Appl Microbiol Biotechnol (2015) 99:9203–9213
DOI 10.1007/s00253-015-6761-4

ENVIRONMENTAL BIOTECHNOLOGY

Engineered *Deinococcus radiodurans* R1 with NiCoT genes for bioremoval of trace cobalt from spent decontamination solutions of nuclear power reactors

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Received: 8 April 2015 / Revised: 12 May 2015 / Accepted: 8 June 2015 / Published online: 26 June 2015

Abstract The aim of the present work was to engineer bacteria for the removal of Co in contaminated effluents. Radioactive cobalt (^{60}Co) is known as a major contributor for person-sievert budgetary because of its long half-life and high γ-energy values. Some bacterial Ni/Co transporter (NiCoT) genes were described to have preferential uptake for cobalt. In this study, the NiCoT genes *nxiA and nvoA* from *Rhodopseudomonas palustris* CGA009 (RP) and *Novosphingobium aromaticivorans* F-199 (NA), respectively, were cloned under the control of the *groESL* promoter. These genes were expressed in *Deinococcus radiodurans* in reason of its high resistance to radiation as compared to other bacterial strains. Using qualitative real time-PCR, we showed that the expression of NiCoT-RP and NiCoT-NA is induced by cobalt and nickel. The functional expression of these genes in bioengineered *D. radiodurans* R1 strains resulted in >60 % removal of ^{60}Co (≥5.1 nM) within 90 min from simulated spent decontamination solution containing 8.5 nM of Co, even in the presence of >10 mM of Fe, Cr, and Ni. *D. radiodurans* R1 (DR-RP and DR-NA) showed superior survival to recombinant *E. coli* (ARY023) expressing NiCoT-RP and NA and efficiency in Co remediation up to 6.4 kGy. Thus, the present study reports a remarkable reduction in biomass requirements (2 kg) compared to previous studies using wild-type bacteria (50 kg) or ion-exchanger resins (8000 kg) for treatment of ~10^5-l spent decontamination solutions (SDS).

Keywords Bioengineering · Bioremediation · NiCoT genes · Trace cobalt removal · Spent decontamination solutions (SDS) · Nuclear power reactors

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Introduction

Spent decontamination solutions are generated from power-generating nuclear reactors during the chemical decontamination process (Ayres 1970; Taylor 1976; Cohen 1980; Lejon et al. 1994; Urch 2013). Radioactive cobalt, mainly as an isotopic mixture of ^{59}Co, a stable isotope of elemental cobalt, and ^{60}Co, an activation product of ^{59}Co, are present at trace chemical concentrations (8.5–34 nM of total cobalt) in such solutions (Charlesworth 1971; Kurnaz et al. 2007). Apart from ^{60}Co, the spent decontamination solution can also include radionuclides such as ^{51}Cr, ^{54}Mn, ^{59}Fe, and ^{58}Co, which are relatively minor contributors to the radiation field due to these solutions. The radiation field arising from these solutions is mainly controlled by ^{60}Co due to its long half-life of 5.27 years and the high energy (1.17 and 1.33 MeV) gamma ray emission from its nucleus. Hence, the removal of this cobalt at trace

chemical concentrations will make the solution benign from the radiation field control point of view. The conventional method of employing cationic exchange column is mainly for regenerating the complexants in the decontamination solution by way of metal ion removal. However, the sorption of soluble Co (as Co^{2+}) from the spent decontamination formulation by the cation resin and its subsequent displacement from the resin due to the sorption of other metal ions like Fe, Cr, and Ni, which are present at much higher chemical concentrations but which are only minor contributors to the radiation field problem, create the requirement of a large number of cation columns to avoid the Co elution. This necessitates an inefficient use of large amount of cation-exchange resin. The large amount of solid radioactive waste thus generated also requires prolonged safe storage (for tens of years) in tile holes/trenches (Venkateswaran et al. 2003; Prakash et al. 2013; Frišták et al. 2014a, b).

Recent developments in microbiology have facilitated the use of fungi and bacteria for bioremediation of toxic metals and radionuclides from soils and waters (Akthar et al. 1996; Daly and Minton 1996; Brim et al. 2000; Amachi et al. 2010; Green et al. 2012). Microorganisms take up metal ions by two broad mechanisms referred to as biosorption and bioaccumulation. Biosorption is a rapid process of metal ion binding to surface of the cell and is independent of cell metabolism. In comparison, bioaccumulation is a slower energy-dependent process involving cell membrane transporters. Both approaches have been employed in bioremediation strategies, for the removal of heavy metals and radionuclide from simulated and natural environments (Gadd and White 1989; Akthar et al. 1996; Rama Rao et al. 1996; Naveena et al. 2005; Satinder et al. 2006; Kumar et al. 2007). Therefore, bioremediation of radionuclides or radioactive waste by using methods such as biotransformation, bioaccumulation, biosorption, biostimulation, and bioaugmentaion is only limited. Bioremediation of trace cobalt from simulated spent decontamination solutions of nuclear power reactor aims at the use of biological agents as such or genetically engineered (Gunjan et al. 2005; Lloyd and Renshaw 2005; Kumar et al. 2007; Prakash et al. 2013; Shih and Shen-Long 2014). This can be a viable and relatively inexpensive cleanup option. However, such organisms are generally radiosensitive and cannot be used effectively for bioremediation of radionuclides from the spent decontamination solutions. To overcome this problem, we have selected a highly radiation resistant organism known as *Deinococcus radiodurans* strain R1, a gram-positive nonpathogenic bacterium that can grow at a chronic exposure of 60 Gy/h (a dose rate that exceeds those in most solidified spent decontamination waste). *D. radiodurans* can also survive acute exposure to more than 10–20 kGy without undergoing mutations and have been successfully bioengineered for environmental biotechnology (Daly and Minton 1995, 1996; Battista 1997; Lange et al. 1998; Brim et al. 2000, 2003; Daly 2000; Kulkarni et al. 2013; Shih and Shen-Long 2014).

Previous studies from our laboratory screened and tested several wild type and mutant fungal strains as well as wild-type bacteria for removal of total cobalt *Co ($^{60}Co+^{59}Co$) from spent decontamination solutions. These studies demonstrated significant *Co removal ranging from 20 to 40 ng/g biomass by fungi and 1 μg/g biomass bacteria from simulated spent decontamination effluents containing 34 nM (2 ppb) of total cobalt (Rashmi et al. 2004, 2007). Genetically bioengineered *E. coli*, with Ni/Co transporter (NiCoT) genes from *Rhodopseudomonas palustris* CGA009 (RP) and *Novosphingobium aromaticivorans* F-199 (NA), were successfully used for removal of trace cobalt (*Co) from aqueous solutions (Raghu et al. 2008; Duprey et al. 2014). These recombinant *E. coli* strains showed significant *Co pickup capacities of up to 12 μg/g biomass under optimal conditions. However, bacteria did not survive beyond 20-Gy exposure to ^{60}Co γ rays. The present study aims at developing microbes capable of surviving in high-radiation environments. Therefore, we chose an inherently radioresistant, solvent tolerant, and nonpathogenic *D. radiodurans* for this purpose. Moreover, its genome is capable of expressing foreign genes with no effect on either its growth or survival at highly extreme radiation doses (Carroll et al. 1996; Daly 2000; Misra et al. 2014).

