*ejpmr, 2016,3(5), 379-386*

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

# MECHANISM OF STRONTIUM UPTAKE AND TRANSPORT IN *NEUROSPORA CRASSA*

**Anupama M., Ashok Kumar K., and Naveena Lavanya Latha J.**

Department of Biotechnology, Krishna University, Machilipatnam-52001.

***Corresponding Author: Dr. Naveena Lavanya Latha, J.**

Department of Biotechnology, Krishna University, Machilipatnam-52001.

**Article Received on 11/03/2016** **Article Revised on 31/03/2016** **Article Accepted on 20/04/2016**

## ABSTRACT

Strontium uptake and sub cellular compartmentation were investigated in the model filamentous fungus *Neurospora crassa*. $Sr^{2+}$ uptake by the mycelium was found to be a function of time involving three compartments corresponding in series to cell wall (52%), cytoplasm (19%) and vacuole (25%), when mycelia were exposed to a 10 mM $Sr^{2+}$ concentration. At 4 ˚C, and in alkali killed biomass only ~50% of the $Sr^{2+}$ taken up by the mycelia was recovered in cell wall-bound fraction, suggesting it is a metabolically mediated process. Inhibitors of IP3 pathway and CaM pathway inhibited $Sr^{2+}$ accumulation in *N.crassa* mycelia by up to 88%. $Sr^{2+}$ uptake into cytoplasm is through the $Ca^{2+}$ transporters. Binding of $Sr^{2+}$ onto cell walls, accumulation of into the cell and displacement of calcium by strontium reveals the ability of the organism to substantiate $Ca^{2+}$ by $Sr^{2+}$. These data represent a first step towards the understanding role of $Sr^{2+}$ as a substituent in place of $Ca^{2+}$ in fungi.

**KEYWORDS:** $Sr^{2+}$ uptake, $Sr^{2+}$ transport, cell wall bound, filamentous fungi, *Neurospora crassa.*

## 1 INTRODUCTION

Strontium is a trace element that has no known essential biological role.[1-3] The divalent cation, $Sr^{2+}$, displays physicochemical properties similar to those of the abundant and biologically essential cations $Ca^{2+}$ and $Mg^{2+}$[4], and it has been shown that $Sr^{2+}$ may substitute for one or both of these ions in binding processes at biological cell surfaces as well as in active uptake via divalent cation transport systems. For example, cell wall binding of metal ions like $Co^{2+}$, $Cu^{2+}$ and $Ni^{2+}$ resulted in a stoichiometric release of $Ca^{2+}$ and $Mg^{2+}$ from the *N.crassa* indicating binding of these ions to equivalent functional groups at the cell surface.[5] Furthermore, a common system is known to mediate active uptake of $Sr^{2+}$, $Ca^{2+}$, and $Mg^{2+}$ in the yeast *Saccharomyces cerevisiae*, albeit with a lower affinity for $Sr^{2+}$ than for $Ca^{2+}$ or $Mg^{2+}$.[6, 7] Fungi maintain low intracellular levels of free $Ca^{2+}$ ions and $Ca^{2+}$ transport across plasma membrane in fungi is suggested to occur through gated channels sensitive to membrane potential.[8] Transient elevations of the [$Ca^{2+}$] play a central role in intracellular signal transduction in plant[9-11] and animal cells.[12, 13] Because of its chemical similarity with calcium, it is easily incorporated into bone and continues to irradiate localized tissues with the eventual development of bone sarcoma and leukemia.[14-19] Vezzoli *et al.*,[20] effectively used stable strontium as a tracer of calcium absorption in their study of normocalciuric subjects with calcium kidney stones. The urinary excretion of absorbed $Sr^{2+}$ was also examined but found not to correlate with calcium excretion. Due to their chemical similarity, the biological behaviours of caesium and strontium resemble those of potassium and calcium, respectively. The uptake of $Cs^{+}$ is well characterized in plants, whereas only few data are available for the uptake of $Sr^{2+}$.[18] Early studies with a variety of plants systems, algae and yeasts have implicated on the close relationship between $Ca^{2+}$ and $Sr^{2+}$.[21] However there is no specific reported mechanism of strontium transport available in living organisms. The purpose of this investigation was to study the strontium binding ability and the mechanisms of $Sr^{2+}$ uptake and transportation in the model fungus *Neurospora crassa*.

