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([1]PROIMI, CONICET, [2]Facultad de Bioquímica, Química y Farmacia (Universidad Nacional de Tucumán), and [3]CERELA)

# Estimation of growth inhibition by copper and cadmium in heavy metal tolerant actinomycetes

MARÍA J. AMOROSO[1, 2, *], GUILLERMO OLIVER[3], and GUILLERMO R. CASTRO[1]

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A new isothermic model of actinomycetes growth in presence of toxic concentrations of $Cd^{2+}$ and $Cu^{2+}$ is described. Microbial growth inhibition, displayed as a decrease of biomass, can be correlated with the increase of cadmium(II) or copper(II) concentration in the medium. The reciprocal dry biomass against metal concentration showed a linear correlation higher than 0.9 in tested strains. The mathematical model can be useful to predict the behavior of actinomycetes at inhibitory concentrations of copper(II) and cadmium(II) in large screening procedures.

Release of heavy metals into the environment is a major source of pollution in soils, aquifers and rivers. In recent years, extensive studies have been carried out to identify microorganisms with high metal resistance and biosorption capabilities due to their potential use in bioremediation processes (VOLESKY and HOLAN 1995, KRATOCHVIL and VOLESKY 1998).

The most useful methods to evaluate metal tolerance in cells are based on measures of growth and metal uptake at several metal concentrations (DUXBURY 1981, Abbas and EDWARD 1989, Farrel *et al*. 1993, MCELDOWNEY 1994). However, these procedures are semiquantitative and time consuming. In addition, they can not be able to predict the behaviour of microorganisms at different metal concentrations, or in different operational conditions. Another approach is the study of heavy metal adsorption by use of isotherms based on the linearized models of LANGMUIR and FREUNDLICH (CHANG and HONG 1994). However, these models can not be used to study the microbial behaviour at inhibitory metal concentrations.

Actinomycetes are chemoorganotrophic microorganisms that can degrade a wide range of substances including cellulose, chitin, paraffin and toxic compounds (OKAMI *et al*. 1988). In addition, actinomycete strains were found to be tolerant and/or resistant to several heavy metals (ABBAS and EDWARD 1989, RAVEL *et al*. 1998). In a previous screening program, fifty-three wild-type strains of actinomycetes resistant to heavy metals showed biosorption higher than 98% between 0.1 to 10.0 mM cadmium(II) and copper(II) concentrations (AMOROSO *et al*. 1998).

The aim of this work was to develop a mathematical model that can predict the behaviour of microorganisms in presence of inhibitory cadmium (II) and copper (II) concentrations using five wild-type actinomycete strains isolated previously.

## Materials and methods

**Microorganisms:** Strains were isolated and grown in broth containing per litre: L-asparagine, 0.5 g; $K_2HPO_4$, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; $FeSO_4 \cdot 7\ H_2O$, 0.01; glucose, 10.0 g; supplemented with 0 to 1.0 mM of $CdCl_2$, or $CuSO_4$ (AMOROSO *et al*. 1998). Heavy metals were added to 30 ml liquid medium

* Corresponding author: Dr. M. J. Amoroso; e-mail: mjamoroso@ciudad.com.ar

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in 125 ml Erlenmeyer flasks after autoclaving. Spores suspension inoculated Erlenmeyer flasks were incubated with shaking (100 rpm) at 25 °C for 48 h.

More than sixty colonies of actinomycetes were isolated, and ten strains were selected considering their multiple resistance to $Cd^{2+}$ and $Cu^{2+}$ (Amoroso *et al*. 1998). A collection strain, *Streptomyces lividans* TK24, was used as a control in all experiments (Ravel *et al*. 1998).

**Analytical procedures:** Culture samples were taken and centrifuged (3,000 × g, 10 min). Biomass was estimated by washing the pellets with 25 mM Tris-EDTA buffer (pH 8.0) and drying to constant weight at 105 °C. Metal concentrations in the cell-free supernatant were quantified by atomic absorption spectroscopy as previously reported (AMOROSO *et al*. 1998). All experiments were carried out in triplicates.

