Application of bacterial biomass as a potential metal indicator

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Bioindicators are biological systems which can be used to indicate the presence of certain elements. The basis of indication are the changes that occur either in their metabolism or in phenotype of the organisms due to the uptake and accumulation of these elements. The threat of environmental pollution due to enhanced availability of both essential and toxic metals is a matter of great concern. Application of any remedial measure requires proper assessment of the contaminated sample. We used a multimetal-resistant organism, *Pseudomonas stutzeri* RS34, to indicate the presence of Cu^{2+} and Co^{2+} in solution as well as in an industrial effluent. Metal resistance mechanism in this organism is based on biosorption and strain RS34 showed preferential adsorption for Cu^{2+}. This accumulation imparted green colour to the biomass in a concentration-dependent manner (2.0 ppm and higher concentration). The colour is seen even in bimetal or multimetal conditions, where Co^{2+}, Zn^{2+} and Mn^{2+} were present along with Cu^{2+} and is also observed in an effluent. Co^{2+} could also be detected by the biomass acquiring a characteristic pink colour. We thus propose that strain RS34 can be employed as bioindicator of certain metals simply based on appearance of colour. This method is not only economical, but also quick and easy to operate.

Keywords: Bioindicator, biomass, heavy metals, *Pseudomonas stutzeri*.

THE concept of bioindicator is not entirely new. Although the complexity of the interactions between organisms and their environment is generally not easily comprehensible, environmental quality assessment using the bioindicator approach offers some convincing advantages compared to direct analysis of soil, water and air[1]. The main advantages of the bioindicator are relatively low cost compared to analytical methods of measurement, and rapidity[2]. Moreover, unlike analytical and electrochemical methods, little pretreatment of samples is required and only the bioavailable concentration of toxic material is measured[3]. These systems are also known as biosensors or bioreporters[4,5].

Presence of heavy metals in the environment is a matter of concern due to their nonbiodegradable nature, thus making them an important class of environmental pollutants. WHO and other environmental agencies have specified the safe limit of these metals in drinking water as well as water used for other purposes[6].

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The general methods used for checking the presence of metals in air, water and soil are chemical-based. These chemicals are not only costly and require exhaustive analysis in specialized laboratories, but in the long term they may cause environmental damages. The use of bioindicators to check the presence of metals in the environment has thus gained significance. Such an application involves both phenotypic and analytical parameters[7–9]. Of the two, the former is quick, inexpensive and eco-friendly.

Among the several systems employed as bioindicators, unicellular microorganisms, in particular bacteria are advantageous. Their large population size, easy maintenance, low cost and rapid growth rate make them a lucrative option for pollution monitoring. They often show altered phenotype in the presence of many metals[10–13]. Other than these natural indicators, some indicators based on metal-binding proteins or metal-induced promoters tagged with reporter genes have also been reported[2,3,14–16]. We have studied metal bioaccumulative properties of a multimetal-resistant bacterium[17] *Pseudomonas stutzeri* RS34. This communication presents data on the detection of metals such as copper and cobalt in water or industrial effluent.

Cells were grown in a nutritionally-defined minimal medium (MM) as described earlier[17], with glucose (0.2%) as carbon source. Complete medium used was LB. Analytical grade metal salts were used to prepare 1M stock solution, and sterilized by autoclaving. Bacterial cells were grown in liquid media at 37°C in New Brunswick Scientific Shaker Incubator (Edison, NJ, USA) at 200 rpm. The dead biomass was prepared by boiling the bacterial suspension at 100°C for 15 min. Loss of viability was checked by streaking a loopful of the treated biomass on LB agar plates. Overnight grown cells (live/dead) were suspended in 20 ml of metal solution of appropriate concentration or industrially polluted soil suspension for 2 h and then reharvested, washed and dried. The dried biomass was used to visualize the colour and then processed for metal estimation by Atomic Absorption Spectrophotometric analysis (AAS, model no. 3110, Perkin Elmer), as described earlier[12]. All experiments were conducted in at least three independent sets and a mean is represented along with standard error.