## 2 MATERIALS AND METHODS

### *2.1 Organism and media*

The model filamentous fungus *Neurospora crassa* wild type strain (FGSC # 2489) and *Cnb-1*, calcineurin mutant of *N. crassa* (G 7416-732) were obtained from Fungal Genetics Stock Centre, Kansas City, Missisipi, USA. Vacuolar mutant of *N. crassa* (*vma-5*) was obtained from Dr. B. J. Bowmann (University of California, Santa Cruz, USA).[22]

### 2.2 *Media and growth conditions*

Media composition, growth conditions and metal ion estimation procedures for *N. crassa* were described in earlier studies.[23, 24] In brief, *N. crassa* was cultured in basal medium containing glucose, 20 g; $KH_2PO_4$, 3 g; $(NH_4)NO_3$, 2 g; $(NH_4)_2C_4H_4O$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g;

NaCl, 0.1 g; $CaCl_2$, 0.1 g; trace elements (pg): Zn, 200; Mn, 200; Cu, 80; Fe, 20; Mo, 20; biotin 5pg; in 1L deionized distilled water and incubated at room temperature.

### *2.3 $Sr^{2+}/Ca^{2+}$ removal screen*

To determine the ability of the fungus to remove strontium and/or calcium from liquid media, the 3 day old mycelial mats of *N.crassa* were incubated in 10 ml basal medium containing 0, 0.1, 0.5, 1, 5, 10, 25, 50 and 100 mM metal ion ($SrCl_2/CaCl_2$) at 100 rpm for 24 h. Metal taken up was quantified, following acid digestion of dried mycelia by atomic absorption spectrophotometry (AAS) using a Perkin Elmer 2380 spectrophotometer. The medium and acid digested samples of dried mycelial mats were analyzed for metal content by AAS. In a time dependent metal removal studies, pre grown mycelial mats (3 d) were incubated in 10 ml basal medium containing metal ion (0.1 and 10 mM) for 24 hrs. At different time intervals, metal taken up by mycelia was quantified, following acid digestion by AAS.

### *2.4 $IC_{50}$ determination*

To determine the 50% growth inhibitory concentration of $CaCl_2$ and $SrCl_2$ on *N.crassa*, conidiospores ($10^6$/ml) were inoculated into 10 ml basal medium containing metal ion and incubated at 28 ± $1^0$ C for 3 d. The mycelia were harvested and washed thrice with glass-distilled water, pressed free of excess moisture with filter paper and dried over night in hot-air oven at 60-$80^0$ C. Growth was quantitated by measuring dry weight of the mycelia.[25] $IC_{50}$ (50% growth inhibitory concentration) value for metal ion was derived from graphical plots of growth verses metal ion concentration.

### *2.5 $Sr^{2+}/Ca^{2+}$ uptake by N.crassa*

*N.crassa* was further assayed for its ability to remove metal from basal medium amended with $SrCl_2 \cdot 5H_2O$ or $CaCl_2 \cdot 7H_2O$ (0 to 1 mM). In further studies to assess possible interaction between $Sr^{2+}$ and $Ca^{2+}$ uptake, both metals were present at equal concentrations at the levels listed above. Also, titration experiments were designed with each metal titrated against the other.

For example, $Ca^{2+}$ was fixed at 5 mM ($IC_{50}$ concentration of $Ca^{2+}$ in basal medium), while $Sr^{2+}$ was varied over a range from 0 to 50 mM (a range including the concentration of calcium in the basal medium). The fungus was grown at 28±1° C, harvested, and the mats and filtrates prepared for analysis as described above.

### *2.6 Mechanism of $Sr^{2+}$ uptake*

To determine if uptake was due to an active or passive process, the removal of $Sr^{2+}$ from $SrCl_2$ by live azide treated and alkali killed biomass of *N.crassa* was compared. *N.crassa* spores ($10^6$ spores/ml) were inoculated into basal medium and cultures were then harvested by filtration and the mycelial mats were then boiled in 5% KOH for 5 minutes, filtered and neutralized with 0.1 N HCl. For azide treatment, the 3 d old mycelial mats were exposed to 10 mM sodium azide for 1 hr and used for experiment. The alkali killed biomass of *N.crassa*, azide treated and control mycelia were then dropped into flasks (three replicates per treatment) containing 100 ml each of the basal medium with 10 mM $SrCl_2$ and incubated at 28±1 °C for 24 h as shake cultures (125 rpm). The cultures were then harvested, and the mats and filtrates were prepared for analysis as described above.

