.B. Kemp, D. Olomolaiye, Thermochim. Acta 399 (2003) 63.
[18] W. David (Ed.), The Physiology and Biochemistry of Prokaryotes, Oxford University Press, NY, 1995.