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## Publication Info

*MethodsX*, 2021, pages 101550-.
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MethodsX 8 (2021) 101550

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

# A method for cultivation, fractionation, and metal determination to evaluate metal removal by the combination of NPs and bacteria

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A B S T R A C T

Metals being released into the environment are posing an increasing risk to both environments and public health. Developing improved approaches to remove these metals from the environment is urgent. A current publication discovered that metal bioremediation was significantly improved by nanoparticles (NPs), and the remediation duration was shortened. However, there is no relevant method for the preparation and evaluation of this novel idea. Hence, we developed this method for bacteria *in-situ*-EPS (Extracellular Polymeric Substances) cultivation, bacteria sub-cellular fractionation, and metal determinations in cultivating solution, EPS and different fractions of bacteria to evaluate metal removal by the combination of NPs and bacteria, including (1) the enhancement of metal bioremediation by NPs, (2) the influence of NPs on bacteria growth and metal toxicity alleviation, (3) the ability of EPS to adsorb metals and the influence of NPs on the EPS metal adsorption, (4) the contribution of bacteria to metal removal in different part, the effects of NPs on metal distribution patterns in bacteria, and the role of NPs in this process.

- The design and experimental procedure for the evaluation of metal removal by combing bacteria and NPs.
- *In-situ* EPS cultivation and separation in the study of bioremediation for metals.

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DOI of original article: 10.1016/j.scitotenv.2019.135378

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https://doi.org/10.1016/j.mex.2021.101550

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ARTICLE INFO
*Method name:* Evaluation method for metals removal efficiency by bacteria and nanoparticles
*Keywords:* Bioremediation, Nanotechnology, Heavy metals, Pollution control, *In-situ*-EPS, Subcellular fractionation
*Article history:* Received 7 August 2021; Accepted 11 October 2021; Available online 14 October 2021

## Specifications table

| | |
|---|---|
| Subject Area: | Environmental Science |
| More specific subject area: | Environmental remediation for metals |
| Method name: | Evaluation method for metals removal efficiency by bacteria and nanoparticles |
| Name and reference of original method: | none |
| Resource availability: | not applicable |

## Method details

### *Preparation of bacteria, metals and NPs*

Bacteria holding the potential to remove metals from solutions were selected to use in this method, and the Extracellular Polymeric Substances (EPS)-producing bacteria are more appropriate. Bacteria was activated by inoculating the stored bacteria into 50 mL tryptic soy broth (TSB) medium and incubating on a shaker table (Excella E24) for 24 h (180 rpm, 28 °C), with minor changes for different bacteria. TSB medium was used in this method, because it is a standard, nutritious medium that will support the growth of a wide variety of microorganisms, especially common aerobic and facultatively anaerobic bacteria and it can be easily used by other researchers when comparing results. While in a defined medium or minimum medium, the growth of bacteria is limited, the secretion of bacteria might be different, thus the results of the remediation efficiency would be influenced, which makes it difficult to evaluate the advantage of the metal remediation method by combining bacteria and nanoparticles (NPs). Fig. 1 shows that the growth efficiency of a bacteria strain *Halomonas* sp. is much higher in TSB than in Marine Broth (MB).

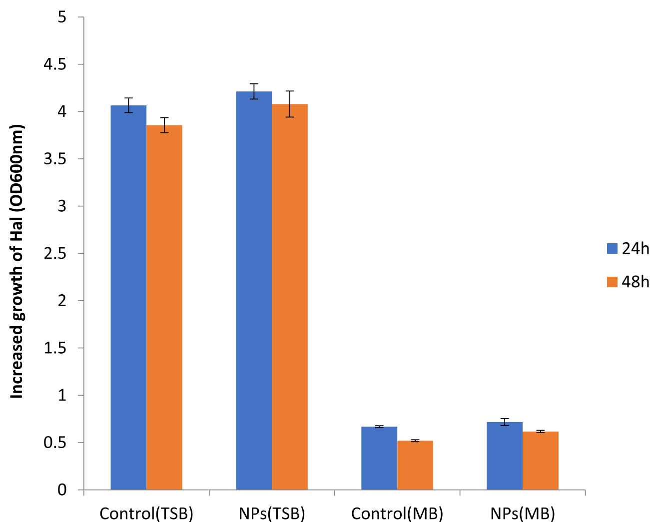


**Fig. 1.** Growth efficiency of bacteria strain *Halomonas* sp. in TSB and Marine Broth (MB).

**Table 1**
The experimental design for the evaluation of metal removal by combining bacteria and NPs.

| Metal-treatment | Bacteria-treatment |
|---|---|
| Control 1 (metal-only)<br>NPs+Me (NPs and metal)<br>Bac+Me (bacteria and metal)<br>Bac+NPs+Me (bacteria, NPs and metal) | **Control 2 (bacteria-only)**<br>**Bac+NPs (bacteria and NPs)** |

