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PhoN-expressing, lyophilized, recombinant *Deinococcus radiodurans* cells for uranium bioprecipitation

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ABSTRACT

Employment of genetically engineered radiation resistant organisms to recover radionuclides/heavy metals from radioactive wastes is an attractive proposition. Cells of recombinant *Deinococcus radiodurans* strain expressing, a non-specific acid phosphatase encoding *phoN* gene, were lyophilized. Lyophilized recombinant *Deinococcus* cells retained viability and PhoN activity and could efficiently precipitate uranium from aqueous solutions for up to six months of storage at room temperature. Batch process for uranium removal using lyophilized cells was more efficient compared to a flow through system, in terms of percent uranium removed, substrate conservation and time taken. Lyophilized recombinant *Deinococcus* cells exhibited high loading of up to 5.7 g uranium/g dry weight of cells in a batch process at 20 mM input uranium concentration. Lyophilization deflated the cells but did not alter gross cell morphology or surface nucleation capability of cells for uranium precipitation. The precipitated uranyl phosphate remained tightly associated with the cell surface, thus facilitating easy recovery.

1. Introduction

Nuclear waste management has been a contentious aspect of nuclear power programs around the world. Radionuclide-containing wastes are produced at all steps in the nuclear fuel cycle from milling and mining of uranium ores to fuel fabrication, reactor operation and fuel reprocessing. Biological treatment strategies of waste have evoked considerable interest (Gadd, 2000) as viable and eco-friendly alternatives, especially at low concentrations of the radionuclide and for *in situ* bioremediation. Naturally occurring organisms such as *Citrobacter* harboring a non-specific periplasmic acid phosphatase PhoN, have been considered suitable for bioremediation of heavy metals (Macaskie et al., 1994). PhoN hydrolyses organic phosphates and the inorganic phosphate, thus released, brings about metal precipitation as insoluble metal phosphate on the cell surface (Kier et al., 1977; Macaskie et al., 1994). In the past, efficient bioprecipitation of uranium with *Citrobacter* strains (Macaskie, 1990; Jeong et al., 1997; Macaskie et al., 1994) and recombinant *Escherichia coli* strains expressing PhoN (Basnakova et al., 1998) have been reported. However, radioactivity restricts the survival, cellular integrity and functionality of such microbes in nuclear wastes (Daly, 2000; Appukuttan et al., 2006).

The bacteria belonging to *Deinococcaceae* family have the extraordinary ability to withstand radiation doses up to 10–15 kGy (Battista, 1997; Daly, 2000). Recently our laboratory genetically engineered *Deinococcus radiodurans* R1 to express *phoN* (Appukuttan et al., 2006). The recombinant strain, DrPhoN survived extreme radiation stress, unlike recombinant *E. coli*-PhoN clones and precipitated uranium from dilute aqueous solutions in high radiation environment. In order to extend the use of the recombinant *Deinococcus* strain for application in nuclear waste, the following factors need to be suitably addressed: (a) the ease of handling and transportation of biomass, (b) shelf-life and storage of cells, (c) mode of application, and (d) substrate conservation. Biological materials often must be dried to reduce bulk volume and to stabilize them for storage or distribution. Drying causes significant loss of activity and viability. But, freeze-drying, significantly reduces such damage (Snowman, 1988; Seetharam et al., 2009).

The present study investigated the utility of lyophilization to achieve aforesaid desirable features for PhoN expressing *D. radiodurans* strains. Our results show that lyophilized recombinant *Deinococcus* cells retained viability, PhoN activity as well as uranium precipitation ability up to 6 months of storage at room

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temperature. Lyophilized cells also retained surface localization property of the precipitate, and facilitated, loading of 5.7 g/g dry weight of biomass in a batch process.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Cells of recombinant *D. radiodurans* strains, DrEV (carrying the shuttle vector pRAD1) (Meima and Lidstrom, 2000) and DrPhoN (carrying *phoN* gene from *Salmonella enterica* serovar *Typhi* cloned downstream of *Deinococcal groESL* promoter) (Appukuttan et al., 2006) were grown aerobically in TGY (1% Bacto Tryptone, 0.1% glucose, and 0.5% yeast extract) liquid medium with 3 μg/ml of chloramphenicol at 32 °C ± 1 °C under agitation (180 ± 5 rpm). Growth was assessed by measuring turbidity (OD_{600nm}) or by determining colony forming units (CFUs) on TGY agar plates (1.5% Bacto Agar) after 48 h incubation at 32 °C ± 1 °C.

2.2. Lyophilization

Overnight grown cells were washed twice with distilled water and re-suspended in distilled water to form a thick suspension which was taken in a Petri plate and frozen in liquid nitrogen. The frozen cells were lyophilized overnight in a Lyospeed (Genevac, United Kingdom, Model Refrigerant R502) at 0.07 mbar for 18 h. The lyophilized cells were scraped off from the Petri plate and stored in Eppendorf vials at room temperature until used for uranium recovery. The survival of cells was measured in terms of the colony forming units (CFUs) before lyophilization or after re-wetting of the lyophilized powder. Fresh and lyophilized cells, equivalent in terms of their protein content were plated onto TGY agar plates containing chloramphenicol for determination of CFUs. The protein content of 0.33 mg lyophilized cells re-suspended in 1 ml distilled water was equivalent to 1 ml of 1.0 OD_{600nm} fresh cells. The lyophilized cells when re-suspended rapidly formed a uniform suspension in acetate buffer and were allowed to equilibrate for 5 min before the PhoN activity and uranium precipitation assays were carried out.

