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Tolerance and accumulation of cesium and strontium in saprotrophic fungi

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Abstract. Soil contamination by nuclear accidents has led to a resurgence of interest in microbe-radionuclide interactions. Soil fungi accumulate radioactive elements from contaminated soil, and it has been hypothesized that this may alter the availability of radionuclides to plants and alter their movement in particular areas. This study intended to demonstrate how soil saprotrophic fungi accumulated Cs and Sr isotopes in both stable and radioactive solution forms. An experiment to determine the tolerance of fungal growth under extremely inhibitory concentrations of Cs and Sr stable isotopes was conducted. The results showed that fungal cells were more sensitive to Cs than to Sr. Accumulations of Cs and Sr were examined through sorption mechanisms using resting cells in the solutions under various conditions. The sorption capacity was indirectly determined by analysing the elements, which decreased in the solution. The equilibrium data were fitted with sorption isotherms to show the best fit with the Langmuir isotherm for both elements, assuming that the sorption sites form a surface monolayer. In addition, pH was examined to investigate its effect on the sorption capacity of Cs and Sr.

1. Introduction

Microbiota (which include fungi, bacteria, actinomycetes, protozoa, microfauna, and algae) are one of the most important components that play a role in radionuclide bioavailability and cycling. Some studies focusing on the interaction of microorganisms with cesium (Cs) and strontium (Sr) removal were reported several years ago. Unicellular organisms (e.g., *Euglena* and *Chlorella*) were the first species of microorganisms to be investigated for their ability to accumulate Cs [1]. Research on freshwater algae, Cyanobacteria, and bacteria (e.g., *Synechocystis* spp., *Rhodococcus* spp. and *Streptomyces* spp.) isolated from the natural environment were also carried out to investigate their accumulation capacity. Moreover, yeasts (e.g., *Saccharomyces cerevisiae*, *Rhodotorula rubra* and *Candida albicans*) [2-5] showed greater accumulation in the logarithmic phase in comparison to the stationary phase of kinetic growth [6]. Fungi are often the major component of soil microbiota that play a critical role in soil ecological function. Fungal biomass can function as a bio-accumulator of soluble forms of inorganic pollutants, including radionuclides. Higher fungi (e.g., lichens and mushrooms) have been investigated as indicators of Cs contamination in the environment and have presented accumulation in their fruitbodies or hyphae. The mechanism has been explained as Cs being immediately and non-specifically adsorbed onto the negatively charged cell surfaces and then taken up into the cytoplasm [5]. Soil fungi have the potential to immobilize radioactive Cs, which may alter availability to plants and radionuclide movement in surface soil layers and reduce migration through the soil [7]. Mycorrhizal fungi are an important group of soil fungi that may have similar effects, thus influencing the uptake of inorganic pollutants including



radionuclides [8]. Saprotrophic fungi are a group of fungi that are free-living in the soil and have an ability to decompose dead organic residues. *Trichoderma* is a genus of saprotrophic fungi that produces various kinds of secondary metabolites. Radionuclides may attach to these fungal cells extracellularly or be actively taken up intracellularly.

Radioactive isotopes, especially Cs and Sr, are present in the soil in their cationic forms, and there are no general differences between their radioactive and stable isotopes with respect to their chemical elements or their behavior in the environment. Cs is a rare type of alkali metal. Its stable isotope is ^{133}Cs , while the fission products include four main Cs isotopes. It exists in the environment as a monovalent cation (Cs^+). Based on the long half-life of radioactive Cs (2 years for ^{134}Cs and 30 years ^{137}Cs), they have become the dominant radionuclides contaminating the environment. In nature, Sr occurs only in the Sr^{2+} oxidation state. Radioactive isotopes can be produced by fission reactions. In particular, ^{90}Sr is a problematic environmental contaminant because of its long half-life (29 years), high transferability, and high fission yield. Currently, soil contamination caused by radioactive elements resulting from nuclear accidents is leading to a resurgence of interest in microbe-radionuclide interactions. However, information on the role of the fungi as a storage organism has been scarce. This study intends to clearly understand how a soil saprotrophic fungi *Trichoderma* spp. accumulates Cs and Sr as both stable and radioactive isotopes.

