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A rapid screening method for the isolation of metal-accumulating microorganisms

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SUMMARY

An agar plate screening method was developed for the rapid isolation of heavy metal-accumulating microorganisms and preliminary estimation of their biosorption capacity. The test is based on the visualization and interpretation of the metal distribution between agar and colonies by chemical precipitation with hydrogen sulphide or ammonium sulphide. The heavy metals silver, thallium, lead, copper, nickel and cadmium have been tested successfully. The efficiency of the method is demonstrated for isolating silver-accumulating bacteria and estimating silver biosorption capacity.

INTRODUCTION

In recent years there has been an increasing global interest in interactions of heavy metals with microorganisms, as demonstrated by the growing number of papers and scientific projects. The field comprises a number of different aspects, and current research relates to: (1) the study of fundamental physiological and genetic relations in uptake and excretion, demand, function, toxicity and resistance to heavy metals [4,6,13,31,35]; (2) ecological and geological aspects of the role of microorganisms in the cycling and speciation of metals [19,20,30,36]; (3) biomonitoring [5,23,25]; (4) metal preconcentration in trace analysis [14,17]; and (5) biotechnology for the treatment of metal-laden water [9,15,21,27,37–39].

These and related topics often involve the necessity to find microorganisms with an extraordinary capability in accumulating heavy metals. Checking the efficiency of laboratory strains as well as the isolation of microorganisms from natural or secondary habitats are often among the basic tasks to be performed. In order to facilitate and accelerate these tasks we developed an agar-plate screening method which enables the selection of metal-accumulating microorganisms from several dozens of colonies. This paper includes a description of the principle behind the method, some possible variations and their specific significance and a demonstration of the efficiency of the method in the case of silver.

TERMINOLOGY

In accordance with the literature [28], the following terms will be used: 'biosorption' to describe physico-chemical processes of metal binding to microorganisms and 'bioaccumulation' to refer to active processes. As bioaccumulation nearly always succeeds a passive biosorptive process, in cases of unknown or mixed mechanisms the term 'accumulation' will be used.

PRINCIPLE AND POSSIBLE VARIATIONS OF THE METHOD

Basic principle

Agar plates with well-developed colonies are overlayed with a further agar layer containing the metal of interest. After incubation the metal is visualized by precipitation and optical effects are interpreted. The main effects are the staining of colonies due to accumulated and precipitated metal and the formation of light haloes around the colonies within the uniformly darkened agar as a result of the diffusion of dissolved metal towards the organisms. In detail, the following steps are performed:

(1) Growth of colonies on agar plates by either spreading an adequately-diluted suspension of mixed microorganisms (e.g. waste water, soil suspension) or by punctual inoculation with lab strains (tooth-pick technique). Evaluable colony numbers depend on their diameter and range up to about 50. Care must be taken in the selection of appropriate media as metal-complexing or precipitating ingredients may hinder metal accumulation by the organisms [24,26].
(2) Replica plating of chosen 'original' plates if different metals or conditions are to be tested, or if strains are to be isolated.
(3) Exposure of microorganisms to the metal. Replica plates with colonies not too large in diameter (1–4 mm) are carefully overlayed with 8–10 ml of soft agar (about 7 g L^{-1}) containing the metal of interest (usually as the nitrate salt). Useful concentrations to give a uniformly coloured agar

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and sufficiently stained colonies are in the range of 1 to 10 mM and mainly depend on the metal and on the recipe of the nutrient agar (Table 1). In order not to kill the organisms and to prevent the colonies from spreading, the metal agar has to be cooled down to about 55 °C prior to use.

(4) Incubation of metal-exposed colonies at a chosen temperature for a minimum of 15 min to allow the organisms to accumulate the dissolved metal (see variations).

(5) Visualization of the metal. The distribution of the metal within the overlayed agar and the colonies is shown by precipitation. Simple and effective formation of dark precipitates is achieved for several metals by treatment with sulphide. The plates are either exposed to gaseous H_2S in a desiccator (e.g. reacting Na_2S with HCl) or overlayed with a few ml of 2% aqueous $(NH_4)_2S$ solution (Table 1). The implementation of colouring complexants as used in photometric metal assays may also be applicable, but was not studied here.

(6) Interpretation of optical effects. Interactions of the microorganisms with the metal supplied may produce colony staining and/or the formation of light haloes around the colonies. A stereo microscope (×10–20) is a very helpful tool in the observation of small colonies.

(7) Isolation of organisms. From the original plates microorganisms of interest can be taken and further cultivated. Their accumulation properties have finally to be confirmed by quantifying assays and also the genetic stability of their features in continual laboratory cultivation.

