J Indian Inst Sci., July–Aug. 1999, **79**, 295–302

Toxic effect of lead on the growth of *Penicillium* species

C. ACHARYA, S. ACHARYA, R. N. KAR AND L. B. SUKLA
Regional Research Laboratory (RRL), Bhubaneswar 751 013, Orissa, India
email: celin_s@yahoo.com and balinki@yahoo.com

Abstract

The growth of *Penicillium* species is studied in mineral salt medium, both in the absence and presence of lead acetate and sucrose at room temperature. A linear and maximum growth takes place in ≤ 4 days. While sucrose favours, lead acetate inhibits fungal growth. The effect of lead shows a two-step inhibition indicating its role in both log and lag phases. The tolerance limit for lead acetate increased with increase in sucrose supplement. Under optimum conditions (8 days, 10% (w/v) sucrose) the tolerance limits, LD_{50}, LD_{100}, were found to be less than 50, 65 and 70 mg l^{-1} of Pb, respectively.

Keywords: *Penicillium*, lead(II) acetate, sucrose, biomass, toxicity

1. Introduction

Filamentous fungi belonging to the genus *Penicillium* are used in industrial fermentation processes and antibiotic production.[1, 2] Metabolic products of this fungus can be used for solubilisation of various metals.[3] Bioprocessing of metals by this fungus is another important aspect of biotechnology that is being actively explored.[4, 5] However, environmental awareness and legislation impose stringent restrictions on the bioprocessing of hazardous wastes, effluents and byproducts.

While leaching lead oxide, a zinc plant waste (obtained from Hindustan Zinc Ltd, Vishakhapatnam, India) using *Penicillium* sp., it was found that the organism did not grow satisfactorily and also there was insignificant leaching of metals.[6] The growth inhibition might have been due to toxic effect of heavy metals such as lead and cadmium. The present investigation was undertaken to study the effect of lead on the growth of *Penicillium* species. The work has direct relevance for fungal leaching of lead-containing minerals and waste materials containing $\geq 0.07\%$ Pb.

2. State-of-the art: Fungi and lead toxicity

There are various ways in which microorganisms interact with metals. Many reviews have dealt mainly with the *in-vitro* studies of biochemical and physiological mechanisms where metals exert their effect on microorganisms.[7] Heavy metals influence microbes by affecting their growth, morphology and biochemical activities. Metals, which are not required for biological functions, are considered as pollutants and are toxic to microorganisms. Lead belongs to this class of metals[7] and is toxic to all microbes. The apparent toxicity of lead to fungi has been attributed to the depletion of phosphate of the cells by precipitation as $Pb_3(PO_4)_2$.[8] About 50 mg l^{-1} of lead reduced the growth of *Rhizoctonia solani* and *Aspergillus gigantus*,

and 500 mg l^{-1} inhibited *Penicillium brefeldianum*.[7, 9, 10] About 70 mg l^{-1} Pb was found to be toxic to *Penicillium* species in the present study. However, repeated adaptation of *Penicillium* sp. to Pb is likely to increase its tolerance level,[7] which would find potential application for lead containing/contaminated materials and wastes. Further work in this direction is worth pursuing.

3. Materials and methods

3.1. *Microorganism*

A laboratory stock culture of *Penicillium* species was used in the present study. The organism was grown in 100-ml batch in 250-ml Erlenmeyer flasks at room temperature (34 ± 2°C) in mineral salt medium (g l^{-1}) containing NH_4NO_3, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5 adjusted to pH 6.5 in the absence and presence of Pb(II) ≤150 mg l^{-1}, and supplemented with sucrose doses of ≤15% (w/v) at 150 rpm.

3.2. *Source of lead*

Lead (II) acetate trihydrate (AnalaR, BDH, India) was used as the source of lead. Concentration of lead was varied from 0 to 150 mg l^{-1} in the growth medium to study its effect on *Penicillium* species.