## Results and discussion

Fifty-three actinomycete strains tolerant to $Cd^{2+}$ and/or $Cu^{2+}$, were isolated in a screening program at Sali River (Tucumán, Argentina). Heavy metal biosorption activities of the wild-type strains in the range of 0.10 to 1.00 mM range were higher than 98% after 48 hours of culture (AMOROSO *et al*. 1998). With increasing heavy metal concentrations in the culture media the metal uptake also increases and it can be correlated to a growth inhibition expressed as a relative biomass decrease in all 53 isolates (data not shown). That means that for a screening procedure of heavy metal resistant strains in absence of an inhibitory activity of the metal, maximal biomass ($x_{max}(t, M_0)$)* can be used under experimental conditions. The growth inhibition by heavy metals has previously been studied using relative growth defined as the ratio between $x(t, M)$ and $x_{max}(t, M_0)$, and the parameter is generally used for qualitative strain comparison (ABBAS and EDWARD 1989, WALKER and HOUSTON 1981).

When relative biomass at defined time points was plotted against inhibitory metal concentrations, hyperbolic curves were observed in all wild-type metal-resistant strains, including in the strain control *Streptomyces lividans* TK24 (Fig. 1). Similar curve profiles were reported for other heavy metal tolerant microorganisms (GODDARD and BULL 1989, DUXBURY 1981, FARREL *et al*. 1993, CHANG and HONG 1994). However, classical models of isotherm adsorption, such as LANGMUIR or FREUNDLICH, not fit well with metal biosorption experimental data, probably because they were developed using chemical compounds in a non-dynamic system (KRATOCHVIL and VOLESKY 1998).

In order to correlate growth inhibition of selected strains with heavy metal concentration, reciprocal wild-type actinomycete biomass was plotted against $Cd^{2+}$ and $Cu^{2+}$ concentrations with results of linear correlation coefficients higher than 0.90. Five selected actinomycete strains showed high adsorption of heavy metal (cadmium(II) or copper(II)), at least 50-folds higher than sensitive reference strain *S. lividans* TK24 (AMOROSO *et al*. 1998). Plots of the five most promising, metal-resistant strains are shown in Figs. 2 and 3. These empirical results of the five most promising isolates resistant to cadmium(II) and/or copper(II) were modelled as follows:

In general, the kinetics of microbial growth can be described by an exponential equation (PIRT 1975):

$$x(t) = x(t_0) \cdot e^{\mu t} \quad (1)$$

where $x(t_0)$ and $x(t)$ are the biomass at $t_0$ and at time t, respectively, and $\mu$ is the specific growth rate.

* **Abbreviations:** $K_i$: growth inhibition metal constant defined as metal concentration that inhibited totally microbial growth; M: metal concentration.; $M_0$: initial metal concentration ($t = 0$); $t$: time; $x_t$: biomass at time $t$; $x_{max}$: maximal biomass; $x_0$: biomass at time point 0; $r$: linear correlation coefficient.

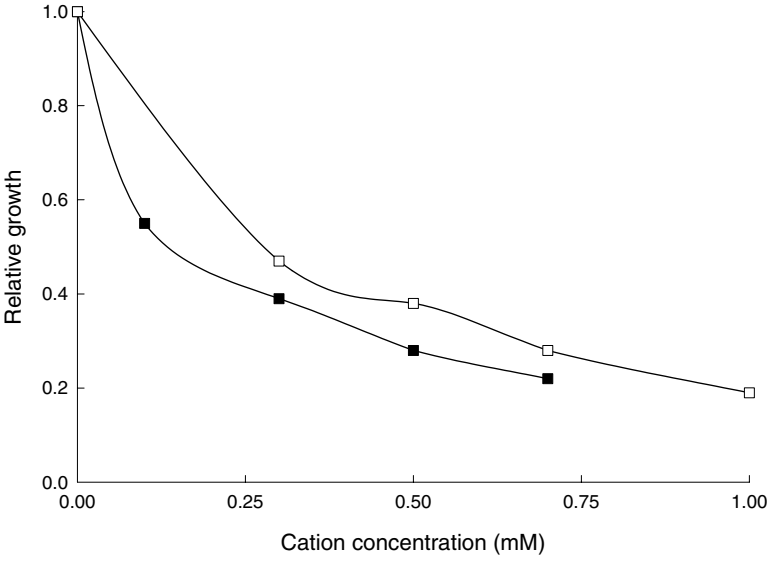


Fig. 1
Effect of different $Cd^{+2}$ (■) and $Cu^{2+}$ (□) concentrations on actinomycete R25 growth at 48 hours and 30 °C