Points of variation

Microorganisms. A lot of information exists on positive and negative correlations between the heavy metal accumulation capacity of microorganisms and their respective resistance or tolerance [1,2,10,16,18,22,29,32,33,41]. Selection of the origins of the microorganisms to be subjected to the test mainly depends on the purpose of the study to be performed. No general instructions can be supplied on where and how to find potent microorganisms, e.g. whether screening should start with a heavy metal contaminated site or with a common soil.

TABLE 1

Screening test conditions for the metals tested

Metal	Compound	Concentration range [mM]	Precipitation with
Ag	$AgNO_3$	5–8	H_2S
Tl	$TlNO_3$	3–7	H_2S
Cu	$Cu(NO_3)_2$	6–10	H_2S
Ni	$Ni(NO_3)_2$	3–7	2% $(NH_4)_2S$
Pb	$Pb(NO_3)_2$	2–6	H_2S
Cd	$Cd(NO_3)_2$	1–4	H_2S

Nutrition. The choice of medium serves as the basis for the selection of the target group of organisms and depends on subsequent aims. For practical reasons the isolation of microorganisms needing vitamins or other expensive compounds is to be avoided in many cases. For biotechnological applications it may be appropriate to take into consideration the availability of cheap waste products. Further adjustable parameters for preselection are medium pH, temperature, and the presence of oxygen or toxic substances such as antibiotics or even heavy metals. As already mentioned extra care has to be taken in the chemical composition of the medium due to complexation or precipitation of the metal of interest. In general the contents of phosphates, organic acids, complexing buffers, and complex nitrogen sources should be minimized for most heavy metals.

Metal exposure. With metal exposure there are several adjustable parameters, which determine the final message of the test. Besides the choice of the element, its speciation which is mainly determined by the pH, the ionic strength and the selected compound, are also of great importance [18,19,30]. In order to select microorganisms with a certain minimum affinity for a metal, one could supply metal complexes with known stability constants. The time of exposure is very important as it permits a distinction to be made between passive biosorption and additional active bioaccumulation. Biosorption usually proceeds within several minutes (about 15–20 min are necessary to gain a light halo due to diffusion), whereas bioaccumulation takes hours and may be seen after one or two days. A further method to exclude active bioaccumulation would be the addition of metabolic blockers.

MATERIALS AND METHODS

Isolation of silver-accumulating bacteria (48-h Ag-exposure)

Samples of soils, sewage sludge, landfill, and river sediment were suspended and diluted with glucose solution (5 g L^{-1}) and plated onto medium PHG-II, composed of 4 g L^{-1} peptone, 2 g L^{-1} glucose, 1 g L^{-1} yeast extract and 15 g L^{-1} agar. The plates were incubated for 48 h at 30 °C and 60% relative humidity. Plates with 30–50 colonies were copied by replica plating. The duplicates were incubated until the biggest colonies reached about 4 mm in diameter and were then gently overlayed with 10 ml soft agar (55 °C) containing 6 mM $AgNO_3$ and 7 g L^{-1} agar (the silver salt was added to the agar from a 100-mM stock solution after cooling the stock down to 55 °C). Plates were then kept at 30 °C, 60% humidity in the dark to allow the bacteria to accumulate the metal. After 48 h they were exposed to gaseous H_2S for 10 min in a desiccator. H_2S was generated by reacting 3 g Na_2S with the stoichiometric amount of 10% HCl. Finally the plates were inspected under a stereo microscope and bacteria yielding black colonies were isolated from the original plates and subcultured on PHG-II agar.

Isotherms of silver biosorption were taken for six selected strains and for the ion exchange resin Dowex 50WX8-100 (Sigma, St Louis, MO, USA). The bacteria were grown in liquid medium PHG-II at 30 °C on a gyratory shaker (200 r.p.m.) for 24 h and the dry weight contents were meas-

ured gravimetrically after filtration (0.2-μm pore size membrane filter) and drying at 80 °C overnight. In preliminary biosorption tests the amount of biomass was determined which gave a decrease of the silver concentration in solution of not more than about 20% in order to maintain nearly constant concentration conditions. Aliquots of the culture broth containing these optimum amounts of biomass were centrifuged in acid-leached and thoroughly rinsed 14-ml Polyallomer tubes (Kontron Instruments, Neufahrn, Germany) at 7000 × *g* for 10 min and the pellets were resuspended in 5 ml unbuffered silver nitrate solutions of 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 mM Ag^+, respectively. The tubes were kept at 30 °C in the dark for 20 min with occasional mixing and finally centrifuged at 10 000 × *g* for 10 min. The supernatant fluid was diluted with 1% HNO_3 as required for the assay or acidified with one drop of concentrated HNO_3. The silver content was measured with an atomic absorption spectrometer (Perkin Elmer, Norwalk, CT, USA; model 2380) under standard conditions. The amount of biosorbed silver was then calculated from the concentration difference between a control without biomass and the test tubes with biomass and related to the dry weight determined. All tests were performed in triplicate.

