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SHORT COMMUNICATION

Evidence for the role of zinc on the performance of dibenzothiophene desulfurization by *Gordonia alkanivorans* strain 1B

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Abstract *Gordonia alkanivorans* strain 1B is able to desulfurize dibenzothiophene (DBT) to 2-hydroxybiphenyl (2-HBP), the final product of the 4S pathway. However, both the cell growth and the rate of desulfurization can be largely affected by the nutrient composition of the growth medium due to cofactor requirements of many enzymes involved in the biochemical pathways. In this work, the effect of several metal ions on the growth and DBT desulfurization by *G. alkanivorans* was studied. From all the metal ions tested, only the absence of zinc significantly affected the cell growth and the desulfurization rate. By increasing the concentration of Zn from 1 to 10 mg L^{-1}, 2-HBP productivity was improved by 26%. The absence of Zn^{2+}, when sulfate was also used as the only sulfur source, did not cause any difference in the bacterial growth. Resting cells grown in the presence of Zn^{2+} exhibited a 2-HBP specific productivity of 2.29 μmol g^{-1} (DCW) h^{-1}, 7.6-fold higher than the specific productivity obtained by resting cells grown in the absence of Zn^{2+} (0.30 μmol g^{-1} (DCW) h^{-1}). These data suggests that zinc might have a key physiological role in the metabolism of DBT desulfurization.

Keywords Dibenzothiophene · Biodesulfurization · *Gordonia alkanivorans* · Zinc · Enzyme cofactors

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Introduction

Sulfur is one of the major contaminants present in a large number of hydrocarbons of fossil fuels. For the coming years, treating crude oil with high sulfur content is the reality for refineries, while at the same time they will face increasing pressure to reduce carbon dioxide emissions and make ultra-low sulfur transportation fuels.

The impetus for biodesulfurization occurred when a highly specific 4S metabolic pathway in the bacterium *Rhodococcus erythropolis* IGTS8 was discovered [4]. This metabolic pathway integrates two monooxygenases, one desulfinase and one flavin reductase. More recently, other bacterial genera were reported with the ability to use the 4S pathway to desulfurize dibenzothiophene (DBT) [1, 5, 7–9, 11, 14, 19]. However, the biodesulfurization studies reported for these new genera are very few compared with the knowledge obtained during the last decade for the well-studied *Rhodococcus* sp. Although the non-*Rhodococcus* genera have a potential to be used in the biodesulfurization of fossil fuels, optimization of the cultivation conditions, as well as using molecular tools to obtain better biocatalysts, are necessary. In fact, the key research needs to upgrade fossil fuels are the development of novel desulfurization biocatalysts with higher specific activity, broader substrate range, higher thermal tolerance [6] and with a lower cost of production. Recently, a study was reported on the costs of different methods for preparing these biocatalysts [12]. The composition of the culture medium is an important parameter to grow desulfurizing bacteria, because some of the metal ions can be necessary as enzymatic cofactors. Ohshiro et al. [17] reported that the DszA activity from *R. erythropolis* D-1 was inhibited by EDTA, suggesting that a metal might be involved in its activity. Molecular characterization of other bacterial monooxygenases revealed that

some metal ions such as zinc, copper, and iron are present in the active site of the enzymes [10, 23, 24]. Metal ions, as part of metalloenzymes, are essential for numerous biocatalytic processes. In many cases, metalloenzymes require specific metal ions to achieve catalytic functionality, e.g., zinc for hydrolytic activities or iron for redox proteins [20]. Zinc is an integral component of a large number and variety of proteins involved in a multiplicity of vital processes accounting for its key role in metabolism, transmission of the genetic message, growth and development. The chemically stable but stereochemically flexible, nontoxic nature of zinc, combined with its amphoteric properties, is the basis for the biochemical organization of a series of zinc binding motifs critical to life and its perpetuation [21]. More than 300 zinc enzymes covering all six classes of enzymes have been discovered, and in most cases it is an essential cofactor for the observed biological function of these metalloenzymes [15].

In this study, the effect of the absence of several metal ions in the culture medium, particularly zinc ion, was examined for growth and dibenzothiophene desulfurization by *Gordonia alkanivorans* strain 1B.