Overall, we engineered recombinant *D. radiodurans* strains expressing NiCoT genes of *R. palustris* CGA009 and *N. aromaticivorans* F-199. The recombinant strains could remove trace cobalt in the presence $>10^5$-fold concentrations of Fe, Cr, and Ni from simulated decontamination solutions, and its capacity to remove cobalt was unaffected up to 6.4 kGy of γ dose.

Material and methods

Bacterial strains and growth conditions

D. radiodurans strain R1 was grown aerobically in TGY (1 % Bacto Tryptone, 0.1 % glucose, and 0.5 % yeast extract) liquid medium over 48 h of incubation at 32±1 °C under agitation (180±5 rpm). *E. coli* (DH5α) and *E. coli* (ARY023) (Rodrigue et al. 2005) cells were grown in Luria-Bertani (LB) medium over 24 h of incubation at 37±1 °C under agitation (180±5 rpm). Bacterial growth was assessed by measuring turbidity (optical density at 600 nm [OD_{600}]) or by determining the number of colony-forming units (CFU)/ml on TGY agar plates (1.5 % Bacto Agar). The antibiotic concentration used for the selection of transformants was 3 μg $ml^{-1}$of chloramphenicol for *Deinococcus* (Meima and Lidstrom 2000) and 100 μg $ml^{-1}$of ampicillin for *E. coli*.

Cloning of groESL promoter and NiCoT-RP and NA genes into *E. coli-Deinococcus* shuttle vector-pRAD1

The shuttle vector pRAD1 was chosen because it can replicate both in *D. radiodurans* R1 and in *E. coli*. pRAD1 is 6.3 kb in size and contains an extended multiple cloning site which will be useful for general-purpose cloning. Since, *Deinococcus* does not often optimally recognize foreign promoters, the NiCoT genes were cloned and expressed under the influence of a *deinococcal* P_{groESL} promoter. The primers designed were based on the published groESL promoter sequences of *D. radiodurans* R1 and NiCoT genes of *R. palustris* CGA009 and *N. aromaticivorans* F-199. Hence, the *groESL* promoter was PCR-amplified from *D. radiodurans* R1 (DR0606) with *Bam*HI and *Eco*RI sites, and the NiCoT genes of RP and NA were PCR-amplified from pCH675RP and pCH675NA (Hebbeln and Eitinger 2004) by using gene-specific primers with *Eco*RI and *Hin*dIII restriction sites (Table 1). The cohesive end NiCoT-RP and NA PCR products (1.05 and 1.10 kb) were ligated to the groESL promoter (0.256 kb), and final ligated products (1.306 and 1.356 kb) were ligated to the *Bam*HI/*Hin*dIII sites of shuttle vector pRAD1 to obtain pRADgroESL-RP and pRADgroESL-NA. The *E. coli* (DH5α) transformants carrying plasmid pRADgroESL-RP and pRADgroESL-NA were selected on LB containing ampicillin (100 μg ml^{-1}), plasmids were isolated, and the inserts therein were confirmed by restriction digestion. Plasmids were isolated from a few of the positive clones and used to transform competent *E. coli* (ARY023), which is a Δ*rcnA* mutant (Rodrigue et al. 2005), and *D. radiodurans* R1 cells as described earlier (Meima and Lidstrom 2000). The transformants were plated on LB agar containing ampicillin (100 μg ml^{-1}) and TGY agar containing chloramphenicol (3 μg ml^{-1}). Further, *D. radiodurans* R1 transformed with pRADgroESL-RP and pRADgroESL-NA transformants were screened by PCR using gene-specific primers. The expected sizes of DNA fragment products of promoter groESL (0.256) and NiCoT genes of RP and NA are 1.05 and 1.1 kb, respectively. The same plasmids were then used for the gene sequencing and PCR reactions using pRAD1 primers P5 and P6 which, respectively, bind upstream and downstream of the multiple cloning site. The expected DNA inserts (promoter groESL plus NiCoT-RP and NA) indicate the presence of gene upstream of the groESL promoter.

RNA Isolation from bioengineered *Deinococcus* expressing NiCoT genes of *R. palustris* CAG009 and *N. aromaticivorans* F-199 and quantitative RT-PCR

Overnight-grown recombinant *Deinococcus* (DR-NA and DR-RP) cultures (10 ml) in the absence or presence of 10 μM (Co^{+2} or Ni^{+2}) were subjected to centrifugation at 9000*g* for 5 min. The pellet (size ~100 μl) was washed twice in chilled ethanol, followed by one to two washes in DEPC-treated distilled water to remove all the ethanol. The pellet was next resuspended in 0.5 ml of RNAWiz (Ambion) and subjected to four cycles of freeze (in liquid nitrogen) and thaw (37 °C). The frozen cell suspension was stored at −70 °C overnight. The cell suspensions were then thawed on ice; then, 0.5 ml of autoclaved glass beads (~500 μ) and 0.5 ml of RNAWiz were added. The mixture was subjected to vigorous vortexing for 3 min followed by 3 min on ice. Total RNA derived from each sample was treated with 10 units DNase I (Ambion, Austin, TX, USA) and purified using RNeasy Minikit columns (QIAGEN, Valencia, CA, USA). Then, absorbance of the isolated RNA at 260 nm (1 OD=40 μg of RNA) was taken. For checking the quality of RNA, electrophoresis was carried out with a 2 % agarose gel with 1× TAE as the running buffer. The total RNA (2 μg) was reverse transcribed to complementary DNA (cDNA) using Revert Aid™ H minus M-MuLV reverse transcriptase and random hexamer primers (Fermentas Life Sciences). The target genes selected in this study were NiCoT-NA and NiCoT-RP, and the primer pairs that were used for qRT-PCR were designed using the primer3 program (Rozen and Skaletsky 2000) and are shown in Table 1. The above synthesized cDNAs were quantitatively measured, and real-time PCR was performed with an Applied Biosystems 7500 Fast machine using Real Master Mix SYBR ROX from 5 PRIME, USA. Quantitative real-time PCR conditions employed were as follows: initial denaturation at 95 °C for 60 s followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s, and extension at 68 °C for 20 s. Reaction specificity was determined for each reaction using the melt-curve analysis of the PCR product. To calculate the fold change in gene expression, the $2^{-\Delta\Delta Ct}$ method was used (Livak and Schmittgen 2001). Expression levels of the target genes (NiCoT-NA and NiCoT-RP) were normalized to the housekeeping 16S rRNA gene (AE000513) of *D. radiodurans* R1 (Lin et al. 1999). Analyses were performed in three different independent experiments and in triplicate for both target and normalize reactions.