2. Materials and methods

2.1. Soil saprotrophic fungi

Trichoderma spp. is the one of the soil saprotrophic fungi representatives that was isolated from a rhizosphere soil that had abundant microbial activity. The sampling sites were located in a conifer forest at Takizawa Research Forest of Iwate University, Japan (39°46'32N, 144°9'25E). *Trichoderma* spp. was isolated by making a dilution series of soil solution from which 1 mL of each dilution was spread onto a potato dextrose agar (PDA) plate. The plates were incubated for 7 days at 25 – 30 °C. Colonies were selected and re-cultured on fresh PDA plates, and DNA extraction and PCR amplification were conducted. Entire internal transcribed spacer (ITS) regions that were potentially useful in rapid and accurate diagnosis of fungal isolation were sequenced [9-10]. ITS1 (5'-TCC GTA GGT GAA CCT TGC GG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used as forward and reverse primers. Sequences of DNA fragments were identified using Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, USA).

2.2. Cell preparation

2.2.1. Active cell preparation

Trichoderma spp. was cultured in potato dextrose broth (PDB) with shaking at 110 rpm at 25°C until it reached the late exponential growth phase.

2.2.2. Resting cell preparation

Trichoderma spp. cells at the late exponential growth phase derived from the active cells were washed three times with sterile purified water to stop cell growth.

2.3. Growth kinetics and tolerance

To investigate the effect of Cs and Sr on the growth kinetics of *Trichoderma* spp., Cs^+ and Sr^{2+} were each prepared in 50 mL of fresh PDB with concentrations of 5, 10, 25, 50, and 100 mM. 1 mL of the active cell culture was then inoculated into the PDB solution. After that, they were incubated with shaking at 110 rpm at 25°C until the experiment was completed (168 h). Approximately 5 mL of the suspension was sampled at each time interval with a sterilized syringe. All the experiments were conducted in duplicate. The cell concentration was determined indirectly by measuring the optical

density at 600 nm (OD_{600}), using a spectrophotometer (PhotoLab 6100 VIS, Wissenschaftlich-Technische Werkstätten, Germany).

2.4. Accumulation

To investigate the effect of Cs and Sr in both stable and radioactive isotopic forms on sorption, the resting cells were resuspended in 25 mL of sterile purified water. The experimental conditions are shown in table 1. The cells were incubated with shaking at 110 rpm at 25°C for 3 h. They were then filtrated through dried filter paper (Whatman no.1). The remaining Cs and Sr in the aliquot and dry weight of cell pellets were measured.

Table 1. Experimental Conditions.

No.	Elements	Concentration	pH
1	^{133}Cs	1, 10, 20, 25, and 50 ppb	4 ^a
2	^{88}Sr	1, 10, 20, 25, and 50 ppb	4 ^a
3	$^{133}\text{Cs}/^{88}\text{Sr}$	2.5, 5, 10, 25, and 50 ppb	4 ^a
4	$^{134}\text{Cs}/^{85}\text{Sr}$	^{85}Sr ; 20, 40, 80, 200, and 400 Bq ^b ^{134}Cs ; 100, 200, 400, 1000, and 2000 Bq ^b	4 ^a
5	$^{133}\text{Cs}/^{88}\text{Sr}$	10 ppb	3, 5, 7 and 9
6	$^{134}\text{Cs}/^{85}\text{Sr}$	^{85}Sr ; 80 Bq [*] ; and ^{134}Cs ; 400 Bq	3, 5, 7 and 9

^a pH was not adjusted. The initial pH was approximately 4.

^b Activity was measured from 25 mL of the samples.