Application of the screening test for biosorbing bacteria (20-min Ag-exposure)

Fifteen bacterial strains from VITO (Vlaamse Instelling voor Technologisch Onderzoek, Mol, Belgium) were grown on modified minimal medium (Schlegel, 1992; modification: glucose replaced by sodium gluconate, and all chloride replaced by nitrate) at 30 °C, 60% humidity for 48 h. Plates were then overlayed with 6 mM $AgNO_3$ soft-agar as above and incubated at 30 °C in the dark for just 20 min. H_2S exposure was performed as already described. All colonies were inspected under the stereo microscope and changes in the colour of the colonies and the formation of haloes were noted. The parameters 'colour of the colony', 'diameter of a halo', and 'transparency of a halo' were each classified into categories (0–2) by increasing intensity.

Quantification of silver biosorption capacity was performed at an initial concentration of 1 mM as described for the isotherms, but for optimum accordance of growth conditions the bacteria were not cultivated in liquid medium, but on agar plates under the same conditions as with the screening test. The biomass was scraped off after 2 days and the dry weight determined from an aliquot by drying at 80 °C overnight. Weighed portions of the wet biomass were placed into 14-ml Polyallomer centrifugation tubes, resuspended in 1 mM metal nitrate solution and further treated as above.

RESULTS

In the isolation of silver-accumulating bacteria, six strains were finally gained from river sediment, aerobically-digested sewage sludge and landfill. All these strains exhibited dark colouring of the colonies after treatment with silver nitrate and hydrogen sulphide, pointing to heavy loading with silver. As exposure to silver lasted 48 h, no distinction between biosorption and bioaccumulation could be made.

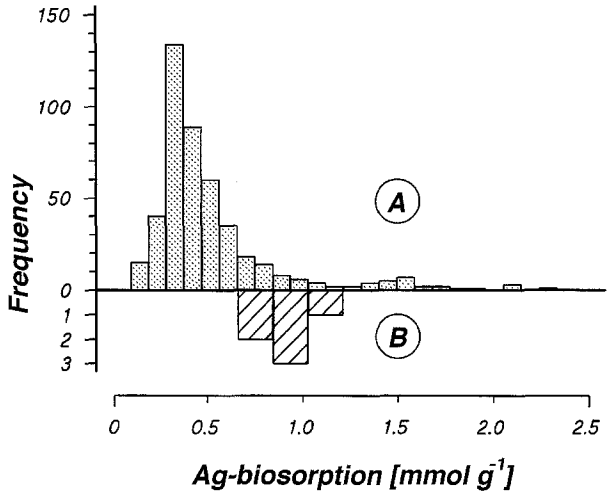

Fig. 1. Frequency histograms of silver biosorption data of (A) 459 bacterial strains [7] and (B) six new isolates received from the screening method.

The capacity of the bacterial strains to biosorb silver was quantified in suspension and the results achieved at the 1 mM silver concentration were compared with previous data taken for more than 400 bacterial isolates [7]. As shown in Fig. 1, the average capacity of the new isolates exceeds about threefold the average calculated from the older study, which was carried out under comparable conditions. Interpreted from a statistical point of view the data show that about 100 randomly selected strains would have to be examined to obtain six isolates within the same capacity range as immediately found with the screening test. For the six new strains, extended range silver biosorption isotherms were also taken over three orders of magnitude. Figure 2 shows the isotherm of the strain with the highest capacity in comparison with the isotherm (same conditions) of the commercial ion exchange resin Dowex 50WX8–100.

The aim of the second experiment was to correlate optical effects with the measured biosorption capacity of selected strains. The obvious optical changes of colonies after 20 min exposure to silver are as follows:

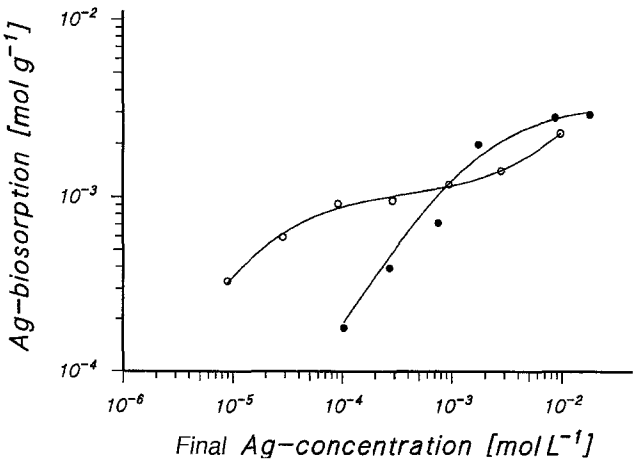

Fig. 2. Silver biosorption isotherms for one isolated strain 89/1/1 (–○–) and for the cation exchange resin Dowex 50WX8-100 (–●–).