*Co removal studies

In the present study, different physicochemical, chemical, and biological factors have been optimized for *Co removal from simulated effluent. They are pH of simulated effluent (2.8, 5.8, and 7.0); Fe-to-Co molar concentration ratio of 1.25, 2.5, 5, and 10×10^5; and duration of treatment (15, 30, 60, 90, and 120 min). To determine dose response to viability for *Deinococcus*, selected doses were 3.2, 4.8, 6.4, 8.0, 9.6, 12.8, 16.0, and 19.2 kGy. Different culture densities (0.5, 1.0, 2.0, 3.0, and 4.0) were also tested. Typically, the simulated decontamination effluent (SE) used to suspend the pelleted

Table 1 Primers, plasmids, and bacterial strains used for cloning of NiCoT genes

Primers, plasmids, or bacterial strains	Sequence (5′ to 3′) or description	Source or reference
Primers		
RP^+	5′-GCGGAATTCATCACCGATCTCGTTCTG-3′	This study
RP-	5′-GCGAAGCTTATTTCTGCACGGCCGCGGC-3′	This study
NA^+	5′-GCGCGGAATTCACCTCACGAAGTCACTGCCATCC-3′	This study
NA-	5′- CGAAAGCTTCACCGACCGAAACCTCGATTTCATCG-3′	This study
$RP\text{-}RT^+$	5′-GCTGTACTACAACATGACGA-3′	This study
$RP\text{-}RT^-$	5′-AAATTGGAATTGAGTTCTTC-3′	This study
$NA\text{-}RT^+$	5′-TTACCATTACCCTGATTAGC-3′	This study
$NA\text{-}RT^-$	5′-AATTTCATCAAAGCGTTTC-3′	This study
P5	5′-GGAGCGGATAACAATTTCACACA-3′	This study
P6	5′-AACGCGGCTGCAAGAATGGTA-3′	This study
$groESL^+$	5′-GCCGGATCCCATGTTCAG-3′	This study
$groESL^-$	5′-GGTTTCAGGAATTCGGGT-3′	This study
$16S\ rRNA^+$	5′-ATGGAGAGTTTGATCCTGGCTCA-3′	This study
$16S\ rRNA^-$	5′-GGAGGTGATCCAACCGCACCTTC-3′	This study
Plasmids		
pCH675-RP	pBluescript SK (+) containing NiCoT gene of *R. palustris CGA009*	Hebbeln et al.
pCH675-NA	pBluescript SK (+) containing NiCoT gene of *N. aromaticivorans* F-19	Hebbeln et al.
pRAD1	*E. coli- D. radiodurans* shuttle vector; $Ap^r\ Cm^r$, 6.28 kb	Meima et al.
pRAD-V	pRAD containing *Deinococcal* groESL promoter	This study
pRAD-RP	pRAD containing NiCoT gene of *R. palustris CGA009* tagged to *Deinococcal groESL promoter*	This study
pRAD-NA	pRAD containing NiCoT gene of *N.aromaticivorans* tagged to *Deinococcal* groESL promoter	This study
Bacterial strains		
E. coli -DH5α	*F^- recA41 endA1 gyrA96 thi-1 hsdR17 (rk^- mk^+) supE44 relAλ lacU169*	ATCC: 47076
E. coli (ARY023)	MC4100 (wt) F-*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR [rcnA:: uidA]*	Rodrigue et al.
D. radiodurans	R1 Wild-type strain	ATCC® 13939™

bacterial cultures was composed of iron (9.3 mM of Fe as $FeSO_4$ $(NH_4)_2SO_4 \cdot 6H_2O$), chromium (3 mM of Cr as $CrCl_3 \cdot 6H_2O$), nickel (0.93 mM Ni as $NiSO_4 \cdot 7H_2O$), and cobalt in the concentration range of 4.25–34 nM (0.25–2 ppb) Co as $CoCl_2$ traced with ^{60}Co to yield a solution specific activity of 40 Bq/ml (1.08 nCi/ml), and the tagged cobalt (represented as *Co) was used in the presence of disodium EDTA (13.5 mM). The initial pH of SE was adjusted to 2.8, 5.8, or 7.0 with dilute HCl/NaOH as required. An optimum culture density of 1 OD unit at 600 nm was used in the kinetic experiments. *D. radiodurans* cultures were grown in TGY medium for 48 h with 3.0 μg/ml chloramphenicol. The bacterial cells were pelleted by centrifugation at 7000*g* for 5 min and resuspended in 20-ml volume of SE to give a density of o1 OD unit (0.5-mg dry weight) and incubated in rotary shaker incubator (120 rpm at 37 °C) for various periods up to 2 h. The cells were then harvested by centrifugation at 14,000*g*, at 22 °C, for 10 min. *Co remaining in clear supernatant was determined by an integral γ-counter coupled to a well type (2×2 in. NaI (Tl) detector.

Post-irradiation and during irradiation *Co removal studies

Early-stationary-phase cultures of *Deinococcus* clones were washed twice and resuspended in fresh TGY and SE at an OD_{600} of 1.0 for the above mentioned studies. Then, the cultures were exposed to 3.2 to 19.2 kGy and 1.6 to 8 kGy of ^{60}Co gamma rays at a dose rate of 3.2 kGy h^{-1} for post-irradiation and during irradiation studies, respectively (^{60}Co Gamma Cell 5000 irradiation unit; HIRUP, Bhabha Atomic Research Centre, Mumbai, India). An aliquot of the culture kept outside the radiation source served as the control. The irradiated and control cells were washed, serially diluted, plated in triplicate on TGY plates containing chloramphenicol in the case of *Deinococcus* clones, and incubated under optimum growth conditions. Colonies were counted, and the survival curve was plotted. Irradiated cells were also assayed for their *Co removal studies as described earlier.

Statistical analysis

Data shown in most of the experiments are average values of triplicates from three independent batch experiments (± SD).