2.3. Acid phosphatase activity assays

The cell-bound PhoN activity was estimated by the liberation of *p*-nitrophenol from di-sodium *p*-nitrophenyl phosphate (*p*NPP), as described earlier (Bolton and Dean, 1972), and expressed as nmol of *p*-nitrophenol (*p*NP) liberated min^{-1} mg^{-1} bacterial protein. Protein concentration was determined by Lowry's method (Lowry et al., 1951) using a protein estimation kit (Bangalore Genei Pvt. Ltd., India).

2.4. Batch precipitation of uranium

Uranium precipitation assays were performed as described previously (Macaskie et al., 2000) with certain modifications (Appukuttan et al., 2006). About 80 mg lyophilized cells were re-suspended in 100 ml (equivalent to $O.D_{600nm}$ of 2.4) of 2 mM acetate buffer (pH 5.0) for 5 min followed by addition of uranyl nitrate and β-glycerophosphate at concentrations specified for each experiment. At different time intervals, aliquots were subjected to centrifugation and the uranium in the supernatant was estimated by Arsenazo method as described previously (Appukuttan et al., 2006). Appropriate controls were included to determine (a) spontaneous chemical precipitation of the metal (assays without any cells), (b) biosorption of uranium on the surface of the lyophilized cells (assays with DrEV cells) and (c) uranium sorption to the surface of the container (assays carried out in different containers which were washed post assay extensively to ascertain that no uranium bound to the surface).

Table 1
Viability and PhoN activity of lyophilized DrPhoN cells. Fresh and lyophilized cells equivalent in terms of their protein content were suitably diluted and plated onto TGY agar plates containing 3 μg/ml chloramphenicol. The viable counts reported are the average values from three independent lyophilization experiments. PhoN activity is expressed as nmol of *p*-NP liberated min^{-1} mg^{-1} protein.

Treatment	cfu/ml	PhoN activity
Before lyophilization	$(1.1 \pm 0.07) \times 10^8$	190 ± 10
After lyophilization	$(1.0 \pm 0.056) \times 10^8$ (90%)	167 ± 5 (87.8%)

Values in parentheses indicate percent of fresh cells.

2.5. Column-based uranium precipitation

About 5 ml of 15% polyacrylamide gel was prepared in distilled water (2.4 ml of distilled water, 2.5 ml of 30% Acrylamide–bisacrylamide solution, 50 μl of 10% APS and 50 μl of TEMED) with 150 mg of lyophilized cells and allowed to set at 4 °C for 45 min. The gel containing the cells was washed four times in 10 ml distilled water and then shredded into small pieces using a sieve (8 pores cm^{-1}). The shredded gel pieces were allowed to swell in distilled water for 5 min, following which they were mixed with 2.5 ml of acid washed sand and packed into a plastic column (1.4 cm inner diameter × 9 cm). The column was equilibrated and allowed to stand overnight. Following this, the input solution containing 1 mM uranyl nitrate and 5 mM β-glycerophosphate in 2 mM acetate buffer (pH 5.0) was allowed to continuously flow through the system at a flow rate of 5 ml h^{-1}. The uranium concentration in the flow through was recorded at different time intervals. A similar column set-up with no added cells served as a negative control where no change in uranium concentration was recorded in the flow-through.

2.6. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray spectroscopy (EDX) of lyophilized cells

Deinococcus cells were processed for electron microscopy as described earlier (Heather et al., 2006). Briefly, cells were washed in cacodylate buffer (100 mM, pH 7.4) and fixed in Karnovskys fixative (Karnovsky, 1965) for 2 h at 4 °C. Samples were then washed in 100 mM cacodylate buffer, dehydrated in a graded series of 20–100% ethanol, spotted on glass slides and dried at 37 °C for 1 h. The dried samples were gold coated by thermal evaporation technique and analyzed by SEM and EDX using a VEGA 40, Tescan Microscope, Czechoslovakia and INCA energy 250 EDX System, Oxford Instrument, United Kingdom.

3. Results

3.1. Effect of lyophilization on survival, cell morphology, PhoN activity and uranium precipitation ability of recombinant D. radiodurans cells

Lyophilization of recombinant DrPhoN cells converted them into a fine powder which could be more easily handled and stored in vials at room temperature. The lyophilized powder was weighed and directly used, after 5 min of wetting in 2 mM acetate buffer, in all experiments to compare their performance against equivalent fresh cells.

Lyophilized DrPhoN cells retained 90% viability and 88% PhoN activity (Table 1) of fresh cells. Scanning Electron Microscopy (Fig. 1a and b) showed no gross morphological damage to cells except that they looked deflated as compared to fresh cells. Lyophilized DrPhoN cells were introduced into a typical reaction