Materials and methods

Bacterial strain and growth conditions

G. alkanivorans strain 1B was previously isolated in our laboratory from oil-contaminated soil [2]. This bacterium was cultured in sulfur-free mineral (SFM) medium supplemented with a trace element solution as described by Alves et al. [1]. For the assays in which a single metal ion was removed, the culture medium was prepared adding the metal compounds separately instead of adding the trace element solution. Filter sterilized glucose (10 g L^{-1}) was used as the only carbon source. A concentration of 500 μM DBT or DBT sulfone (both dissolved in dimethylformamide) or sulfate was added as the sulfur source to the sterilized culture media. All *G. alkanivorans* cultivations were performed in shake-flasks at 30 °C, pH 7.5, and 150 rpm shaking, at least in duplicates.

Resting cells

In order to investigate the effect of Zn^{2+} ion in desulfurization activity of resting cells of *G. alkanivorans* strain 1B, 500 mL of culture broth (SFM medium supplemented with 10 g L^{-1} glucose and 150 μM DBT), with and without zinc, was taken at a late growth phase (7 days after inoculation). Cells were harvested by centrifugation at 7,500*g* and 4 °C for 10 min. The harvested cells were washed twice with a saline solution (0.85% NaCl) and kept in 0.1 M phosphate solution (pH 7.0) at 4 °C. Some of the resting cells pregrown without zinc were incubated overnight with 1 mg L^{-1} zinc and then used in a desulfurization assay.

Analytical methods

The cell growth was monitored by measuring the optical density of the culture broth samples at 600 nm. Dry cell weight (DCW) was determined using 0.22 μm cellulose acetate membranes after 18 h at 100 °C. The determination of the organic compounds involved in desulfurization was performed as described by Alves et al. [2]. In all GC measurements, anthracene was used as an internal standard to minimize the variation.

Chemicals

DBT (99%) was obtained from Acros Organics, DBT sulfone (97%) from Aldrich, 2-HBP from Sigma, DMF from Riedel-deHaën (Seelze, Germany), anthracene and ethyl acetate from Merck. All other materials were of the highest purity commercially available and were used without further purification.

Results and discussion

The effect of the absence of several metal ions in the culture medium on growth and production of 2-HBP by *G. alkanivorans* strain 1B was investigated. The metal ions studied were Ca^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, MoO_4^{2-}, Cu^{2+} and Zn^{2+} (Table 1). As positive and negative controls, the culture medium contained none and all of the metal ions tested, respectively. The absence of all metal ions resulted in a decrease in biomass and 2-HBP production of about 64 and 32%, respectively. In addition, the absence of Fe^{3+} and Mn^{2+} enhanced DBT desulfurization, while Co^{2+} had no effect. Furthermore, a decrease in production of 2-HBP was detected in the absence of Ca^{2+} and MoO_4^{2-}. However, the most significant decrease in production of 2-HBP was detected in the absence of Zn^{2+} and Cu^{2+} even though the absence of the latter metal did not significantly decrease the production of biomass, compared to the value obtained without any metal ions. The effect of zinc on both growth and 2-HBP production suggests that this metal might have an important role in the metabolism of *G. alkanivorans* strain 1B; this was further investigated (Fig. 1). The increase of Zn^{2+} concentration in the culture medium enabled both a higher bacterial growth (Fig. 1a) and a higher 2-HBP production (Fig. 1b). The addition of 1 mg L^{-1} zinc increased both μ_{max} by 32%, from 0.019 h^{-1} to 0.025 h^{-1}, and DCW around 2-fold (data not shown), compared with the absence of zinc. Further increases of Zn^{2+} did not affect the growth rate. The 2-HBP productivity,

Table 1 Effect of removal of some metal ions on *G. alkanivorans* strain 1B growth and production of 2-HBP after 7 days of culture

Metal ion	Abs_{600}	Final pH	DCW (g L^{-1})	2-HBP (μM)
Negative control	9.29	5.69	3.6	133.2
Positive control	2.66	6.64	1.3	91.1
Fe^{3+}	6.61	6.06	2.2	159.9
Co^{2+}	9.30	5.76	3.3	131.1
Cu^{2+}	9.35	6.02	3.4	97.8
Ca^{2+}	8.99	6.02	3.4	107.6
Mn^{2+}	7.92	5.99	2.7	153.0
MoO_4^{2-}	10.25	5.89	3.7	115.4
Zn^{2+}	3.60	6.58	1.4	97.2

G. alkanivorans was grown in SFM medium supplemented with 10 g L^{-1} glucose as carbon source and 500 μM DBT as sulfur source. The results presented are the mean values of duplicates with a standard deviation less than 5%