Results

Bioengineering of *D. radiodurans* R1 with NiCoT genes of *R. palustris* CGA009 and *N. aromaticivorans* F-199

The present study aims to generate recombinant clones capable of *Co bioremediation in radioactive environments. Thus, the above mentioned NiCoT-RP and NiCoT-NA genes, coding for highly specific cobalt transporters from *R. palustris* CGA009 (RP) and *N. aromaticivorans* F-199 (NA), were successfully cloned and expressed under the control of T7 promoter in *E. coli* and a strong *deinococcal groESL* promoter in both *E. coli* and *D. radiodurans* R1 (Table 1).

Quantitative real time-PCR analysis of NiCoT genes of *R. palustris* CGA009 and *N. aromaticivorans* F-199 expression in bioengineered *D. radiodurans* R1

The expression of two NiCoT gene (NiCoT-NA and NiCoT-RP) messenger RNA (*m*RNA) in bioengineered *D. radiodurans* R1 was examined by reverse transcriptase PCR (data not shown) and quantitative real-time-PCR. There was no apparent difference between the levels of *m*RNA expressed in these two clones, and the expression pattern of the NiCoT-NA transcripts was similar to that of NiCoT-RP transcripts. *m*RNA levels were significantly enhanced (6.4–7.3 or 1.8–1.9-fold) upon addition of Co^{+2} or Ni^{+2} (10 μM), respectively (Fig. 1). The NiCoT-NA and NiCoT-RP transcripts were found to be higher in Co^{+2} than in Ni^{+2}-treated cells (≈4-fold). The *groESL* promoter is thus induced by Ni or Co.

*Co removal efficiency by transformed *E. coli* and *Deinococcus* cells

The Co removal efficiency of recombinant *D. radiodurans* strains was optimized using overnight-grown cultures. The cells were incubated in presence of SE containing 8.5 nM of Co. The results showed that *Co removal reached a maximum (>60 %) at 60-min incubation in SE for *E. coli* recombinants, whereas 90 min was required for getting the similar efficiency removal for *Deinococcus* transformants (Fig. 2a). Beyond 90 min the Co removal efficiency was decreased to 30–40 %. In case of vector controls, about 5 or 25 % of Co removal was achieved for *E. coli* (ARY023) and *Deinococcus*, respectively. The 20–30 % Co removal capacity exhibited by pRAD-V (DR1) indicates that the basic organism itself seems

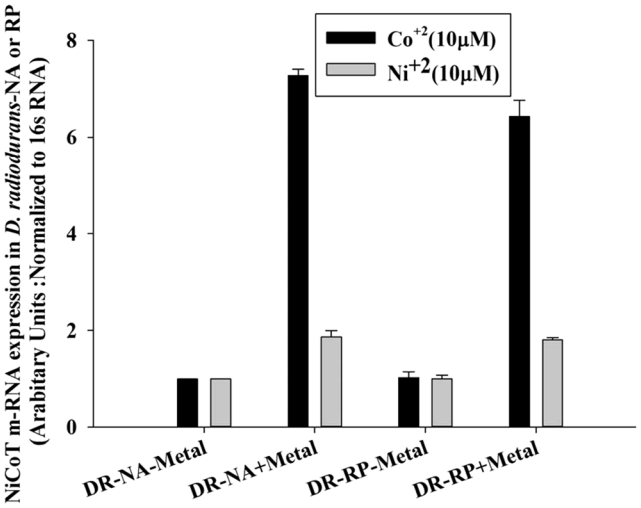

Fig. 1 NiCoT gene expression in recombinant *D. radiodurans* R1-NA/RP by quantitative RT-PCR analysis: NiCoT-NA, NiCoT-RP relative gene expression in DR-NA and DR-RP. Total RNAs extracted from DR-NA and DR-RP cultured in the absence or presence of 10 μM Co^{+2} or Ni^{+2} as its chlorides. Samples were reverse transcribed to cDNA and *m*RNA expression was measured by qRT-PCR. The expression of 16S rRNA was used as the internal control

to be containing specific (i.e., ABC cobalt transporter) and or nonspecific transporters (i.e., CDF and CorA transporters), some cobalt binding proteins unlike in the case of pRAD-V (ARY023). Increased incubation time even up to 24 h (usual decontamination run duration) had no further effect on *Co removal (data not shown). Toxic effects of other metal ions present in simulated effluent could lead to cell lysis and subsequent release of Co from the biomass into the solution. Hence, in a separate experiment, the chemical toxicity caused by SE to the bacteria during different periods of incubation was determined by a viable counting method (Fig. 2b). Up to 90-min incubation in SE, ≈20 % cell lysis was noted, but beyond 90 min, there was a drastic decrease in cell viability. Long incubation periods result thus in severe chemical toxicity due to other metal ions and chelators present in the decontamination solutions (Fig. 2b).

In subsequent experiments of *Co removal, the influence of concentrations of Co (i.e., 4.25, 8.5, 17, and 34 nM in SE) was studied. It may be noted that SE also contains large excess of iron (9.3 mM), chromium (3 mM), and nickel (0.93 mM). The results shown in Fig. 3a clearly depicted that the percentage of Co removal efficiency was 20–25 %, 50–60 %, 35–40 %, and 32–33 % at 4.25, 8.5, 17, and 34-nM initial Co concentrations, respectively. However, the specific Co removal capacities of bioengineered DR1 strains were 40–50, 260–280, 190–200, and 180–200 nmol g^{-1} of biomass at 4.25, 8.5, 17, and 34-nM initial Co concentrations, respectively (Table 2). The chemical process exhibited by the bioengineered *D. radiodurans* in picking up *Co from simulated spent decontamination solutions is expected to be concentration dependent. Starting with an initial ≥8.5-nM *Co