Biosorptive capability of dead and live biomass for the metals Cu^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} (2 mM each) was compared individually. As shown in Figure 1, the dead biomass accumulated more Zn^{2+} and Co^{2+} than live biomass. However, the situation is just the reverse in case of Cu^{2+} and Mn^{2+}. In case of cobalt- and copper-exposed cells, it was observed that the biomass acquired distinctive colour in 2 h of contact time in the metal salt solution (pink with cobalt and green with copper; Table 1, Figure 2), which otherwise appeared colourless. Interestingly, the colour was more intense in dead and dried biomass than in live biomass. Similar results, where dead biomass of different microorganisms has been found to be a more efficient biosorbent of heavy metals, have been reported earlier also[18–20]. The difference could be explained on the basis of active metabolizing cells possess-

ing energized plasma membrane generating competing H^+ ions[21]. Alternatively, in dead state, the cells become more permeable and allow the metal to enter and bind to the internal surfaces and components as well, thus increasing metal biosorption. In case of copper, although the dead cells took up less metal in comparison to live cells, colour of the biomass was more intense in the former case. According to Gadd *et al.*[22], biosorption by dead cells is easy to manipulate, safe and also confers immunity from metal toxicity and other adverse operating conditions.

It is known that many potential anionic sites are available in and on the cell wall[23]. These sites behave as nonspecific ionospheres, and can bind to a variety of metals. Our work has established that the dead bacterial biomass is a suitable material for metal binding, which showed concentration-dependent accumulation of metals. The increasing intensity of colour in the biomass reflects this property in the presence of copper and cobalt. It was observed that even at 2 ppm colour developed in both cases (Figure 2 *A*). After a certain concentration this increase became insignificant, as at that concentration anionic sites and metal ions perhaps reached equilibrium. It was thus inferred that the dead biomass could be used to detect a metal concentration as low as 2 ppm.

The use of this biomass as a potential bioindicator was not diminished in bimetal as well as multimetal conditions (Table 1). It was found that Cu^{2+} was the most preferred metal, except in the presence of Zn^{2+}. When Cu^{2+} is present in the solution, the dead biomass invariably showed a green colour irrespective of the presence of other metal(s) (Figure 2 *B*). In the case of Cu^{2+}-Co^{2+} and Cu^{2+}-Mn^{2+}, preferential sorption of Cu^{2+} over Co^{2+} and Mn^{2+} respectively, has been demonstrated confirming the colour response. However, in case of Cu^{2+}-Zn^{2+} combination, even though biosorption of Zn^{2+} is approximately equal to that of Cu^{2+}, green colour of the biomass is not masked. Our results thus suggested that binding sites for Cu^{2+} and Zn^{2+} are independent. Similarly, in the case of binary mixture of Co^{2+} and Mn^{2+}, colour of the biomass was pink due to preferential sorption of Co^{2+}, while in the case of Co^{2+} and Zn^{2+} combination the colour was light pink, perhaps because the preferential sorption of Zn^{2+} reduced Co^{2+} adsorption (Figure 2 *C*). In the presence of bimetal cations, the decreased biosorption of individual metals can be explained in terms of competition between these ions for the same metal-binding sites[19]. In all binary mixtures, this ionic competition could be easily visualized.

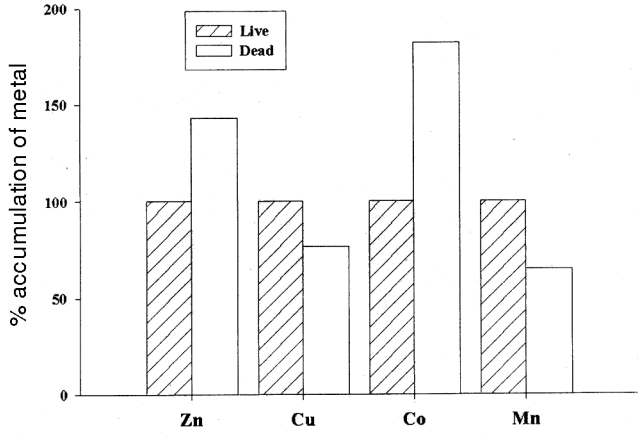

Figure 1. Biosorptive efficiency of live and dead biomass of strain RS34 for different metal ions [Co^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+}, 2 mM each]. Biosorption by live biomass was taken as 100%.