### *2.7 $Sr^{2+}$ compartmentation on cell wall and sub cellular organelle*

**a) Cell wall isolation**

The method of Schmit *et al*.,[26] was used for cell wall preparation. Dried cell wall preparations was ground to powder, weighed and used as suspension.

**b) Isolation of nuclei**

Sub-cellular fractionation was carried out as per the reported *Neurospora* protocol guide downloaded from FGSC site (http://www.fgsc.net/Neurospora/ *Neurospora* ProtocolGuide. htm). The nuclei pellet was suspended in lysis buffer containing Tris-SDS and the amount of $Sr^{2+}$ present was estimated by AAS.

**c) Isolation of mitochondria and vacuoles**

For isolation of mitochondria and vacuoles, the mycelial suspension of *N.crassa* in 10 mM TES buffer pH-7.2 with 1mM EDTA was centrifuged at 600g to remove cell wall debris. The supernatant was centrifuged at 15000 g for 20 minutes. The supernatant was subjected to step-gradient using 1.6 M sucrose (lower) and 1.2 M sucrose (upper) in 50 ml tubes for 2 h at 43000 g. Mitochondria collect at the interface of 1.2/1.6 M sucrose and vacuoles are pelleted down.

### *2.9 Effect of inhibitors of calcium transport on strontium uptake*

To check whether the uptake and transport of strontium is through the same channels of calcium transport, the 3d old mycelial mats of *N.crassa* were incubated in medium containing 10 mM Strontium with specific for 24 hr and then suspended in 10 ml of EDTA (10 mM) to remove cell wall bound metals and strontium taken up by mycelium (intracellular) was estimated by AAS.

### *2.10 Strontium uptake by mutants of N.crassa*

To confirm the results of strontium transport from the above experiment, the transport of strontium was studied in the mutants of *N.crassa*, *vma-5* [22] and *CNB-1*.[27] These are the mutants of *N.crassa* defective in vacuolar calcium transport and calcineurin path way respectively.

The mycelial mats (3d old) of wild type and mutant strains were incubated in 10 ml of strontium solution (10 mM) for 24 hrs and the $Sr^{2+}$ taken up (cell wall bound and intracellular) by the mycelia were determined by AAS followed by acid digestion.

***2.11 $Sr^{2+}$ /$Ca^{2+}$ uptake and metal displacement studies with isolated cell wall preparations of N.crassa***

Next, isolated cell wall preparations of *N.crassa* were assayed for metal removal from basal medium amended with $SrCl_2$ or $CaCl_2$ (0 to 1 mM). In order to assess possible competitive interaction between $Sr^{2+}$ and $Ca^{2+}$ with cell wall components during uptake, both metals were added at equal concentrations as listed above. Also, metal displacement experiments were designed to determine the ability of each metal to displace the other cell wall bound metal. For example, a set of cell walls were first allowed to bind $Ca^{2+}$ for 24 hrs and then incubated in the medium containing strontium for 6 hrs. The calcium displaced/strontium bound to cell walls was estimated by AAS.

***2.12 Sample analysis***

Metal content in all the samples were analyzed by atomic absorption spectroscopy. Filtered fungal mats dried and weighed prior to analysis. The dry mycelial mats or isolated cell wall preparations after processing were subjected to wet acid digestion by dissolving them in 10 ml of concentrated nitric acid with 3-4 drops of perchloric acid on a hot plate in fume hood. On complete dissolution and drying of the sample, a mixture of HCl and $HNO_3$ (2+2 ml) was added and heated until dryness. Lastly, 1 ml of HCl was added and again heated until dryness. At this condition all the organic matters will get degraded as $CO_2$ and $H_2O$ and get evaporated. Only the inorganic matter will remain in the flask and by adding 5 ml of double deionized water, the metal ions trapped in water sample were estimated by AAS. All glass ware used in these experiments was rinsed with a solution of 25% ACS grade nitric acid to remove any metal contaminants. Statistical analysis was performed on data using the Microsoft Excel 2007 software package (Microsoft Inc., Redmond, WA). The experiments where the radio active calcium was used was normalized to get ppm from the respective dpm to obtain required concentration.