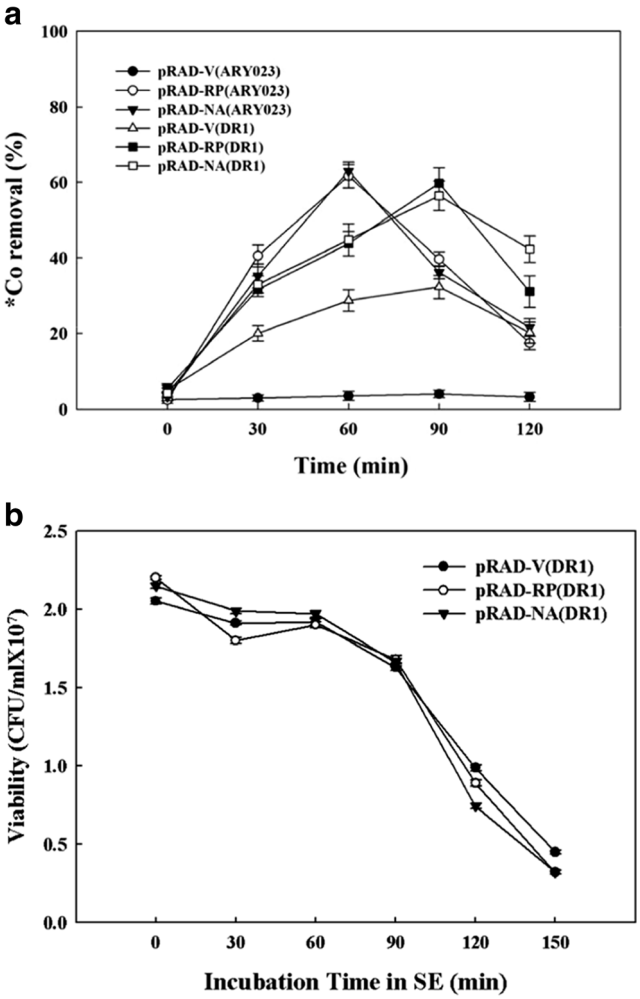

Fig. 2 **a** Cobalt removal kinetics in control as well as bioengineered *E. coli* and *D. radiodurans* with NiCoT-RP and NA: *filled circles on solid line* indicate *E. coli* ARY023 (pRAD-V), *empty circles on solid line* indicate *E. coli* ARY023 (pRAD-RP), *filled inverted triangles on solid line* indicate *E. coli* ARY023 (pRAD-NA), *empty triangles on solid line* indicate DR1 (pRAD-V), *filled squares on solid line* indicate DR1 (pRAD-RP), and *empty squares on solid line* indicate DR1 (pRAD-NA). **b** Bioengineered *E. coli* and *D. radiodurans* viability with incubation time in SE (concentration of Co is 8.5 nM): *filled circles on solid line* indicate pRAD-V, *empty circles on solid line* indicate pRAD-RP, and *filled inverted triangles on solid line* indicate pRAD-NA

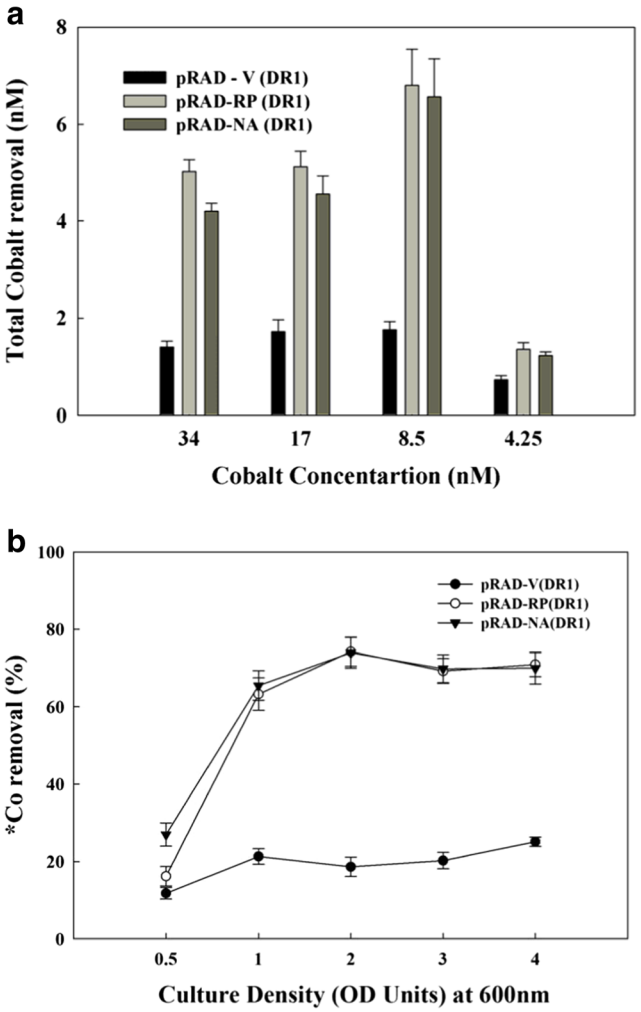

Fig. 3 **a** Total Co removal by control and bio-engineered DR1 species. **b** Effect of culture density on cobalt removal: *filled circles on solid line* indicate pRAD-V, *empty circles on solid line* indicate pRAD-RP, and *filled inverted triangles on solid line* indicate pRAD-NA

solution, the decrease in Co concentration observed is >5 nM. However, the sharp decrease in pickup of Co at a concentration of 4.25 nM under similar duration of exposure implies a change in the kinetics. The rate constant seems to get lowered by more than a factor of 5, and at <4.25 nM the rate constant seems to become independent of *Co concentration; a zero-order kinetics comes into play. The availability of cobalt ions in solution becomes critical, and this critical concentration seems to be 8–10 nM. Thus, the NiCoT-RP and NiCoT-NA genes known as high-affinity NiCoT transporter genes have a functional expression at an optimal cobalt concentration in the range of 8–10 nM (Hebbeln and Eitinger 2004); this could be the possible reason for the sudden drop in cobalt removal capacity observed in the present study. This suggests that the efficiency of Co removal is optimum when the initial concentration is around 10 nM. However, the amounts of removed Co (0.93, 5.03, 6.09, and 9.93 nmol) increased with the increasing initial concentration of Co. The strain with the vector removed constant amounts of Co independently of the initial Co concentration. This suggests that, in this case, passive mechanisms are responsible for Co capture. Co removal capacities were also established by using different culture densities of *D. radiodurans* transformants (Fig. 3b). About 18–22 % removal was achieved at 0.5 OD units; it increased to more than 65–75 % removal efficiency at ≥1.0 OD units. Beyond 1 OD unit, no significant change in *Co removal was observed; hence, 1 OD unit seems to be optimum. Both DR-RP and DR-NA showed similar *Co removal capacities. DR alone showed similar removal capacities all along the tested OD range. At 1.0 OD unit, maximum removal was obtained; this condition was employed in further experiments.