In multimetal ion condition, the present study showed that dead cells of *P. stutzeri* RS34 could accumulate appreciable amounts of metals tested in the order $Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+}$ (Table 1). This result is in accordance with the order of selectivity shown by *Chlorella regularis, Rhizopus arrhizus* and *Aspergillus nodosum*[24–26]. The selectivity is based on the ability of the metal ion to complex a ligand, which itself depends upon its polarizing power, i.e. upon the ratio of the charge and ionic radius. In the present study, copper appears to bind more strongly among the four transition metals. Brady *et al.*[27] also reported the same trend. These results suggest that biosorption of metals could be selective as well as competitive[28]. In such a case the colour developed in the dried biomass was light green, due to possible interference by the other metals (Figure 2 *D*, Table 1).

We also investigated the capability of bacterial biomass to biosorb metals from an industrial effluent to indicate the presence of metal(s). For this, the dead biomass was exposed to a soil suspension, which was prepared by suspending 1 g of soil from dump-site of a brass factory in 5 ml of deionized double-distilled water. The suspension was centrifuged (6000 rpm, 5 min) and supernatant was used as soil suspension. This soil suspension contained 70 ppm of Cu^{2+} (as determined by AAS analysis). We then tested the strain RS34 to detect the presence of Cu^{2+} in this suspension. It was found that the treated biomass accumulated 24 μg Cu^{2+} per mg of dry biomass and gave a light green colour which could be clearly distinguished from untreated biomass (Figure 2 *A*, *E*). The difference in the colour as observed in Figure 2 *D* and *E* could be attributed to the complex constitution of the polluted suspension. Our results with industrially polluted sample clearly established the usefulness of this strain as a metal indicator even under complex situation, where not only multimetal ions but also the occurrence of other chemicals is expected.

While the colour response provided a simple, easy and quick test for the detection of metals, our indicator system also appeared to be cheaper and non-polluting. The desired results were obtained in ~3–4 h, which is less in comparison to other indicator systems, for example[2], 6 h in case of Ni-indicating *Ralstonia eutropha* AE2505. With gene-based biosensors, additional problems have been identified. Since the sensor component consists of viable cells, its usage is restricted by the conditions that affect survival such as pH, temperature and presence of other toxic compounds. The efficiency of binding protein-based sensor is