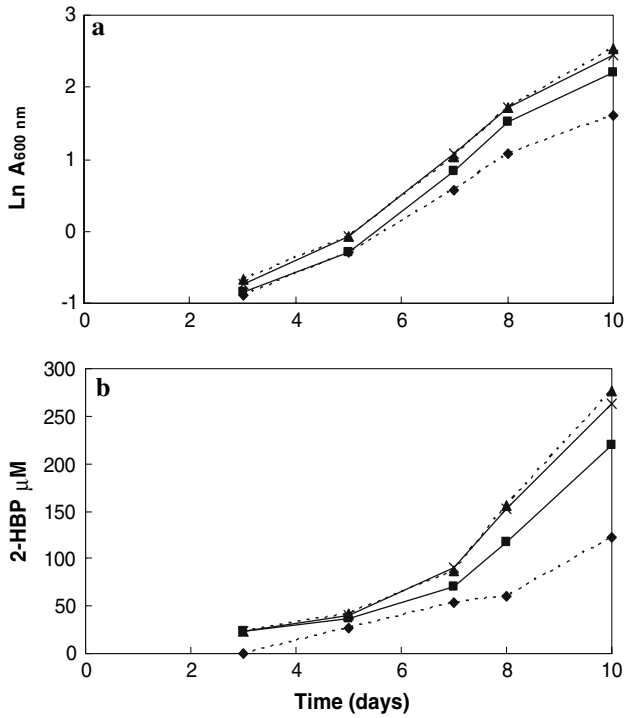

Fig. 1 Time course of growth (**a**) and production of 2-HBP (**b**) by *G. alkanivorans* strain 1B. The strain was grown at 30 °C and 150 rpm, in SFM medium with DBT as sulfur source and several zinc concentrations: *cross symbol*—25 mg L^{-1} Zn^{2+}; *filled triangle*—10 mg L^{-1} Zn^{2+}; *filled square*—1 mg L^{-1} Zn^{2+}; *filled diamond*—without Zn^{2+}. The results presented are the mean values of duplicates with a standard deviation less than 5%

after 10 days of culture (Fig. 1b) in the absence of zinc, was only 0.51 μM h^{-1}. The addition of 1, 10, and 25 mg L^{-1} Zn^{2+} improved the 2-HBP productivity to 0.91, 1.15, and 1.09 μM h^{-1}, corresponding to an increase of about 78, 126, and 114%, respectively. The slight decrease in 2-HBP productivity observed with 25 mg L^{-1} Zn^{2+} is possibly due to an inhibitory effect of this Zn^{2+} concentration in the culture medium. Although zinc is an essential trace element used in the majority of the bacterial culture media, it is known to be a potent inhibitor of the respiratory electron transport system [3]. Previous studies in our laboratory, using *G. alkanivorans* strain 1B [2], have shown a 2-HBP specific productivity of 1.03 μmol g^{-1} (DCW) h^{-1} when cultured with 0.5 mg L^{-1} Zn^{2+}, 20% higher than the specific productivity obtained without zinc in the present work (data not shown). In addition, the same effect was observed in the presence and absence of Zn when DBT sulfone was used instead of DBT (data not shown).

Moreover, the effect of zinc absence was analyzed during growth of strain 1B using either DBT or sulfate as the only sulfur source (Fig. 2). Only when DBT was used as the only sulfur source, *G. alkanivorans* strain 1B demonstrated a higher growth in the presence of the zinc ion. After 7 days, the bacterial growth was 38% higher in the Zn-containing medium compared to the growth medium without Zn^{2+}. As expected, strain 1B grew better in the sulfate-containing growth medium compared with the one with DBT, since sulfate is more easily assimilated as sulfur source.