Table 2 Bioengineered DR1 strain *Co removal capacity in terms nanomoles per gram of biomass

Initial Co concentration (nM)	Observed decrease in Co concentration (nM)			Specific Co removal capacity (nmol Co/g of biomass)		
	pRAD-RP (DR1)	pRAD-NA (DR1)	pRAD-V (DR1)	pRAD-RP (DR1)	pRAD-NA (DR1)	pRAD-V (DR1)
34.0	5.00	4.50	1.25	200	180	50
17.0	5.00	4.75	1.75	200	190	70
8.50	7.00	6.50	1.75	280	260	70
4.25	1.25	1.00	0.75	50	40	30

A typical Co removal capacity of 200 nmol/g biomass, i.e., 11.79 μg/g of biomass, would mean that in a typical BWR reactor water volume of 1×10^5 l, with a solution Co concentration of 8.5 nM (i.e., 0.5 ppb), it would require about 4.2 kg of biomass for total Co bioremediation. At 1 OD culture density, application will require suspension of about 2 kg of biomass in 1×10^5 l of reactor water, and hence, a two-stage treatment would be required to remove all the Co from reactor water

This shows that there is an optimal solution volume to culture density (in the present experiment, it is≈20), beyond which it is not beneficial to use high culture densities. The increase in culture density was obtained by using the same growth phase culture. The improvement of cobalt removal from solution with a culture density shows a saturation effect at≥ 1 OD unit (about 65 % removal efficiency). In a heterogeneous system like bacteria culture/metal ion in solution, there can be different concentration gradients from the surface of the bacteria to bulk solution especially at high culture densities. Such a phenomenon could possibly explain the observed saturation pickups. Hence, culture density-solution volume parameter as observed in the present work is important from the application point of view. Finally, we checked the *Co removal at different pH of SE solution. The efficiency values were of 22, 60, and 40 % at pH 2.8, 5.8, and 7, respectively (Fig. 4). Hence, in all subsequent experiments, the optimal pH of 5.8 of simulated effluent was employed. Overall, the optimal conditions for *Co removal were 90-min incubation time with 1.0 OD unit culture density, in SE solution containing an initial concentration of 8.5 nM Co, at pH 5.8.

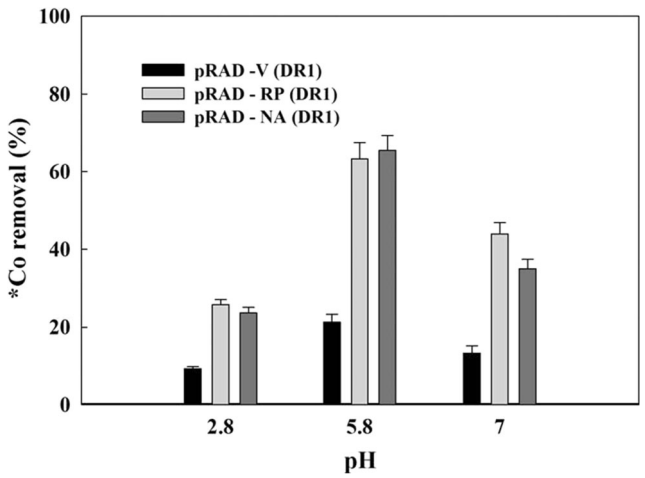

Fig. 4 Effect of pH on Co removal by control and cloned DR1 species

Post-irradiation response and *Co removal efficiency of *D. radiodurans* R1 harboring NiCoT genes of RP and NA

The radiation toxicity was determined on the different *Deinococcus* strains by evaluating their D_{xy} value, where *x* corresponds to 10 % survival, *y* corresponds to 90 % lethality, and *D* is the corresponding dose received (dose allowing 10 % survival and causing 90 % lethality). From the above relationship, this *D* value for DR clones was found to be close to 12.8 kGy (Fig. 5a). It indicates that cloned NiCoT genes of *R. palustris CGA009* and *N. aromaticivorans F-199* genes did not affect the radiation resistance of the host cell. There was no much change in the total Co removal efficiency of *D. radiodurans* clones (DR-RP and DR-NA) up to 6.4-kGy exposure. However, beyond 9.6-kGy dose, Co removal decreased progressively. The Co removal efficiency percentage was 55–60 %, 54–55 %, 52–59 %, 54–60 %, 52–53 %, 51–52 %, 39–42 %, 32–38 %, and 12–15 % at 0, 3.2, 4.8, 6.4, 8, 9.6, 12.8, 16, and 19.2 kGy of radiation dose, respectively (Fig. 5b). The studies on trace Co removal from simulated effluents containing a large excess (>10^5) of heterogeneous other metal ions (Fe, Cr, and Ni) using bioengineered radiation-resistant bacteria reported in this work are indeed very significant advancements over their previous efforts using several species of bacteria, fungi (Rashmi et al. 2007, 2004), and bioengineered *E. coli* (ARY023) with NiCoT gene of *R. palustris* and *N. aromaticivorans* (Raghu et al. 2008). The problem is that bioengineered *E. coli* hardly survives up to 20 Gy during the Co removal, because of γ-radiation toxicity, and the survival values for recombinant *E. coli* and DR at 20 Gy are≈1.125×10^7 and≈2.95×10^7 CFU ml^{-1}, respectively (Raghu et al. 2008). In the present study, we successfully constructed recombinant *D. radiodurans* R1 strains capable of expressing NiCoT genes of RP and NA, which exhibit good capacity to remove trace cobalt from simulated effluents of nuclear power reactors even after exposure of 8 kGy of γ-radiation dose, without changing its radiation resistance.

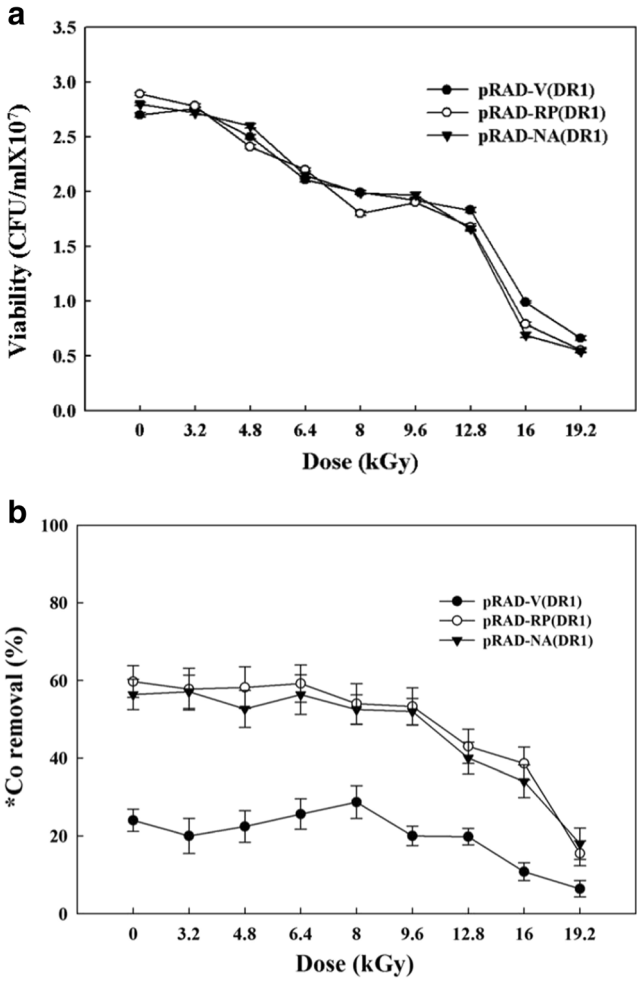

Fig. 5 **a** Radiation toxicity of different *Deinococcus* strains—D_{xy} value evaluation where *x* is 10 % survival and *y* is 90 % lethality: *filled circles on solid line* indicate pRAD-V, *empty circles on solid line* indicate pRAD-RP, and *filled inverted triangles on solid line* indicate pRAD-NA. **b** Post-irradiation percentage Co removal by control and cloned DR1 species with absorbed dose: *filled circles on solid line* indicate pRAD-V, *empty circles on solid line* indicate pRAD-RP, and *filled inverted triangles on solid line* indicate pRAD-NA