2.5. Radioactive materials

^{134}Cs has a radioactive half-life of 2.06 years. It is a β -emitter, and it also emits γ -ray with an energy level of 604.7 keV (97.5%). ^{85}Sr has a radioactive half-life of 64.85 days; it decays by electron capture, emitting γ -ray with an energy level of 514.0 keV (98.3%). The ^{134}Cs and ^{85}Sr stock solutions contained an activity concentration of approximately 2.5 kBq mL⁻¹, the pH was adjusted to pH 7. The radiotracer solution was added to each experiment at 50, 100, 200, 500, and 1000 μL to achieve an activity of approximately 20, 40, 80, 200, and 400 Bq for ^{85}Sr and 100, 200, 400, 1000, and 200 for ^{134}Cs .

2.6. Measurement

^{133}Cs and ^{88}Sr are stable isotopes. They were measured by inductively coupled plasma mass spectrometry (ICP-MS; XSeries 2, Thermo Scientific, USA). Sample preparation followed the standard methods ISO17294-2 and EPA 6020a [11]. ^{134}Cs and ^{85}Sr are radioactive isotopes. The γ -ray spectrum was measured for 30 to 60 min using a gamma spectrometer with a HPGe detector (GMX series, EG&G, Ortec, USA) and a channel analyser.

3. Results and Discussion

3.1. Soil saprotrophic fungi

Colonies on the PDA plates that represented those with major growth and different morphology were selected using the hyphal tip isolation technique. This technique isolates single colonies that represent a strain of soil fungus. These strains were then re-cultured on fresh PDA plates. The ITS regions from the amplified DNA samples were compared against the nucleotide collection database in GenBank by using BLAST. The result showed greater than 95% similarity to *Trichoderma* spp. (data not shown). Therefore, it was concluded that the representative fungus used in this study belonged to *Trichoderma* spp., which is a genus of saprotrophic fungi that produces various kinds of secondary metabolites [12].

3.2. Growth kinetics and tolerance

The effect of Cs and Sr on the kinetic growth of *Trichoderma* spp. was investigated under extreme concentrations (5, 10, 25, 50 and 100 mM) and compared with a control condition under which no elements were added (0 mM). Growth curves were plotted for the logarithm of the number of cells (N), which were indirectly measured using optical density in the unit of $\ln(N/N_0)$ against time in the unit of hours. The results are presented in figure 1.

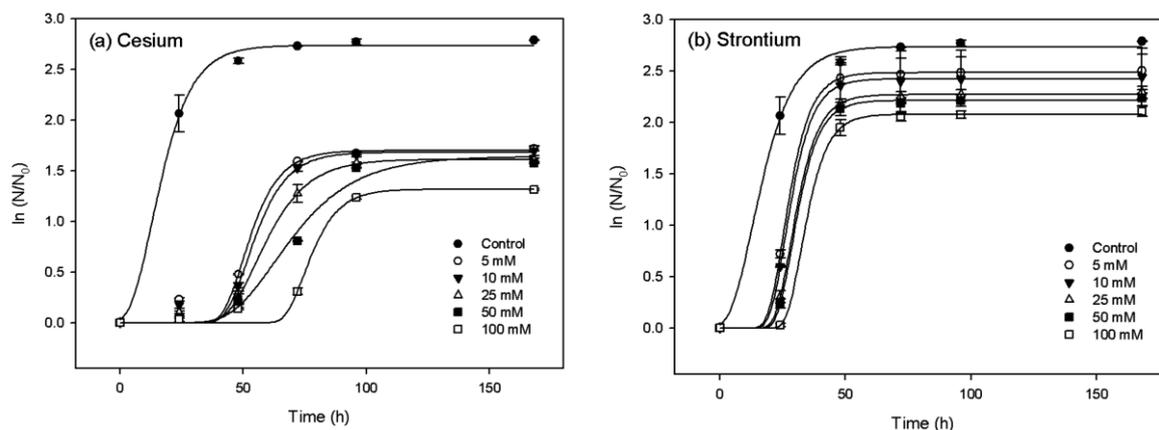


Figure 1. *Trichoderma* spp. cell growth kinetics under various concentrations of (a) cesium and (b) strontium fitted with Gomperts model.