**Reproducibility**

All experiments unless indicated were repeated thrice in duplicates with $^{45}Ca^{2+}$ (specific activity =157mCi/g) with half life of 163 days measeured radio activity in a scitillation counter (Schimadzu) and average values ± S.D. are shown.

## 3. RESULTS AND DISCUSSION

The calcium ions are required for the operation of a number of physiological processes such as bone formation, blood coagulation, signal transduction and muscle contraction. The radionuclide's of calcium that have been widely used with considerable advantage as tracks in the study of calcium and bone metabolism are the beta emitting $Ca^{45}$ and the gama-emitting $Ca^{47}$. Though stable isotopes of calcium are available, the more specialized equipment required for their analysis. Hence, researchers have moved to the use of strontium as a substituent for calcium.[28] In the preliminary studies where we performed few experiments to check the effect of salinity on growth of *N.crassa*[29], it was observed that the fungus was able to grow well in medium containing strontium in place of calcium. This result prone us to focus more on uptake and transportation studies in comparision to calcium in *N.crassa*.

The $Sr^{2+}$ and $Ca^{2+}$ metals are the members of alkaline earth series (group IIB of periodic table), have many properties in common, both having a valence of 2+, similar ionic radii and the ability to form complexes and chelates of various solubilities and binding strengths. In most biological systems, preference in general is given to $Ca^{2+}$ over $Sr^{2+}$.[30] The marine organism, *Acantharia*, constructs its internal skeleton with strontium sulfate. The usefulness of strontium as a potential therapeutic agent for osteoporosis has experimental support. The upswing is in interest in strontium as a useful analogue of calcium made researchers to develop concepts to formalize the relationships between $Ca^{2+}$ and $Sr^{2+}$. Numerous studies have appeared very recently in which $Sr^{2+}$ has been used as an analogue of calcium of which a few are referenced here; High correlations (r=5; 0.9) between the absorbability of calcium and strontium was reported by patients who are idiopathic hypercalciuric stone formers and in patients with osteoporosis etc.,[21, 30]

The present study demonstrate the mechanisms involved in strontium uptake process in the model filamentous fungus *Neurospora crassa*. The better binding and uptake of strontium over calcium depends on the biosorptive capacity and also an inherent physiological response to external conditions. There are several calcium binding proteins reported in *N.crassa* and other fungi and these proteins are activated by calcium and have specific binding sites for calcium.[31]

***3.1 Strontium uptake kinetics in Neurospora crassa***

In the preliminary studies where we performed few experiments to check the effect of salinity on growth of *N.crassa*[28], it was observed that the fungus was able to grow well in medium containing strontium in place of calcium. This result prone us to focus more on uptake and transportation studies in comparision to calcium in *N.crassa*.

In the first experiment, $Sr^{2+}$/$Ca^{2+}$ removal by *N.crassa* was studied over a range of concentrations of $Sr^{2+}$/$Ca^{2+}$ (0 to 100 M) for 24 h. The data obtained indicates that $Sr^{2+}$ removal by *N.crassa* reached 100% at low concentration (0.1 mM) and it was between 70-85% from 0.5 mM to 10 mM. Even at higher concentration (50 and 100 mM) more than 60% removal was observed. Where as in case of calcium there is about 90% of removal at low concentrations (0.1 and 0.5 mM) which gradually decreased to 30% at 100 mM. This result clearly indicates that the fungus is able to distinguish between the essential and non essential metal ions, that is why the calcium removal gradually decreased with increase in concentration.

In the next experiment, the time dependent $Sr^{2+}/Ca^{2+}$ removal was studied at two different $Sr^{2+}/Ca^{2+}$ concentrations. When the 3 day old mycelial mats of *N.crassa* were incubated in medium containing 0.1mM and 10 mM $Sr^{2+}/Ca^{2+}$ metal ions. At different time intervals aliquots were removed and metal content was estimated by AAS. The data obtained indicates that removal of $Sr^{2+}$ reached equilibrium by 2h at both the concentrations. Where as in case of calcium, the metal removal was gradually increased to 90% at 0.1 mM and at 10 mM, the calcium removal was only 40%. However, at both the concentrations, equilibrium was not reached.