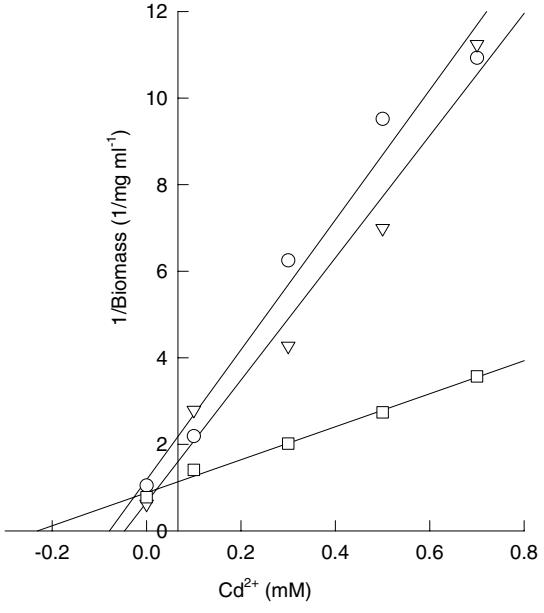


Fig. 2
Effect of $Cd^{2+}$ on growth of actinomycete strains: R06 (○), R16 (▽) and R25 (□)

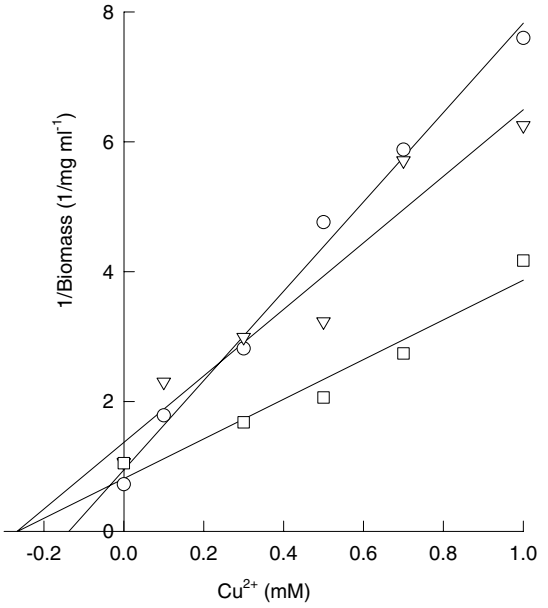


Fig. 3
Effect of $Cu^{2+}$ on growth of actinomycete strains: R10 (△), R25 (□) and R27 (◇)

Strong inhibition of microbial growth was observed when high heavy metal concentrations of cadmium(II) or copper(II), were added to the medium (ABBAS and EDWARDS 1989, AMOROSO *et al*. 1998). At millimolar cadmium(II) or copper(II) concentrations, the predominant mechanism is the inhibition of cell growth. That means that biomass at a time point $t$ ($x$ ($t$, $M$)) can be described as a function ($\boldsymbol{F}$) of the metal concentration in the medium ($M$), considering the metal as the only limiting growth element and as a toxic factor:

$$\frac{1}{x(t, M)} = \boldsymbol{F}(M). \tag{2}$$

If a linear relationship between the reciprocal of microbial growth and metal concentration at time t is hypothesized (Figs. 2 and 3), a linear equation can be obtained considering inhibition mechanisms as major effect:

$$\frac{1}{x(t, M)} = \boldsymbol{A} + \boldsymbol{B} \cdot (M). \tag{3}$$

Where $\boldsymbol{A}$ and $\boldsymbol{B}$ are constants for each metal-strain pairs. In absence of heavy metal (zero concentration) or heavy metal non-inhibitory concentration ($M_0$), $x_{max}(t, M_0)$ can be defined. Using this approach, $x_{max}(t, M_0)$ can be defined as the maximum biomass for the selected strain in the experimental conditions (e.g. medium composition, microorganism, pH, temperature, etc…) at time $t$. Substitution of $\boldsymbol{A}$ by the reciprocal of maximal growth at time t ($1/x_{max}(t, M_0)$) into equation 3 results in:

$$\frac{1}{x(t, M)} = \frac{1}{x_{max}(t, M_0)} + \boldsymbol{B} \cdot (M). \tag{4}$$

If the metal concentration approaches zero, no growth inhibition will be observed, so the inverse of growth approaches to the inverse of maximal growth:

$$\frac{1}{x(t, M)} = \frac{1}{x_{\max}(t, M_0)} \Leftrightarrow (M) = 0. \tag{5}$$

When metal concentration increases to a very high and toxic concentration (for theoretical consideration metal concentration approaches infinity), biomass approaches zero.