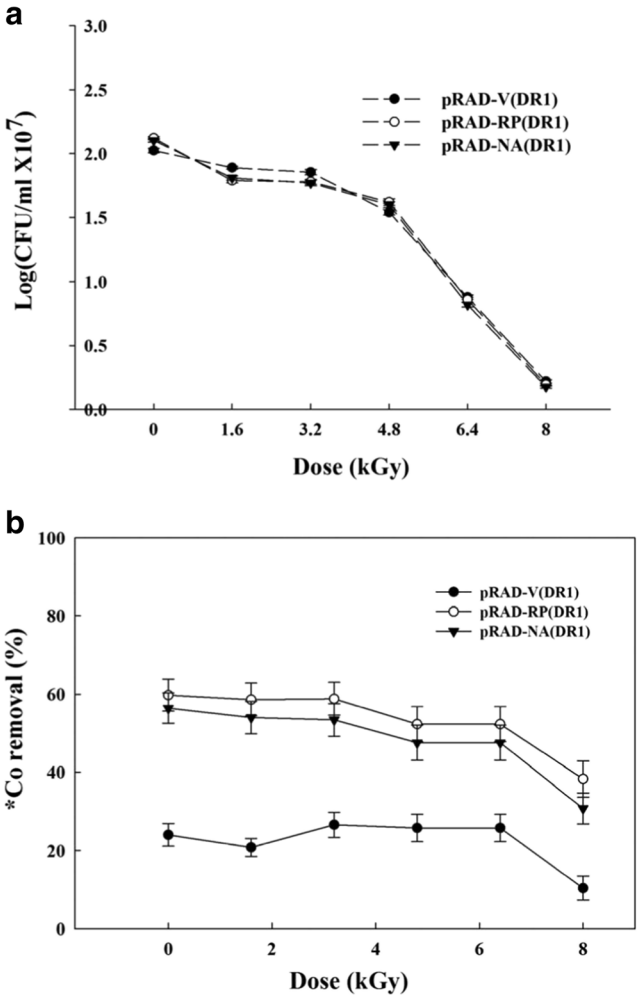

Fig. 6 **a** Survival of control and bioengineered *D. radiodurans* when irradiated in the presence of simulated spent decontamination solution to lower absorbed doses: *filled circles on dashed line* indicate pRAD-V, *open circles on dashed line* indicate pRAD-RP, and *filled inverted triangles on dashed line* indicate pRAD-NA. **b** Cobalt removal during γ-irradiation: percentage cobalt removal by control and cloned *Deinococcus* strains taken in simulated spent decontamination solution and exposed to different doses of γ-irradiation: *filled circles on solid line* indicate pRAD-V, *empty circles on solid line* indicate pRAD-RP, and *filled inverted triangles on solid line* indicate pRAD-NA

*Co removal efficiencies of bio-engineered *D. radiodurans* R1 during irradiation

D. radiodurans R1 clones (DR-RP and DR-NA) were exposed to different doses along with the simulated effluent solution. The results depicted in Fig. 6a clearly point to the fact that there was no change in the Co removal capacity of *Deinococcus* clones up to 3.2-kGy exposure. Up to 6.4-kGy dose received, there was a negligible decrease (≈5 %) in *Co pickup, and beyond this dose, *Co pickup reduced from 60 to 40 % (Fig. 6b). The known advantage with *D. radiodurans* is that it can easily survive at higher doses of γ-radiation, which was also clearly depicted in the present study (Fig. 5a, b). In addition, the Co removal efficiency of irradiated *D. radiodurans* recombinants was more when compared to the unirradiated ones (Table 3).

Discussion

Selective transport of molecules across the cell membrane is a fundamental process in all living organisms. Uptake of metals by various microorganisms broadly involves rapid process of biosorption to the cell wall followed by a relatively slower process of bioaccumulation into the cell (Rama Rao et al. 1996; Naveena et al. 2005). The later process involves both specific and nonspecific transporters located on the cell membrane. Recent advances in genetic engineering revealed that biological metal transporters assimilate and accumulate metals for bioremediation purposes (Maruthi Mohan et al. 2007). The cobalt transporter gene, which was first identified in *Rhodococcus rhodochorus* (*nhlF*) (Komeda et al. 1997), is

Table 3 Comparison of *Co removal by different strains

S. no	Name of the strain	*Co removed (μg/g dry wt biomass)	Reference
1.	*Neurospora crassa*	0.02	Rashmi et al.
2.	*Neurospora crassa* mutant (CSM9)	0.04	Rashmi et al.
3.	*E. coli* (DH5α)	1.00	Rashmi et al.
4.	*E. coli* (BL21)-NiCoT	5.00	Raghu et al.
5.	*E. coli* (JM109)-NiCoT	6.00	Raghu et al.
6.	*E. coli* (MC4100)-NiCoT	1.00	Raghu et al.
7.	*E. coli* (ARY023)-NiCoT	12.0	Raghu et al.
8.	DR1-NiCoT (RP and or NA)	12.0	Present study