(1) *Before* the precipitation of silver by H_2S a blackening of some colonies can be observed, which may be due to enzymatic reduction as well as H_2S formation by the bacteria.
(2) *After* H_2S-treatment light haloes develop around biosorbing colonies within the now uniformly-grey stained agar, as a result of silver diffusion towards the cells.

The determined silver biosorption capacity and all rated optical effects were subjected to a correlation analysis (Statgraphics-Plus). The arithmetical sum of the two parameters 'diameter of a halo' and 'transparency of the halo' (named 'sum') correlates positively ($r = 0.7$ at $\alpha = 0.0035$) with biosorption capacity. Although the optical effects were subjectively rated rather than measured, which reduces the conciseness of the statistical result, transparency and diameter of light haloes are shown to be useful measures for a preliminary estimation of biosorption capacity (Table 2, Fig. 3).

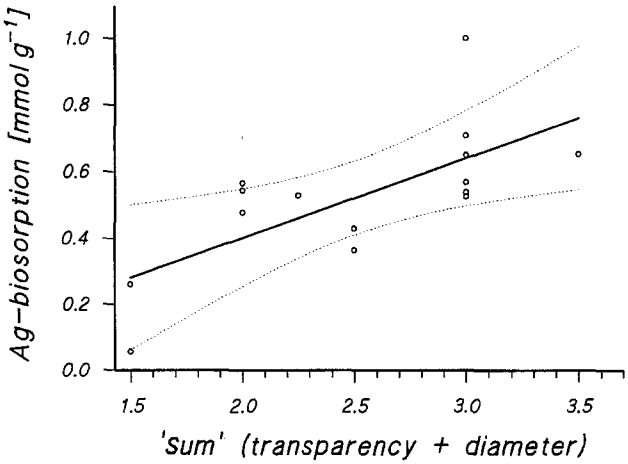

Fig. 3. Linear regression analysis of the optical parameter 'sum' versus silver biosorption capacity at 1 mM.

DISCUSSION

A mechanistic interpretation of silver binding by the isolated bacteria is beyond the scope of this paper, but it should be mentioned that mathematical fitting of the isotherms was performed with a high degree of correlation ($r^2 = 0.997$) with a biphasic Langmuir model [3,8,11,12,34,40,42]. Comparing the isotherm of the highest capacity strain with an isotherm of silver adsorption for a commercial cation exchange resin (Dowex 50WX8–100) demonstrates the advantage of the biomass in the concentration range up to 1 mM. As expected for single site binding of silver by the exchange resin with sulphonic acid as the reactive group, the curve fitted a simple Langmuir model.

As demonstrated by two exemplary results, the screening method presented represents a powerful tool for the isolation of metal-accumulating microorganisms and for the preliminary estimation of biosorption capacity. By chance we isolated a bacterium with a high capacity for silver accumulation, which did not show any optical changes in the test. Hydrogen sulphide was not able to precipitate the silver associated with the cells, but sunlight was. Thus, identification of each potent strain cannot be guaranteed. Nevertheless for common purposes the savings in time and materials are remarkable. Considerable scope for variation exists, permitting the method to be adapted to specific needs.

TABLE 2

Correlation matrix of optical effects and silver biosorption capacity

	Biosorb[a]	Diam[b]	Trans[c]	Sum[d]	Colcol[e]
Biosorb	1.0000	0.3205	0.5373	0.7018	−0.0986
	(15)	(15)	(15)	(15)	(15)
	0.0000	0.2441	0.0389	0.0035	0.7266
Diam		1.0000	−0.2321	0.5468	0.1252
		(15)	(15)	(15)	(15)
		0.0000	0.4052	0.0349	0.6565
Trans			1.0000	0.6875	−0.2172
			(15)	(15)	(15)
			0.0000	0.0046	0.4368
Sum				1.0000	−0.0935
				(15)	(15)
				0.0000	0.7403
Colcol					1.0000
					(15)
					0.0000

Coefficient, (sample size), significance level.
[a]Silver biosorption capacity.
[b]Diameter of a light halo.
[c]Transparency of a halo.
[d]Arithmetical sum of b and c.
[e]Staining of the colony.

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