The slope $\boldsymbol{B}$ can be estimated supposing this hypothesis:

$$x(t) \to \infty \Rightarrow \frac{1}{x(t, M)} \to 0,$$

Therefore,

$$0 = \frac{1}{x_{\max}(t, M_0)} + \boldsymbol{B} \cdot (M_x) \tag{6}$$

$$\Rightarrow -\frac{1}{x_{\max}(t, M_0)} = \boldsymbol{B} \cdot (M_x) \tag{7}$$

$$\Rightarrow \boldsymbol{B} = -\frac{1}{x_{\max}(t, M_0) \cdot (M_x)}. \tag{8}$$

Where $(M_x)$ is the concentration of heavy metal $M$ necessary to completely inhibit microbial growth ($x(t, M) = 0$). The concentration $M_x$ is a constant for each relationship between microorganism and heavy metal under given experimental conditions, and arbitrarily it can be defined as growth inhibition metal constant: $K_i$. Substitution of equation (8) into equation (4) follows:

$$\frac{1}{x(t, M)} = \frac{1}{x_{\max}(t, M_0)} - \frac{1}{x_{\max}(t, M_0) \cdot K_i} \cdot (M) \tag{9}$$

The reciprocal of equation (9) is:

$$x(t, M) = x_{\max}(t, M_0) - \frac{x_{\max}(t, M_0) \cdot K_i}{(M)} \tag{10}$$

which rearranged can be to:

$$\Rightarrow x(t, M) = x_{\max}(t, M_0) \cdot \frac{\left[1 - K_i\right]}{(M)} \tag{11}$$

In consequence, the ratio between $K_i$ and $(M)$ determines the cessation of growth. Also, constant $K_i$ can be considered as a measure of "microbial sensitivity" to the metal under the experimental conditions.

For the five selected metal resistant actinomycetes, values of linear correlation coefficients (r) in the range of 0.91 to 0.98 for first-order equation 9 were found (Table 1). Comparison between experimental results and mathematical approach using the most promising metal-resistant wild-type actinomycete strains are in good agreement and are shown in Table 1.

Table 1
Microbial growth inhibition by $Cd^{2+}$ and $Cu^{2+}$ in five wild-type actinomycete strains

| Metal | Strain | $\frac{1}{x_{max}(t, M_0)}$ | | $r$ | $K_i$ | $\frac{1}{K_i \cdot x_{max}(t, M_0)}$ |
|---|---|---|---|---|---|---|
| | | Exp | Calc | | | |
| $Cd^{2+}$ | R06 | 1.05 ± 0.25 | 1.18 | 0.96 | 0.078 | 15.01 |
| | R16 | 0.62 ± 0.11 | 0.6 7 | 0.95 | 0.047 | 14.31 |
| | R25 | 0.78 ± 0.16 | 0.88 | 0.97 | 0.23 | 3.80 |
| $Cu^{2+}$ | R10 | 0.73 ± 0.26 | 0.60 | 0.98 | 0.057 | 10.53 |
| | R25 | 1.05 ± 0.25 | 0.81 | 0.91 | 0.27 | 3.06 |
| | R27 | 1.06 ± 0.30 | 1.36 | 0.95 | 0.27 | 5.10 |

Abbreviations: Exp., experimental; Calc., calculated; *r*, correlation coefficient

Analysis of growth inhibition metal constant ($K_i$) for cadmium in the three cadmium-resistant strains showed 3 to 5 times higher values for strain R25 as compared with R06 and R16 (Table 1). Also, for R25 the slope is more than 3 times lower than that of the other two strains, R06 and R16. In conclusion, strain R25 is more resistant to $Cd^{2+}$ than R06 and R16 strains. However, even the strains R06 and R16 show approximately the same capacity for cadmium uptake as high Known for cadmium-biosorbents *Ascophyllum* sp., *Eclonia radiata, Sargassum* sp. and commercial resins (VOLESKY and HOLAN 1995, KRATOCHVIL and VOLESKY 1998).