To confirm the role of zinc, production of 2-HBP was assessed using resting cells pre-grown either in the presence or absence of zinc (Fig. 3). Using resting cells pre-grown in the presence of Zn^{2+}, a 2-HBP specific productivity of 2.29 μmol g^{-1} (DCW) h^{-1} was obtained, which is 7.6-fold higher than the specific productivity obtained using resting cells pre-grown in the absence of Zn^{2+} (0.30 μmol g^{-1} (DCW) h^{-1}). Recently, a higher 2-HBP specific productivity (56.58 μmol g^{-1} (DCW) h^{-1}) was

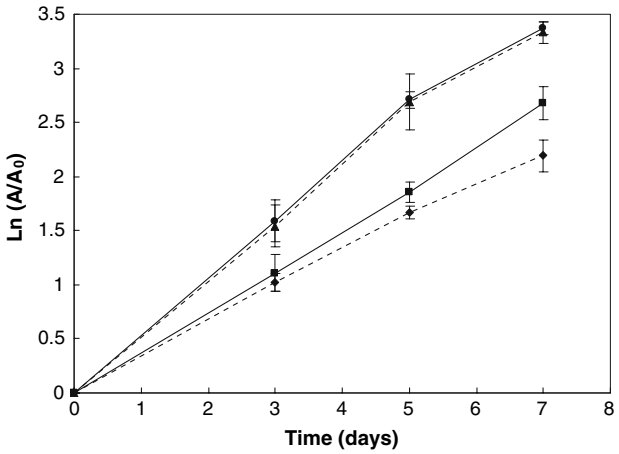

Fig. 2 Time course of growth of *G. alkanivorans* strain 1B. The strain was grown at 30 °C and 150 rpm, in SFM medium with DBT or sulfate as sulfur source and with or without zinc: *filled circle*—sulfate without Zn^{2+}; *filled triangle*—sulfate with Zn^{2+}; *filled square*—DBT with Zn^{2+}; *filled diamond*—DBT without Zn^{2+}

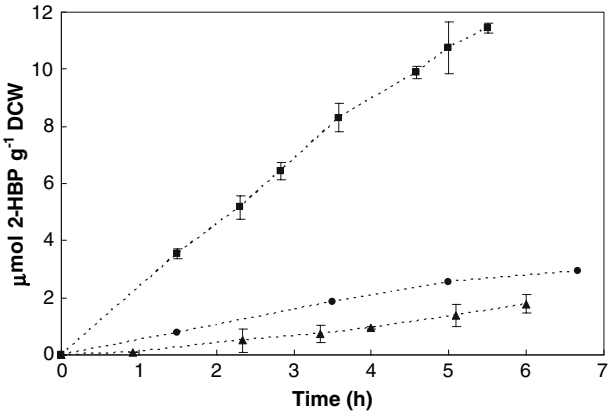

Fig. 3 Time course of 2-HBP production by resting cells of *G. alkanivorans* strain 1B: *filled triangle*—pre-grown in the absence of zinc; *filled square*—pre-grown in the presence of zinc; *filled circle*—pre-grown in the absence of zinc + overnight incubation with 1 mg L^{-1} Zn^{2+}

reported with resting cells of another strain of *G. alkanivorans*, but using an optimized biphasic system [14].

An additional assay was carried out by overnight incubation of the resting cells pre-grown in the absence of zinc with 1 mg L^{-1} Zn^{2+} (Fig. 3). There was an increase of 70% in the production of 2-HBP. However, production of 2-HBP was not fully restored since the productivity was lower than that observed using resting cells pre-grown in the presence of Zn^{2+}.

There are numerous reports in the literature on the characterization of the desulfurizing enzymes from *R. erythropolis* [13, 17, 18, 22]. Ohshiro et al. [18] showed that the metal ions Cu^{2+}, Zn^{2+}, and Mn^{2+} significantly inhibited the DBT monooxygenase activity of *R. erythropolis* D-1. Conversely, a positive effect on DBT sulfone monooxygenase activity was reported when Al^{3+}, Cd^{2+}, and Zn^{2+} were added [17]. These results suggested that SH group(s) and metal(s) might be involved in these enzyme structures. On the contrary, the enzyme 2-(2′-hydroxyphenyl)benzenesulfinate desulfinase from *R. erythropolis* does not require a metal cofactor for catalysis [16, 22]. In addition, Watkins et al. [22] showed that the presence of zinc reduced the activity of DszB by 50%, due to the interference of substrate binding or catalysis. To date, there are no reports on the purification and characterization of Dsz enzymes from *Gordonia* sp.

This work shows the importance of zinc for the growth and DBT desulfurization by *G. alkanivorans* strain 1B. A maximal desulfurization rate by *G. alkanivorans* strain 1B was obtained using a growth medium containing 10 mg L^{-1} Zn^{2+}. Further research on purification and characterization of the Dsz enzymes by *G. alkanivorans* strain 1B is necessary to understand the role of zinc and other metal ions in the biological desulfurization systems.

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