Trichoderma spp. yielded a higher number of cells at lower Cs and Sr concentrations and the number of cells decreased as the concentration increased. *Trichoderma* spp. growth mostly exhibited an exponential phase up to 48 h. However, when the Cs concentration was increased to 50 mM, the exponential phase extended to 72 h before reaching the stationary phase. Cell growth at the stationary phase decreased maximally to 52% for Cs and 24% for Sr compared with the control at 100 mM for both elements. These results showed that *Trichoderma* spp. was more sensitive to Cs than Sr. Similar results were also observed by various kind of microorganisms in previous studies. For instance, the filamentous actinomycetes also could not grow in the presence of Cs concentrations higher than 25 mM, which is more sensitive to Cs than planktonic bacteria [13]. Cyanobacterium demonstrated considerable toxicity, as shown by changes in various parameters such as dry weight, protein and carbohydrate content as well as acid phosphatase activity, which also decreased with increased Sr concentrations [14]. In addition, Sr inhibited the growth of *Micrococcus* spp. and *Cupriavidus metallidurans* when the concentration reached 300 mM [15].

The phases of the growth curve can be described by three parameters. Maximum specific growth rate (μ_m) is defined as the specific growth rate in a certain period of time, resulting in values for lag time (λ) and the asymptote (A) which is defined as the specific growth rate at time ∞ ; $A = \ln(N_\infty/N_0)$. Those parameters were obtained for each concentration. Gomperts model is a sigmoidal mathematical model that has been used to estimate these parameters. The Gomperts function (equation 1) was rewritten by substituting the mathematical parameters with biological parameters: μ_m , λ , and A (equation 2) [16].

$$y = a \cdot \exp[-\exp(b - cx)] \quad (1)$$

$$y = A \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A} (\lambda - t) + 1\right]\right\} \quad (2)$$

The data-fitting equations obtained using Gomperts model were statistically evaluated for the residuals and the goodness of fit (table 2). Serial correlations for the residuals were examined using the Durbin-Watson (DW) statistics, which was used to test residuals for their independence to each other. The DW values were obtained by fitting the models to the growth data. If the residuals do not correlate, the

DW values will be in the range 1.5 to 2.5, which is obtained when the serial correlation is small and the residuals are randomly distributed around the zero line when plotted against time. There were fewer curves for DW values, indicating that the errors were positively autocorrelated which was shown by DW values that were lower than the lower bound or negatively autocorrelated that were indicated by DW values higher than the upper bound. There were more DW values, indicating that the errors were not autocorrelated. The R^2 values for most curves were greater than 0.98, indicating that this statistics can be used as a basis for model evaluation.

Table 2. Statistics and parameter values obtained when the models were fitted to the growth data.

	Cesium (Cs)					Strontium (Sr)				
	DW	R^2	A	λ	μ_m	DW	R^2	A	λ	μ_m
Control	1.77	0.997	2.74	0.05	8.90	1.77	0.997	2.74	0.05	8.90
5 mM	2.01	0.983	1.70	0.62	5.11	1.50	1.000	2.48	0.55	5.44
10 mM	2.02	0.990	1.68	0.55	5.44	1.51	1.000	2.42	0.55	5.36
25 mM	1.99	0.996	1.61	0.33	6.85	1.50	1.000	2.27	0.61	5.06
50 mM	3.16*	0.986	1.64	0.14	10.72	1.53	1.000	2.21	0.65	4.82
100 mM	1.50	0.989	1.32	1.14	3.72	1.50	1.000	2.08	0.81	4.40

*DW value was higher than the upper bound

3.3. Accumulation

Data were gathered using several sorption isotherms. The best fitting model was indicated by high values of the correlation coefficient of determination (figure 2).