Stock solutions of metals (Cd, Pb, Cu, Zn in this study) were obtained by dissolving their corresponding salts in ultrapure water and stocked at 4 °C. NPs were usually suspended in ultrapure water by vortex and sonication. Based on the stability of different NPs, it should be prepared in stock solutions or freshly made to avoid aggregation. Take polyvinylpyrrolidone (PVP) coated iron oxide NPs for instance, although it is mono-dispersed and sterically stabilized by the PVP after freshly synthesized, this NP is prone to agglomerate during long-term storage [1,2]. The agglomerated NPs were not applicable to evaluate metal removal by the combination of NPs and bacteria, since the bioavailability was substantially reduced. Hence, the freshly made PVP coated iron oxide NPs were suggested to be used for further analysis.

*Preparation of mixed culture*

The experiment should contain at least 6 treatments: Control 1 (metal-only), Control 2 (bacteria-only), Bac+Me (bacteria and metal), NPs+Me (NPs and metal), Bac+NPs+Me (bacteria, NPs and metal), Bac+NPs (bacteria and NPs). The experimental design was shown in Table 1. TSB is the basic medium in all treatments for better comparison and at least three replicates for each treatment were concluded. Adjust the concentrations of the stock solution for metals and NPs according to their working concentrations in the mixed culture. 20 mL total media of each mixed culture is enough for this experiment. The total solution added other than TSB should be less than 2 mL, and make sure they have the same adding content of solution other than TSB in each treatment, with ultrapure water as replacement. For that without inoculation of bacteria (Control 1 and NPs+Me), added the same volume of TSB instead. After mixing each culture thoroughly, the activated bacteria were added into each treatment (obtain 20 mL of total media) to achieve a density of $1 \times 10^6$ cells $mL^{-1}$. All the treatments were then incubated for 48 h while shaking (180 rpm, 28 °C).

*Bacteria growth determination*

At predefined time points (0, 4, 8, 12, 24, 36, 48 h), 0.5 mL samples for each bacteria-treatment (Control 2, Bac+Me, Bac+NPs, Bac+NPs+Me) were collected. Carefully clean the mouth of the culture bottle with ethanol before collecting the cultivated samples in the super clean workbench to make sure the mixed cultures are not contaminated. All the samples were processed immediately after collected. 0.1 mL sample was diluted into 1 mL solution by adding 0.9 mL ultrapure water. Make 3 repeats for each sample. By reading the OD value at 600 nm with a UV-visible spectrophotometer (Shimadzu UV-2401), the growth curve for each bacteria-treatment was obtained. Compare the bacteria growth among the treatments to explore the influence of NPs on bacteria growth and metal toxicity alleviation.

*Metal removal determination*

1 mL samples for each metal-treatment (Control 1, Bac+Me, NPs+Me, Bac+NPs+Me) were collected at predefined time points (0, 4, 8, 12, 24, 36, 48 h). All the samples were immediately centrifuged at 4000 rpm (4 °C) for 10 min. Then the supernatant (carefully obtained the upper 0.8 mL) was ultra-filtered using centrifugal filter units (3 kDa, Pall Corporation) at 4000 rpm for 1 h (Centrifuge 5810R, Eppendorf). The obtained filtrates were stored at 4 °C before acid digestion. Prepare fresh aqua regia by carefully adding concentrated nitric acid into hydrochloric acid (1:3).

Added 0.4 mL fresh aqua regia into 0.2 mL filtrates, mixed thoroughly, and digested overnight in the fume hood. The next day, added ultrapure water to get 8.6 mL of total solution. Prepare at least 6 concentrations of metal standard solutions according to the estimated metal concentration. Run the standard solutions in the inductively coupled plasma-optical emission spectroscopy (ICP-OES, Varian 710-ES) before sample determination. By calculating the determined data, metal removal ability in each treatment was compared to study the enhancement of metal bioremediation by NPs. Control 1, without additions of bacteria, were used to evaluate metals combined by the TSB medium.