the NiCoT family of secondary transporters. NiCoT genes have been characterized from various bacterial species such as *R. palustris* CGA009 (RP); *N. aromaticivorans* F-199 (NA) were shown to have preferential uptake for cobalt than for Ni (Hebbeln and Eitinger 2004; Raghu et al. 2008; Deng et al. 2013; Duprey et al. 2014). The tight homeostatic control mechanisms, which could limit metal influx or efflux of accumulated metals inside the cell, can be used for the improvement of bioremediation systems to remove or reduce the concentration of radionuclides from spent decontamination solutions (Liu and Duu-Jong 2014; Won et al. 2014). Recent developments in microbiology and molecular biology have been applied for metal bioremediation through construction of bioengineered microorganisms (Gadd and White 1989; Daly 2000; Gunjan et al. 2005; Lloyd and Renshaw 2005; Kumar et al. 2007; Prakash et al. 2013; Shih and Shen-Long 2014). Recently, the extremely radioresistant bacteria such as *Deinococcus geothermalis* and *D. radiodurans* have been bioengineered to reduce the organic solvents and uranium from nuclear waste (Lange et al. 1998; Appukuttan et al. 2006; Kulkarni et al. 2013; Misra et al. 2014). Interestingly, these bioengineered microbes survived against acute exposures of more than 10–20 kGy of γ-irradiation (Daly and Minton 1995; Battista 1997; Daly 2000; Appukuttan et al. 2006; Kulkarni et al. 2013; Misra et al. 2014). As *Deinococcus* does not often optimally recognize foreign promoters, the NiCoT genes were cloned and expressed in the present study under the influence of a *deinococcal* P_{groESL} promoter. Although the *groESL* promoter is involved in the heat shock response in most of the bacteria, the *deinococcal groESL* is a strong promoter at normal growth temperature of *Deinococcus* (Meima and Lidstrom 2000). Therefore, it was chosen to drive the expression of NiCoT genes of RP and NA. The NiCoT-RP and NiCoT-NA genes were fused to the *groESL* promoter and transformed into both *E. coli* and *D. radiodurans* using the shuttle vector pRAD1. These vectors are present in the cell at approximately the same copy number as the chromosome, which is present at 7 to 10 copies per cell (Meima and Lidstrom 2000); hence, there could be minimal possibility of plasmid curing or lost. During the time of the cobalt removal assay or of the process, there are no subcultures of *D. radiodurans*; hence, there will be no chance to lose the plasmid, because, first, the bacteria are grown in the presence of an antibiotic followed by harvesting and packing for the mean of remediation. Previous results showed that the *m*RNA expression of the *groESL* gene was induced by a wide range of divalent metal ions like Cu^{+2}, Zn^{+2} (at concentrations ranging from 10 to 100 μM), and Cd^{+2} (as low as 1 μM) (Ybarra and Webb 1999). However, Co^{+2} or Ni^{+2} have not been tested previously on the induction of the *groESL* promoter. Hence, in the present study, the metal response of the *groESL* promoter of *D. radiodurans* R1 was studied at *m*RNA level and was found to be activated under Ni or Co stress. These findings indicate that Ni or Co present in SE solution can regulate the expression of both NiCoT-RP and NiCoT-NA in *Deinococcus* recombinants.

Efforts have been made in the past to remove *Co effectively from simulated effluent decontamination solution of nuclear power reactors (Ayres 1970; Venkateswaran et al. 2003; Tišáková et al. 2013; Urch 2013). The major objective of our study is to address the problem of solid waste generation in the hitherto adopted ion-exchange methodology, which exhibits only a limited selectivity in metal ion pickup during the treatment of spent decontamination solution arising from the decontamination of primary heat transport systems of nuclear power reactors of the water-cooled type. In a typical boiling water reactor (BWR) system, the decontamination solution volume is 1×10^5 l (Taylor 1976; Bradbury et al. 1986) with a total Co concentration of≈8.5 nM (0.5 ppb) and a total exposure dose rate of >60 Gy h^{-1}. A typical Co removal capacity of 200 –nmol g $biomass^{-1}$ (i.e., 11.79 μg g^{-1} of biomass) would require about 4.2 kg of biomass for total Co bioremediation from a BWR system. At 1 OD culture density, application will require suspension of about 2 kg of biomass of *D. radiodurans* R1 expressing NiCoT genes of RP and NA in 1×10^5 l of reactor water, and hence, a two-stage treatment would be required to remove all the Co from reactor water.

The presently observed removal capacities are much higher than that observed in our previous studies using bioengineered *E. coli* with NiCoT genes of RP and NA (Raghu et al. 2008;

Duprey et al. 2014) and mutant variety of simple bacteria and fungi (Rashmi et al. 2007, 2004). In conclusion, this study reports for the first time the efficient *Co removal by recombinant DR-RP and DR-NA (12.0 μg of cobalt) when compared to fungi *Neurospora crassa*, *N. crassa* CSM-9, and *E. coli* DH5α (0.02, 0.04, and 1.0 μg, respectively). NiCoT gene efficiency in various host systems like *E. coli*-BL21, JM109, MC4100, and ARY023 and *D. radiodurans* showed removal efficiency values of 5.0, 6.0, 1.0, 12.0, and 12.0 μg/ g dry wt biomass, respectively. Though the ultimate total Co removal capacities observed with bioengineered *D. radiodurans* and *E. coli* is about the same, the radiation stability of *D. radiodurans* is a significant advantage over the *E. coli* species. In vitro *D. radiodurans* R1 had shown efficiency up to 6.4-kGy radiation. As a part of future work, we are in a process to clone and overexpress a suitable cytoplasmic cobalt metal binding protein in *D. radiodurans* R1, helps in excess metal sequestration entered through influx transporter, which, in turn, maintains the intracellular homeostasis and uncontrolled metal uptake. Further, it necessary to evaluate the efficiency of these engineered stains under field conditions enabling design of large-scale bioreactor for radioactive decontamination form nuclear reactors. The rationale of the present study was to demonstrate the advantages of using *D. radiodurans* for the bioremediation of *Co from spent decontamination solution in a radiation-ridden ambience. The batch experiments carried out in the present study successfully demonstrated the same. Nevertheless, more laboratory experiments especially under flow conditions and column parameters are essentially needed as well in order to translate into viable biotechnological applications of nuclear power reactors. Moreover, scaled-up studies are required before taking the methodology to actual application.

Acknowledgments The authors dedicate this manuscript to late Prof P. Maruthi Mohan. The authors thank Dr. Thomas Eitinger, Humboldt University, Germany, for providing the plasmids (pCH675-RP and pCH675-NA), K. W. Minton and M. J. Daly, Uniformed Services University of the Health Sciences, Bethesda, MD, for providing the *D. radiodurans* R1 strain, and M. E. Lidstrom, Departments of Chemical Engineering and Microbiology, University of Washington, Seattle, for providing the *E. coli*-*Deinococcus* shuttle vector pRAD1. We thank Deepti Appukuttan, who provided technical information related to *Deinococcus* transformation. We thank Dr. Venkata Prasuja Nakka, Department of Biotechnology and Bioinformatics, University of Hyderabad, Dr. Abdul Qadeer Mohammed, Department of Biochemistry, Osmania University, Hyderabad for their helpful suggestions and critical evaluation of the manuscript. The research work was supported by grants from the Department of Atomic Energy (No: 2004/37/17/BRNS), IFCPAR (3709-1), UGC-SAP (DRS-II), and INSPIRE Faculty Award (DST) IFA12-LSPA-11 to Raghu Gogada.

Conflict of interest The authors declare that they have no conflict of interest.

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