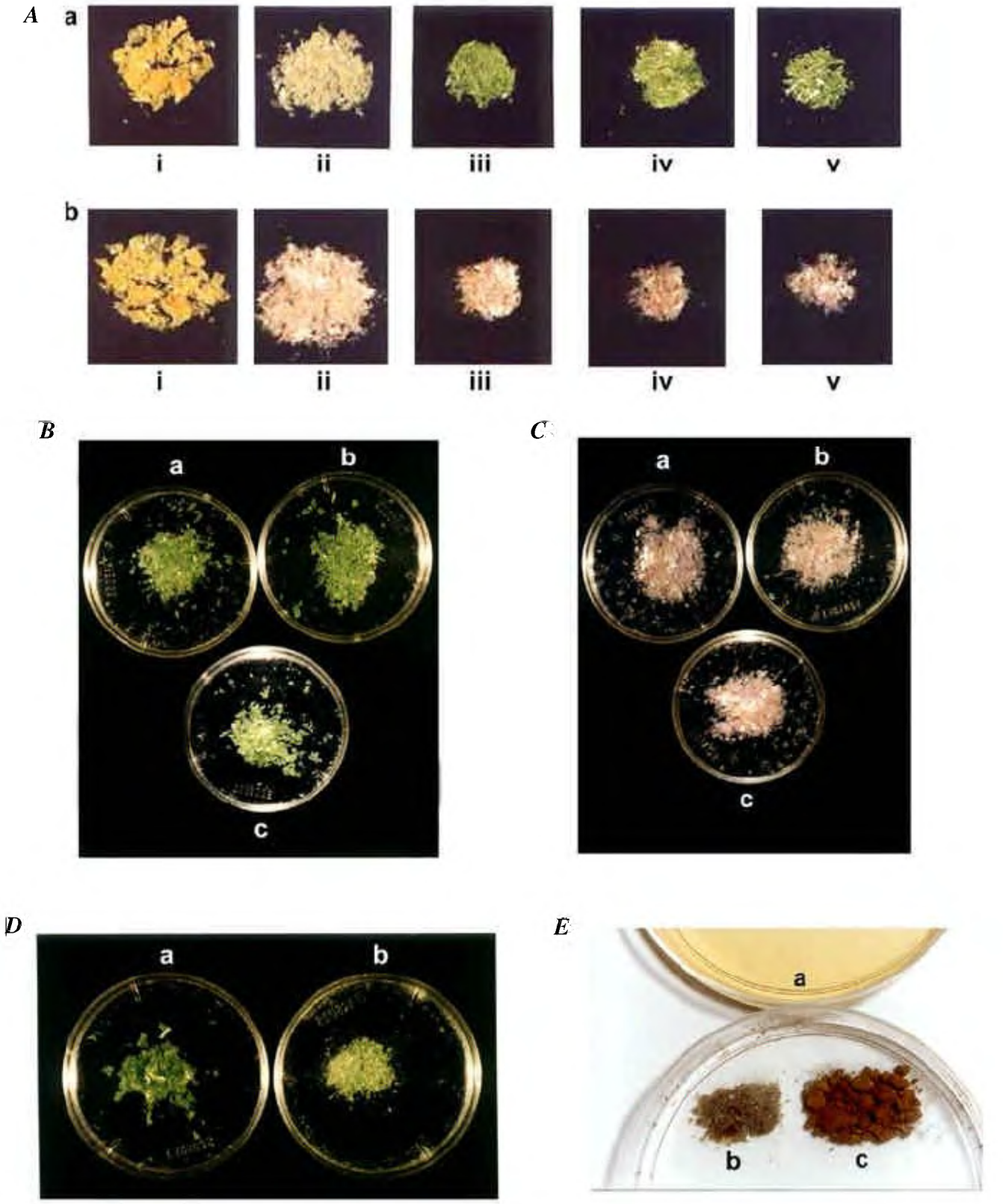

Figure 2. Colour development in dead dried biomass of strain RS34 on exposure to metal(s). (***A***) a/b(i). Unexposed biomass; a(ii-v), Biomass exposed to increasing concentration of Cu^{2+} (2, 10, 100, 200 ppm). b(ii-v) Biomass exposed to increasing concentration of Co^{2+} (2, 10, 40, 80 ppm). (***B***) Biomass exposed to bimetal solutions (2 mM each). a, $Cu^{2+} + Co^{2+}$; b, $Cu^{2+} + Mn^{2+}$; c, $Cu^{2+} + Zn^{2+}$. (***C***) Biomass exposed to metal solution (2 mM each); a, Co^{2+}; b, $Co^{2+} + Mn^{2+}$; c, $Co^{2+} + Zn^{2+}$. (***D***) a, Biomass exposed to Cu solution (2 mM). b, Biomass exposed to multimetal [$Co^{2+} + Cu^{2+} + Mn^{2+} + Zn^{2+}$, 2 mM each] solution. (***E***) Biomass exposed to soil suspension from a dump-site of brass factory. a, Soil suspension; b, Biomass exposed to soil suspension; c, Soil.

Table 1. Accumulation of metals by dead dried biomass after exposure to metal solution for 2 h, and its assessment by colour

Metal (2 mM each)	Colour of biomass	Amount of metal adsorbed (μg/mg DW)
Cu(II)	Green	26.64 ± 2.8
Zn(II)	Colourless*	48.0 ± 2.9
Mn(II)	Colourless	6.66 ± 0.62
Co(II)	Pink	19.3 ± 1.6
Cu(II)/Co(II)	Green	24.4 ± 1.8/1.7 ± 0.18
Cu(II)/Mn(II)	Green	28.4 ± 2.1/2.27 ± 0.3
Cu(II)/Zn(II)	Green	22.15 ± 2.1/25.0 ± 2.3
Co(II)/Mn(II)	Pink	15.34 ± 1.1/1.7 ± 0.2
Co(II)/Zn(II)	Light pink	2.27 ± 0.35/36.36 ± 3.1
Mn(II)/Zn(II)	Colourless	2.84 ± 0.4/35.79 ± 3.0
Cu(II)/Co(II)/Mn(II)/Zn(II)	Light green	22 ± 3.1/5 ± 0.8/0.7 ± 0.2/17 ± 3.7

*Colourless means no colour difference with unexposed biomass.

also expected to go down when applied to natural samples containing mixture of metals[1]. The dead biomass-based sensor employed by us will not be affected by such conditions. The limitation in our system presently is that it is restricted to metals which provide a colour. A suitable counter-staining protocol can be developed to detect other metals.

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