### *EPS extraction and metal determination*

In bacteria-treatments, 2 mL of the cultivating media was removed after 48 h. The separating procedure was conducted according to the previous study [3] with modification. Samples were centrifuged at 5000 g for 15 min; the obtained supernatant was then filtrated through a 0.22 $\mu$m syringe filter (PVDF, Sterile). 100% ethanol was pre-stored at -20 °C at least 4 h before use. Three equal volumes of 100% cold ethanol were added into the filtrate. After storage at -20 °C overnight, the solution was centrifuged at 5000 g for 15 min. After removing the supernatant, the EPS in the pellet was re-suspended in ethanol to wash three times. The EPS pellet was allowed to air-dry in the fume hood. Prepare fresh aqua regia the same as the above. Added 0.5 mL fresh aqua regia into the air-dried EPS and digested overnight in the fume hood. The next day, added 10.25 mL ultrapure water to dilute the solution and mixed them thoroughly. Metal determination procedure are conducted according to the above. The metal concentration in each bacteria-treatment was calculated to compare the ability of EPS to adsorb metals and the influence of NPs on the EPS metal adsorption.

### *Bacteria sub-cellular fractionation and metal determination*

After cultivating for 48 h, aliquots in bacteria-treatments were taken to extract and separate bacterial fractions according to previous procedures [4,5] with modification. 5 mL cultivating media were used for the separation of bacterial sub-cellular components as soon as possible. 0.03 mol $L^{-1}$ Tris buffer containing $2.5 \times 10^{-3}$ mol $L^{-1}$ EDTA (pH8.0) was prepared and stored at 4 °C before use.

The mixed culture was centrifuged at 3000 g for 25 min (4 °C) to harvest cells in the pellet. The raw cells were washed with 1 mL Tris buffer, centrifuged at 5000 rpm for 15 min twice. Prepare lysozyme solution by adding lysozyme (Sigma-Aldrich) to the Tris buffer to get a final concentration of 10 mg $mL^{-1}$. The twice-washed cells were then incubated in the freshly prepared lysozyme solution (total volume 0.5 mL) at 25 °C for 30 min. All subsequent steps were performed at 0–4 °C. After incubation, the cells were centrifuged at 3000 g for 15 min, the supernatant obtained was the periplasmic fluid consisting of a peptidoglycan layer, which is the cell wall containing metals in this study. While spheroplasts in pellets were re-suspended in the same Tris buffer to get a total volume of 0.5 mL. Then the spheroplasts were disrupted by two 15 s bursts (Vibronic Ultrasonic processor) in ice bath and centrifuged at 2000 g for 10 min to remove debris and unbroken cells. Finally, the supernatant was centrifuged at 3000 g for 150 min. The pellet consisting of both outer and inner membrane envelopes were collected as crude membrane, while cytoplasmic fractions were obtained in the supernatant.

All the bacterial sub-cellular fractions were stored at -20 °C before acid-digestion. After thawing, 0.5 mL, 0.5 mL, 1 mL fresh aqua regia were added to the cell wall, crude membrane and cytoplasmic fractions of the bacteria, respectively. After digested overnight in the fume hood, ultrapure water was added to get a total volume of 10.75 mL, 10.75 mL, 21.5 mL for cell wall, crude membrane and cytoplasmic fractions of the bacteria, respectively. All samples were centrifuged at 5000 rpm for 10 min before metal determination. Metal concentrations in different bacteria fractions were analyzed to explore the contribution of bacteria to metal removal in different part, the effects of NPs on metal distribution patterns in bacteria, and the role of NPs in this process.

*Application potential of this method*

This method for bacteria *in-situ*-EPS cultivation, bacteria sub-cellular fractionation, and metal determinations in cultivating solution, EPS and different fractions of bacteria is appropriate and useful to evaluate metal removal efficiency by the combination of NPs and bacteria. The successful application of this method in the recent publication [1] is a good proof. To our knowledge, there are studies on how extracted EPS, free EPS or bound EPS affect metal binding by bacteria [6–8]. However, these studies separated EPS from bacteria first, and then used the separated EPS to adsorb metals. Separating the EPS from cells changes the physical conformation of the EPS, and likely the availability of metal-complexing functional groups on the EPS. These studies differ from the *in-situ* EPS study, because (1) the effects of the separated EPS on the heavy metal binding of bacteria might be different from that of the *in-situ* EPS, and most bacterial cells in the environment exist predominantly in biofilms (EPS) attaching to mineral surfaces, which makes the research on *in-situ* EPS necessary; (2) the distribution of heavy metals in different components of the bacterial cells was investigated in the recent publication, including *in-situ* EPS, during the process of bioremediation to better explore these distribution mechanisms (use of separated EPS would have complicated the interpretations of this); (3) according to the *in-situ* EPS study, more precise information was acquired on whether the EPS acted as a barrier or filter to influence passage for different metals into cells. From this point, the present method should also be applicable to explore the mechanisms of metal bioremediation with *in-situ* EPS.

## Acknowledgment

This work was supported by the National Natural Science Foundation of China (42007112, U20A20115) and Smartstate Center for Environmental Nanoscience.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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