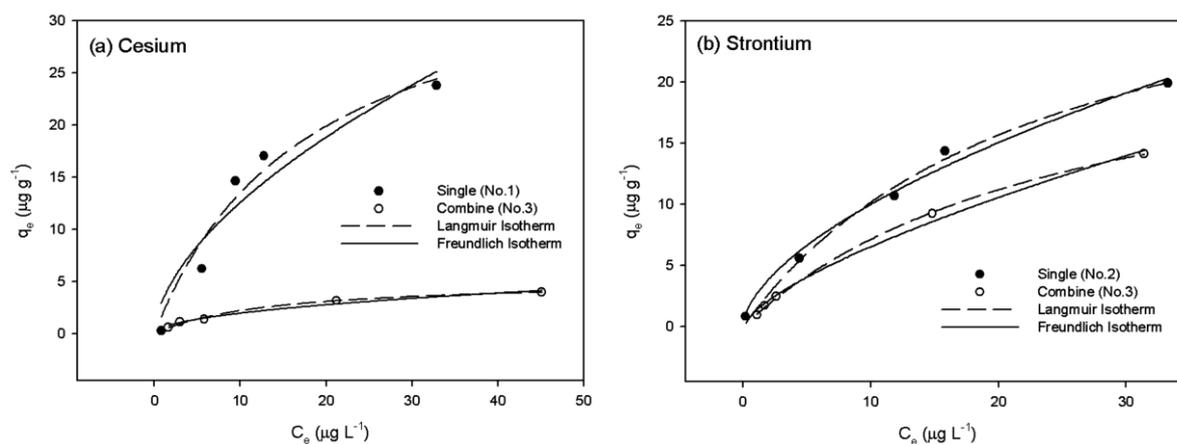


Figure 2. Data fitting with the Langmuir and Freundlich isotherm for (a) cesium and (b) strontium.

The results indicated that Cs and Sr were adsorbed under all conditions in agreement with the Langmuir isotherm ($R^2 = 0.96 - 0.99$), which was theoretically derived supposing no interaction between the elements and the cell surface and that adsorption occurred on fixed homogenous adsorption sites forming a monolayer surface coverage (equation 3).

$$q_e = \frac{q_m K_L C_e}{1 + C_e} \quad (3)$$

where q_e is the amount of elements adsorbed ($\mu\text{g g}^{-1}$), C_e is the amount of element concentration in the solution ($\mu\text{g L}^{-1}$), q_m is the maximum amount adsorbed ($\mu\text{g g}^{-1}$) and K_L is an equilibrium constant representing the affinity between adsorbate and adsorbent.

However, for Sr, there was also strong agreement with the Freundlich isotherm for both single and combined conditions ($R^2 = 0.99$). The Freundlich isotherm is an empirical equation based on the adsorption on the heterogeneous surface (equation 4).

$$q_e = K_F C_e^{1/n} \quad (4)$$

where q_e is the amount of elements adsorbed ($\mu\text{g g}^{-1}$), C_e is the amount of element concentration in the solution ($\mu\text{g L}^{-1}$), K_f is a constant that is related to sorption capacity and n is an empirical parameter that varies with the degree of heterogeneity.

The results show the same trend for both elements. For the stable isotopes (^{133}Cs and ^{88}Sr), the sorption capacity increased when the initial concentration of elements was increased and showed a specific trend pertaining to approaching a plateau. Lan *et al.* (2014) reported a similar result that assumed that the biosorption of Cs onto *Rhodospiridium fluviale* (initial Cs concentration varied from 0.01 to 2.0 mg/L) was a monolayer adsorption [17]. Furthermore, ^{88}Sr was also found to highly agree with the Freundlich isotherm, which assumes that heterogeneity occurred and that the adsorption capacity of ^{88}Sr was higher than ^{133}Cs . The effect of the presence of competitive ions occurred under combined conditions whereby each element was less adsorbed than under the single condition. This suggests that Sr^{2+} and Cs^+ ions were adsorbed onto similar sites. A similar result was observed by Ofomaja *et al.* (2013), who studied the effect of Cs sorption in the presence of Na^+ and Ca^{2+} [18], which have an ionic radius of 0.99 Å, and was found to be similar to that for Sr^{2+} 1.12 Å. To determine the mechanistic parameters associated with stable isotope (^{133}Cs and ^{88}Sr) sorption, results were obtained from sorption experiments for both single and combined conditions. The isotherm parameters are shown in table 3.