Regression analysis of copper growth inhibition curves in the most promising copper-resistant wild-type actinomycete strains showed $x_{max}(t, M_0)$ differing two times between the three strains (Table 1). On the contrary, differences on slopes and $K_i$ were approximately five times between strains R10 and R25–R27 (Table 1, Fig. 3). Also, the two selected wild-type actinomycetes R25 and R27 displayed higher tolerance to $Cu^{2+}$ than other strains of actinomycetes selected, and other biosorbents reported previously (ABBAS and EDWARD 1989, VOLESKY and HOLAN 1995).

Taking in account $K_i$, the slope ($1/K_i \cdot x_{max}(t, M_0)$) as a measures of metal toxicity or resistance, the model presented here systematized and confirmed previous empirical data of wild-type R25 actinomycete. The R25 actinomycete could be very useful for bioremediation of contaminated places, especially for sites containing simultaneously cadmium(II) and copper(II) at toxic level. However, the model presented here was developed considering the growth toxic effect of one single parameter. Simultaneous presence of two or more toxic compounds on wild-type actinomycetes are under study in our laboratory.

In conclusion, the described model provides a simple technique that can be used to predict the behaviour of actinomycetes in presence of inhibitory concentrations of metals under different physical and/or chemical conditions (e.g. T and pH). It can be useful to compare several growth curves of different microorganisms in presence of inhibitory concentration of one heavy metal, and to reduce measured data to a limited number of parameters.

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## References

ABBAS, A. and EDWARD, C., 1989. Effects of metals on a range of *Streptomyces* species. Appl. Environ. Microbiol., **55**, 2030–2035.

AMOROSO, M. J., CASTRO, G. R., CARLINO, F. J., ROMERO, N. C., HILL, R. T. and OLIVER, G., 1998. Screening of heavy metal-tolerant actinomycetes isolated from the Salí river. J. Gen. Appl. Microbiol., **44**, 129–132.

CHANG, J. S. and HONG, J., 1994. Biosorption of mercury by the inactivated cells of *Pseudomonas aeruginosa* PU21 (Rip64). Biotechnol. Bioeng., **44**, 999–1006.

DIXON, M. and WEBB, E. C., 1979. In: Enzymes (M. DIXON, and E. C. WEBB, Eds.), pp. 60–61. Academic Press, Florida.

DUXBURY, T., 1981. Toxicity of heavy metals to soil bacteria. FEMS Microbiol. Lett., **11**, 217–220.

FARREL, R. E., GERMIDA, J. J. and HUANG, P. M., 1993. Effects of chemical speciation in growth media on the toxicity of mercury (II). Appl. Environ. Microbiol., **59**, 1507–1514.

GODDARD, P. A. and BULL, A. T., 1989. Accumulation of silver by growing and non-growing populations of *Citrobacter intermedius* B6. Appl. Microbiol. Biotechnol., **31**, 314–319.

KRATOCHVIL, D. and VOLESKY, B., 1998. Advances in biosorption of heavy metals. Tibtech., **16**, 291–300.

MCELDOWNEY, S., 1994. Effect of cadmium and zinc on attachment and detachment interactions of *Pseudomonas fluorescens* H2 with glass. Appl. Environ. Microbiol., **60**, 2759–2765.

OKAMI, Y., BEPPU, T. and OGAWARA, H., 1988. In: Biology of Actinomycetes (Y. OKAMI, T. BEPPU and H. OGAWARA, Eds.). Japan Scientific Press, 38–47.

PIRT, S. J., 1995. In: Principles of Microbe and Cell Cultivation (J. S. PIRT, Ed.), pp. 4–14. Blackwell Scientific Publications, London.

RAVEL, J., AMOROSO, M. J., COLWELL, R. and HILL, R. T., 1998. Mercury-resistant actinomycetes from Chesapeake Bay. FEMS Microbiol. Lett., **162,** 177–184.

VOLESKY, B. and HOLAN, Z. R., 1995. Biosorption of heavy metals. Biotechnol. Prog., **11**, 235–250.

WALKER, C. W. and HOUSTON, C. W., 1981. Toxicity of cadmium to bacteria. Biotechnol. Lett., **3**, 437–442.

Mailing address: Dr. M. J. AMOROSO, PROIMI, PJE Bel Grano y Caseros, 4000 San Miguel de Tucumán, Argentina
E-mail: mjamoroso@ciudad.com.ar