3.3. *Method*

Flasks with media were sterilized by autoclaving (120°C, 20 min) and were inoculated with 1 ml of spore suspension of *Penicillium* containing about 10^6 spores/ ml. Incubation was carried out for different durations (≤20 days). pH measurement and harvesting of fungal biomass were done after inactivation by autoclaving. Biomass was obtained by filtration using pre-weighed Whatman No.1 filter paper. Weights of the biomass, w (an indicator of fungal growth), were measured using a single pan balance after drying in an oven at 100°C for 1h.[11]

4. Results and discussion

Penicillium sp. grows in mineral salt media in the presence of sucrose (Figs 1 and 2). Usually, fermentation of sugars is carried out under similar condition.[12] The pH of the medium decreases from 6.5 to as low as 2.8 due to the release of various carboxylic acids as metabolites.[13] The growth is significantly lower in the presence of lead acetate (Fig. 2).

Pb(II) is likely to be precipitated out a) as $PbSO_4$ and $Pb_3(PO_4)_2$ due to the presence of SO_4^{2-} and PO_4^{3-} in the mineral salt media (Table 1), b) on the cell wall as $Pb_3(PO_4)_2$ due to bio-accumulation process.[13] The soluble part of the Pb(II) remains as Pb^{2+}, $PbOH^+$ and PbA_n^{2-n}, where A^- is acetate ion in the media. Due to increase in fungal growth, the concentration of carboxylic acid (such as oxalic, citric, succinic, etc. acids) metabolites increases resulting in the decrease of pH and binding of Pb(II) by carboxylate anions through chelation (Table I) leading to a) resolubilisation of $PbSO_4$ and $Pb_3(PO_4)_2$ and b) less possibility of damage by phosphate to functional groups of fungal cell wall.[7]

$$PbA_2 = Pb^{2+} + 2\,A^- \qquad \log K_{sp} = 2.75 \tag{1}$$

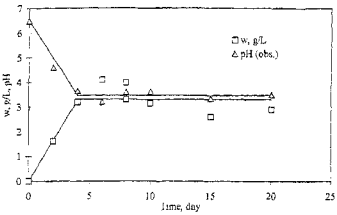

FIG 1. Effect of time on biomass and pH

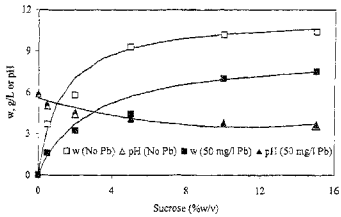

FIG. 2. Effect of sucrose and 50 mg/l Pb on biomass and pH

$$Pb^{2+} + H_2O = PbOH^+ + H^+ \qquad \log K_{h1} = -7.71 \qquad (2)$$

$$Pb^{2+} + SO_4^{2-} = PbSO_4 \qquad \log K_{sp} = 5.54 \text{ to } 7.79 \qquad (3)$$

$$\text{Glycerol 2-phosphate} \xrightarrow{\text{Phosphatase}} \text{Glycerol} + PO_4^{3-} \qquad (4)$$

$$Pb^{2+} + PO_4^{3-} = Pb_3(PO_4)_3 \qquad \log K_{sp} = 42.1 \qquad (5)$$

$$Pb^{2+} + H_nL = [PbH_mL]^{(n-m-2)-} + (n-m)\,H^+ \qquad \log K_1 \text{ (see Table I)} \qquad (6)$$

where H_nL are carboxylic acids (fungal metabolites).

4.1. *Factors affecting the growth of Penicillium species*

4.1.1. *Effect of time*

The growth, w, vs time, t is, linear (≤ 4 days) and remains constant beyond this period in mineral salt media in the presence of 2% (w/v) sucrose:

$$w\ (\text{g l}^{-1}) = At \approx 1.1t\ (\text{day}) \qquad (7)$$

Such type of growth kinetics was already observed in many other studies.[19, 20] With increase in time, the weight of the fungal biomass (or growth) increases almost linearly till about 4 days at 2% (w/v) sucrose in the presence of 50 mg l^{-1} Pb (Fig. 2) similar to the trend in the absence

Table I
Stability constant and solubility product of some selected Pb(II)–ligand species at 25°C[4-18]