Table 3. Isotherm parameters for cesium and strontium sorption onto *Trichoderma spp.*

Element	Langmuir constants			Freundlich constants		
	q_m	K_L	R^2	K_f	$1/n$	R^2
^{133}Cs (single)	37.72	17.87	0.9604	3.32	0.58	0.9174
^{133}Cs (combined)	5.10	12.96	0.9914	0.63	0.49	0.9758
^{88}Sr (single)	34.01	23.38	0.9940	2.58	0.59	0.9901
^{88}Sr (combined)	25.85	26.15	0.9995	1.34	0.69	0.9925

Figure 3 demonstrates the effect of the initial activity of Cs and Sr, which was derived from the experiments in combined conditions between ^{134}Cs and ^{85}Sr to investigate the sorption capacity (Q), which was calculated based on equation 5.

$$Q \text{ (Bq/mg)} = \frac{(A_i - A_f)}{M} \quad (5)$$

where A_i and A_f are the initial and final activities in the solution (A) and M is the mass of *Trichoderma spp.* (mg).

Both Cs and Sr showed the same trend (figure 3): the sorption capacity of Sr was higher than Cs at the same initial activity, which corresponded with the stable isotopes mentioned above. However the ability to accumulate Cs and Sr was dependent on the different species of microorganisms, according to reports from previous studies. For example *Pseudomonas fluorescens* and *Rhodococcus spp.* isolated from the soil were grown in the presence of Cs, whereas no detectable Cs accumulation was observed for *Pseudomonas fluorescens*[4].

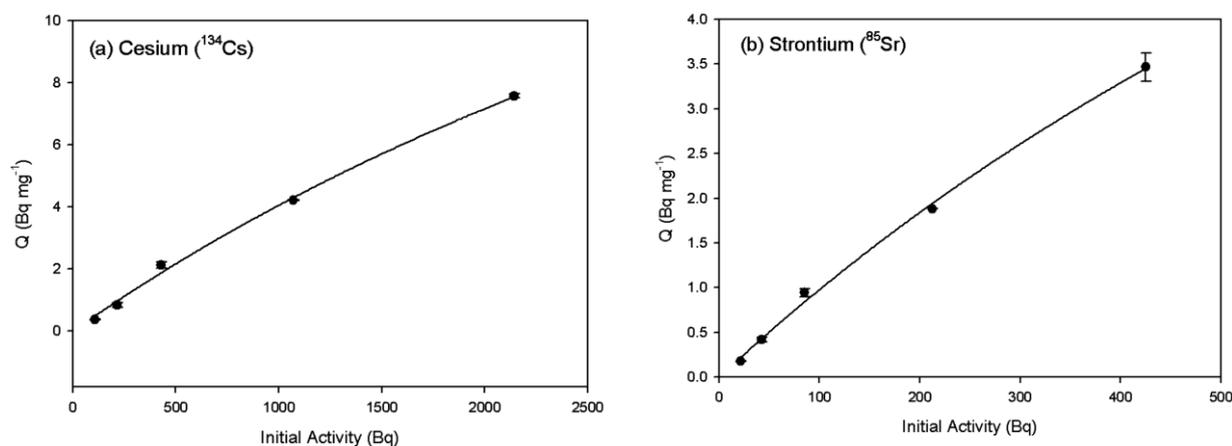


Figure 3. Effect of the initial activity of (a) cesium (^{134}Cs) and (b) strontium (^{85}Sr) on the sorption capacity.