Ligand	Log K_{sp}	Log K_1	Log K_2	Log K_3	Log K_4
Hydroxide	17.12	–7.71	–17.12	–28.06	
Sulphate	5 54–7.79	2.4			
Phosphate	42.1				
Acetate	2.75	2.1–2.17	2.98	3.18	3 4
Oxalate	9.32				
Succinate		2.4–2.8	3.73	4.11	
Malonate		2.6–3.1	6.7–8.9		
Citrate		4.08–4.34	3.62	4.32	
D-gluconate		2.6			

of Pb. However, both lag and log phases ($\lesssim$ 4 days) could not be distinguished. After 4 days, the growth vs time attains more or less a steady constant value of 3.3 g l^{-1} (Fig. 1).

$$w\ (\text{g l}^{-1}) = A't \approx 0.82t\ (\text{day}). \tag{8}$$

The steady weight of biomass with time ($\gtrsim$ 4 days) may be due to the exhaustion of sucrose. To study the effect of other parameters such as sucrose and lead acetate concentrations, the growth (in terms of biomass weight, w) at 8th day is monitored.

4.1.2. *Effect of sucrose*

The growth of the fungus was found to increase about 250-fold with increase in sucrose concentration, [S] $\leq$ 15% (w/v) (Fig. 2). However, beyond 10% (w/v) sucrose dose, there was only a marginal increase in growth. The above trend can be expressed in a Michael Menten's-type relation (or for simplicity in an empirical power equation) as:

$$w\ (\text{g l}^{-1}) \approx (a + b[\text{S}])/(1 + c[\text{S}]) \approx p + q[\text{S}]^r \approx 0.044 + 4.98[\text{S}]^{0.3}\ R^2 = 0.97 \tag{9}$$

where a, b, c, p, q and r are empirical constants. In the absence of sucrose, we get eqn (10).

$$w\ (g\ \text{l}^{-1}) \approx a \approx p \approx 0.044. \tag{10}$$

At [S] $\leq$ 15% (w/v), eqn 9 can be rearranged as:

$$(w-a)/[\text{S}] = X\ (\text{say}) \approx b - cw \approx (10.88 \pm 0.07) - (0.973 \pm 0.012)w, \qquad R^2 = 0.9996. \tag{11}$$

The values of b and c could be obtained as 10.88 and 0.973, respectively, from the intercept and slope of the X vs w plot.

4.1.3. *Effect of sucrose in the presence of 50 mg l^{-1} lead*

Similar trend of growth vs sucrose concentration, [S] (Michael Menten's type) is observed both in the absence and presence of lead(II) at 50 mg l^{-1} (Fig. 2)

$$w\ (\text{g l}^{-1}) \approx (a' + b'[\text{S}])/(1 + c'[\text{S}]) \approx p' + q'[\text{S}]^{r'} \approx 0.017 + 2.06[\text{S}]^{0.5} \qquad R^2 = 0.986 \tag{12}$$

where a', b', c', p', q' and r' are empirical constants. In the absence of sucrose and lead(II), eqn (12) reduces to eqn (13).

$$w\ (\text{g l}^{-1}) \approx a' \approx p' \approx 0.017. \tag{13}$$

At sucrose $\lesssim$ 15% (w/v), eqn (12) can be rearranged as

$$(w - a')/[\text{S}] = X'\ (\text{say}) \approx b' - c'w \approx (3.2 \pm 0.1) - (0.36 \pm 0.02)w \qquad R^2 = 0.988. \tag{14}$$

The plot of X' vs w yields the values of b' and c' as 3.26 (intercept) and 0.36 (slope), respectively. The growth vs pH_f is related as $pH = pH_i - w^2$ indicating their direct relationship.

4.1.4. *Effect of lead(II) acetate*

The growth of fungus is low in the absence of sucrose and it became very low in the presence of added lead acetate, making it difficult for measurement. However, the effect of lead could be studied conveniently in the presence of added sucrose supplement. For this purpose, the

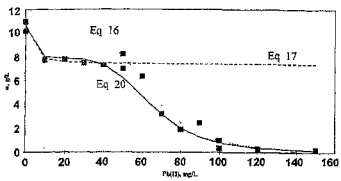

FIG. 3. Effect of Pb(II) on biomass.

dose of sucrose was fixed at 10% (w/v), which is the maximum level in growth vs sucrose plot (Fig. 3). It can be seen that the growth of the fungus is found to decrease almost to a constant level of about 25–30% lower than the control in the Pb level of 5 to 50 mg l^{-1} in the first step. Further increase in lead concentration showed a very high rate of growth decrease in the range of 50 to 100 mg l^{-1} (Fig. 3). The fungal growth became more or less constant at 0.22 g l^{-1} above 150 mg l^{-1} Pb. Both the overall LD_{50} and LD_{100} of the second phase fall in lead level of 60–70 mg l^{-1} (or more accurately at 62 and 68, mg l^{-1}), respectively, coinciding with LD_{50}. The growth inhibition due to the presence of lead acetate can be expressed as:

$$w\ (\text{g l}^{-1}) \approx (f + \Sigma g_i[\text{Pb}]^i)/(1 + \Sigma h_j[\text{Pb}]^j) \qquad (15)$$

where f, g_i, h_j are empirical constants, and $i, j = 1, 2, \ldots, n$.

The two-step decrease in growth with increase [Pb] can be treated as follows:

a) The first portion of growth vs [Pb] ≤ 50 mg l^{-1} could be fitted (Fig. 3) as:

$$w\ (\text{g l}^{-1}) \approx (f + g_1[\text{Pb}])/(1 + h_1[\text{Pb}]) \approx (10.6 + 3.8[\text{Pb}])/(1 + 0.55[\text{Pb}]). \qquad (16)$$

However, more data at [Pb] ≤ 10 mg l^{-1} is needed to precisely determine the exact values of g_1 and h_1 since this type of data would be close to the permissible level of lead contamination and its toxicity relevant to potable water system and aquatic environment (0.05 mg l^{-1} Pb). In this concentration range, most probably a major portion of Pb(II) precipitates or forms complex precipitates as $PbSO_4$ and/ or $Pb_3(PO_4)_3$ as the mineral salt media contains these anions.[16] The data in Table I indicate that the K or $K_{sp} > [\text{Pb}]^m[\text{X}^{m-}]^n$ for Pb_nX_m where $X^m = SO_4^{2-}$, PO_4^{3-}, etc. and hence most of the Pb(II) is likely to be present as $PbX_{m'}$ complex species and/ or Pb_nX_m solid species. So the decrease in growth may be due to decrease in these nutrients and/ or toxicity of very low concentration level of soluble Pb(II) species.

b) The second portion of growth vs [Pb] ≥ 50 mg l^{-1} could be fitted (Fig. 3) as:

$$w\ (\text{g l}^{-1}) \approx f'/(1 + h'_k[\text{Pb}]^k \approx 10.6/(1 + 1 \times 10^{-9}[\text{Pb}]^5]. \qquad (17)$$

Comparing eqns (16) and (17), we observe that $f \approx f' \approx 10.6$ g l^{-1}. The very high concentration range of lead is relevant to the bioleaching of lead a) containing minerals and ores and b) contaminated industrial byproducts and wastes. This concentration range most probably exceeds the threshold level of anions (SO_4^{2-} and PO_4^{3-}) of the media responsible for the precipitation of Pb(II) and hence the toxicity of all the soluble Pb(II) species (such as Pb^{2+}, $PbOH^+$,

PbA_n^{2-n}, etc. contribute to decrease in fungal growth. The high value of $K \approx 1 \times 10^{-9}$ indicates the possible multiple interaction of Pb(II) species with the fungal cell wall.

c) The whole range of [Pb] ≤ 150 mg l^{-1} studied for its effect on fungal growth can be approximated as the simple factor contribution of the above two concentration ranges:

$$w\ (\text{g l}^{-1}) \approx (10.6 + 3.8[\text{Pb}])/\{(1 + 0.55[\text{Pb}])(1 + 1 \times 10^{-9}[\text{Pb}]^5)\}. \tag{18}$$

However, eqn (18) is found to be overestimating the decrease of growth in the range of 20 < [Pb] < 80 mg l^{-1}. By incorporating another factor, $(h' + h_1''\ [\text{Pb}])$ in the numerator, the fitting is found to be satisfactory.