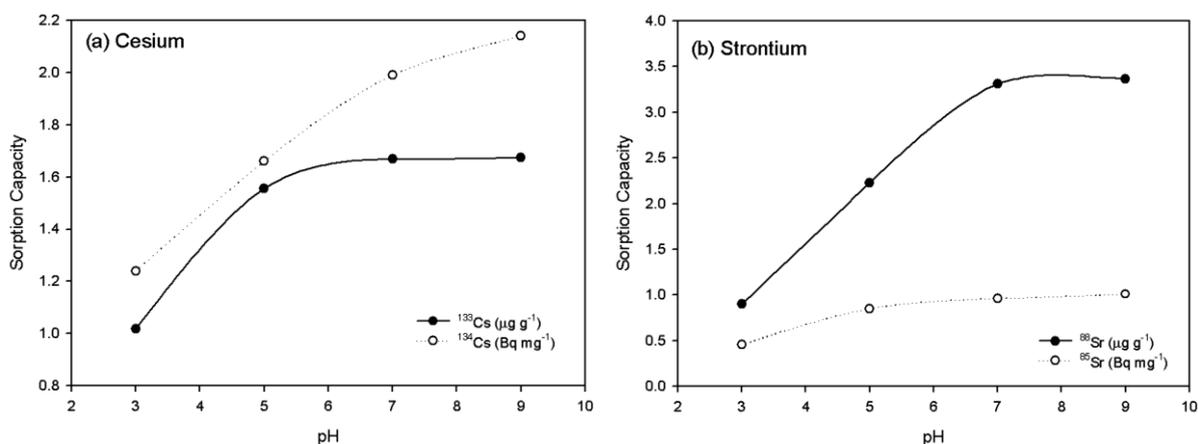


Figure 4. Effect of pH on the sorption of (a) cesium and (b) strontium.

External physico-chemical factors, such as pH, can be a major factor that influences sorption of Cs and Sr. Figure 4 shows the effect of pH on the sorption of Cs and Sr onto *Trichoderma* spp. where pH was plotted against the sorption capacity in the unit of $\mu\text{g g}^{-1}$ for stable isotopes and Bq mg^{-1} for radioactive isotopes. The results demonstrated that pH had a greater effect on the sorption of both elements with the same trend for stable and radioactive isotopes. When the pH was increased from 3 to either 7 or 9, the sorption capacity onto *Trichoderma* spp. greatly increased for both Cs and Sr. Meanwhile, at lower pH (pH 3) the sorption capacity decreased to its lowest capacity, probably because H^+ also interacts with sites mediating the monovalent and divalent cation transport system. Then, at the lower pH, binding of H^+ inhibits the sorption of other cations such as Cs^+ and Sr^{2+} [19-20]. Thus, during the experiment, the sorption ratio of Cs and Sr onto *Trichoderma* spp. increased when pH was increased from 3 to 9. In addition, other species, for example the marine bacteria, *Vibrio alginolyticus* and yeast (*Saccharomyces cerevisiae*), have also shown the same mechanism [6,19, 21].

4. Conclusion

Trichoderma spp. was isolated from the soil. Experiments were conducted with two main approaches: growth kinetics and accumulation. An experiment to investigate the growth kinetics was conducted under extreme concentrations of stable Cs and Sr isotopes. The results showed that *Trichoderma* spp. was more sensitive to Cs than to Sr. For Cs, *Trichoderma* spp. grew at lower concentrations (5 to 25 mM) and an inhibition of growth appeared at 50 mM, which became more significant when the

concentration increased to 100 mM. The growth rate decreased evenly when the concentration of Sr increased. The accumulation of Cs and Sr were conducted under various conditions using both stable and radioactive isotopes. The results showed the same trend for both elements and both isotopes; the sorption capacity increased when the initial concentration and initial activity increased. Then, the effect of pH, showed a decrease in sorption capacity at lower pH because H⁺ also interacts with sites mediating the monovalent and divalent cation transport system. The radioactive accumulation in fungi was found to be the most difficult to predict. This work did not intend to provide a comprehensive result of biological processes, but rather to study the possibility of soil saprotrophic fungi to accumulate the radioactive elements. The experimental results indicated that *Trichoderma* spp. effectively contributed substantially to the long-term retention of Cs and Sr in an organic layer which is essential to reduce the migration in soil layers by directly bind fungal cells extracellularly or actively taken up intracellularly which should be completed by future studies.

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