$$w(1 + h_1[\text{Pb}])(1 + h_6[\text{Pb}]^6)/(f + g_1[\text{Pb}]) \approx f' + g'_1[\text{Pb}] \approx 1 + 5 \times 10^{-3}\ [\text{Pb}] \tag{19}$$

$$w\ (\text{g l}^{-1}) \approx (10.6 + 3.8[\text{Pb}])(1 + 5 \times 10^{-3}[\text{Pb}])/\{(1 + 0.55[\text{Pb}])(1 + 1 \times 10^{-9}[\text{Pb}]^5)\}$$

$$\approx (f + g_1[\text{Pb}] + g_2[\text{Pb}]^2)/(1 + h_1[\text{Pb}] + h_5[\text{Pb}]^5 + h_6[\text{Pb}]^6)$$

$$\approx (10.6 + 3.8[\text{Pb}] + 1.9 \times 10^{-2}[\text{Pb}]^2)/(1 + 0.55[\text{Pb}] + 1 \times 10^{-9}[\text{Pb}]^5 + 5.5 \times 10^{-10}[\text{Pb}]^6) \tag{20}$$

Equation 20 indicates that the system is very complex due to a) precipitation of Pb(II), SO_4^{2-} and PO_4^{3-} in the minimal salt media through $h_{i \le 1\ 5}$, b) Pb(II) attacks the fungal cell wall in multiple sites as indicated by $h_{i \ge 5}$, and c) the possible fungal tolerance for Pb(II) toxicity due to some other interactions as quantified by the g_i coefficients.

4.1.5. *Combined effect of parameters*

The overall effect of all the parameters in the whole range of study can be expressed as:

$$w\ (\text{g l}^{-1}) = (A + \sum B[\text{Pb}]^i)[\text{S}]t/(1 + C[\text{S}] + Dt + \sum E_i[\text{Pb}]^j) \tag{21}$$

At $t \le 4$ days and [S] ≤ 5% (w/v) the growth shows the following:

a) At [Pb] ≤ 20 mg l^{-1}, $w\ (\text{g l}^{-1}) = A[\text{S}]t/(1 + E_1[\text{Pb}])$ (22)

b) At 20 ≤ [Pb] ≤ 50 mg l^{-1}, $w\ (\text{g l}^{-1}) = (A + B_1[\text{Pb}])[\text{S}]t/(1 + E_1[\text{Pb}])$. (23)

Equations (21–23) would be very useful for scale up and simulation purpose.

Microbe–metal interactions are very complex and the estimation of binding constants for such systems are not easy.[7] Binding of *Penicillium* sp. with Pb in more than one binding/interaction steps is observed similar to metal–ligand systems. Attempts to resolve such complex systems through *Penicillium* sp.–Pb(II) toxicity through eqns (16–23) were dealt with is interesting to note that the initial mild toxicity <50–70 mg l^{-1} Pb gives a binding constant of about 3.8 mg l^{-1}. However, above this level, there is a multiple binding of Pb, viz. ≥ 5 with a binding constant of the order of 1×10^{-9} without any adaptation constant. So multiple adaptation starting with the initial 50–70 mg l^{-1} Pb may increase Pb tolerance in subsequent generations of *Penicillium* sp. which will have potential application for bioprocessing of Pb-containing/contaminated materials. The findings of the above study would be relevant to the fermentation process, bioleaching of lead containing materials and aquatic environment in general.

5. Conclusions

1. The biomass growth of *Penicillium* species increases almost linearly with time and attains maximum level at ≤ 4 days in minimal salt media containing 2% (w/v) sucrose both in the absence and presence of 50 mg l^{-1} Pb. Beyond this period, there is no increase but a marginal decrease in biomass weight.
2. The growth increases with the increase in sucrose concentration <15% (w/v) both in the absence and presence of 50 mg l^{-1} Pb.
3. The growth shows a two-step decrease with increase in [Pb]< 150 mg l^{-1}. The first step at [Pb] ≤ 50 mg l^{-1} shows about 25% decrease in growth where Pb(II) undergoes mainly exogenous precipitation by the media constituents. The second-step decrease of growth at 50 ≤ [Pb] ≤ 150 mg l^{-1} may be due to the crossing of the threshold limit of Pb(II) precipitation by the media resulting in the damage of fungal cell wall phosphate functional groups and bioaccumulation of Pb.
4. Growth vs pH indicated a direct relationship.
5. The effect of parameters such as time, sucrose and lead could be empirically related to the biomass growth.

Acknowledgements

The authors wish to thank Dr H. S. Ray, Director and Dr R. P. Das, Head of the Department, Hydrometallurgy Division, RRL, for permission to publish the work and for valuable suggestions. The first author wishes to thank the Council for Scientific and Industrial Research for awarding a fellowship.

References

1. HOCKENHULL, D. J. D. — *Progress in industrial microbiology*, Vol. 12, Academic Press, 1973, pp. 47–72.
2. SYLVESTER, J. C AND COGHILL, R. D. — The penicillin fermentation. (In *Industrial fermentation*, Vol. II, (L. A. Underfloker and R. J. Hickey, eds), Chemical Publishing Co., 1954, pp. 219–263.
3. THAYER, J. S., OLSON, G. J. AND BRINCKMAN, F. E. — Iodomethane as a potential metal mobilising agent in nature, *Environ. Sci Technol*, 1984, **18**, 726–729.
4. BURGSTALLER, W. AND SCHINNER, F. — Leaching of metals with fungi, *J. Biotechnol.*, 1993, **27**, 91–116.
5. FRANZ, A., BURGSTALLER, W. AND SCHINNER, F. — Leaching with *Penicillium simplicissium*: Influence of metals and buffers on proton extraction and citric acid production. *Appl. Environ. Microbiol.*, 1991, **57**, 769–774.
6. BHATTACHARYA, B. — *Development of a microbial process for the recovery of metal values from industrial wastes*, M.Sc. Dissertation, Orissa University of Agriculture and Technology (OUAT), Bhubaneswar, India, 1997.
7. BEVERIDGE, T. J. AND DOYLE, R. J. — *Metal ions and bacteria*, Wiley Interscience, 1989, pp. 32–44.
8. VALLEE, B. L. AND ULMIS, P. D. — Biochemical effects of mercury, cadmium and lead, *A. Rev. Biochem.*, 1972, **41**, 91–96.

9. BABICH, H. AND STOTZKY, G — Abiotic factors affecting the toxicity of lead to fungi, *Appl. Environ. Microbiol.*, 1979. **38**, 506–509.

10 SEIGEL, S. *et al.* — Biosorption of lead and chromium by *Penicillium* preparations, *Wat. Air Soil Pollution*, 1986, **27**, 69–73

11. SWAMY, K M , SUKLA, L. B , NARAYANA, K. L , KAR, R N AND PANCHANADIKAR, V. V. — Application of ultrasonics in improvement of fungal strain, *Acoust Lett.*, 1993, **17**(3), 45–49.

12. MARK, H. F. *et al.* (EDS) — *Kirk–Othmer, Encyclopedia of chemical technology*, Vol. 9, Wiley, 1980, pp. 861–880.

13 YONG, P. AND MACASKIE, L. L. — Bioaccumulation of lanthanum, uranium and thorium and use of a model system to develop a method for the biologically mediated removal of plutonium from solution, *J. Chem. Technol. Biotechnol.*, 1998, **71**, 15–26.

14. MORTIMAR, C. E. — *Chemistry. Concept, approach*, Appendix B, Reinhold, 1968, pp. 668–687.

15. BAES, C. F. AND MESMER, B. E. — *Hydrolysis of cations*, Wiley, 1976, pp 358–365

16. EILEN, L. G. AND MARTEL, A. E. — *Stability constants of metal ion complexes*, Supplement No.1, Special Publication No.25, The Chemical Society, London, 1977.

17. MARTEL, A. E. AND SMITH, R. M. — *Critical stability constants*, Vol 3· Inorganic complexes and Vol. 4: Other organic ligands, Plenum, 1977.

18. DEAN, J. A (ED) — *Lange's handbook of chemistry*, McGraw-Hill, 1987.

19. KARAVAIKO, G. I. *et al.* — *Biogeotechnology of metals manual*, Centre for International Projects, GKNT, Moscow, 1988

20. CHOUDHURY, S. — *Bioleaching of lateritic nickel ore using fungal strains*, M.Sc. Dissertation, Orissa University of Agriculture and Technology (OUAT), Bhubaneswar, India, 1994.