### *3.2 $IC_{50}$ determination for $Sr^{2+}$ and $Ca^{2+}$*

As the organism is able to remove strontium very well compared to calcium, we aimed to determine the $IC_{50}$ (50% inhibitory concentration) of $Sr^{2+}$ and $Ca^{2+}$ in presence and absence of each other. The results indicate that $IC_{50}$ for strontium in presence and absence of calcium was 10mM and 12.5 mM respectively. For calcium, the $IC_{50}$ concentration in the presence and absence of $Sr^{2+}$ was 3.2mM and 5 mM in the absence of $Sr^{2+}$ ( figures 1a and 1b). In both the cases the $IC_{50}$ concentration was less when both the metals were incorporated in the medium. This effect may be due to the combined effect of both the metals on growth of the fungus.

### *3.3 $Sr^{2+}/Ca^{2+}$ accumulation by N.crassa*

In order to check (1) whether the growth inhibition by $Sr^{2+}/Ca^{2+}$ is due to physical adsorption or an absorption by the fungus, *N.crassa* was grown in medium containing $Sr^{2+}/Ca^{2+}$ as in the previous experiments and the mycelia were subjected to wet-acid digestion analysis for determination of metal accumulated. (2) Further to determine the competitive ability of the metals ($Sr^{2+}$ and $Ca^{2+}$), we have grown the fungus in the medium containing $IC_{50}$ concentration of $Sr^{2+}$ (i.e., 10 mM) and increasing concentrations of calcium (0-50 mM) for 72 h. After the incubation, the mycelial dry weights were recorded and then the $Sr^{2+}/Ca^{2+}$ accumulated was determined by AAS after acid digestion. (3) To determine the growth and transport of the fungus in the medium containing equal moles of $Sr^{2+}$ and $Ca^{2+}$. For his the fungus was grown in medium containing equal concentrations of both $Sr^{2+}$ and $Ca^{2+}$ (0-50 mM). After the incubation time the metal analysis of mycelial mats was performed.

In the first instance, it was observed that there is maximum accumulation of $Sr^{2+}$ at low levels of $Ca^{2+}$ (upto 1 mM), there after a slight decrease in $Sr^{2+}$ accumulation was observed. And a proportional increase in $Ca^{2+}$ accumulation was observed with increase in $Ca^{2+}$ concentration. However the growth of the fungus was found optimum between the 1 mM to 7.5 mM of $Ca^{2+}$ (figure 2).

In second set, the results were very surprising and informative that as the fungus requires very low levels of free $Ca^{2+}$, there was an equal and uniform accumulation over a wide range of concentrations used. Whereas $Sr^{2+}$ accumulation started only after 0.5 mM and there was a drastic increase up to 10 mM and then equilibrium was observed. The reason could be the ability of the fungus to recognize between essential and non essential metal ions and hence calcium accumulated more than strontium at lower concentrations. When the $Sr^{2+}$ was added in high amounts in the medium, and calcium requirement was satisfied, $Sr^{2+}$ started accumulating in high quantities (figure 3).

In the third case, where the fungus was allowed to grow in medium with equal concentrations of both $Ca^{2+}$ and $Sr^{2+}$ (0-50 mM), the fungal growth was less at lower concentrations and it increased steadily up to 5 mM and a sudden drop was observed after 5 mM. The $Ca^{2+}$ and $Sr^{2+}$ accumulation also increased gradually with increase in concentration (figure 4). However because of very high levels of metals than the optimum concentration, the fungus suffered from growth inhibition. In the case of $Sr^{2+}$ uptake, only very few data exist and it is assumed that it occurs via the same pathways that account for $Ca^{2+}$. In the present work, calcium displacement by strontium with live and alkali-killed biomass of *N.crassa* is supported by the earlier reports.[7] Displacement of $Ca^{2+}$ by $Sr^{2+}$ and vice versa suggested that $Sr^{2+}$ was involved in both ionic and covalent bonding with cell wall proteins of *N.crassa*. This observation is contrast to a report on fresh water alga, where the $Sr^{2+}$ intake or binding was purely an ion-exchange process.[32] There is a stoichiometric exchange of $Sr^{2+}$ for $Ca^{2+}$ in *N.crassa* clearly suggests that $Sr^{2+}$ was bound to the sites that are previously occupied by the $Ca^{2+}$ ions. The greater binding of strontium described in the study is because of its larger ionic radius of $Sr^{2+}$ hence it is more polarized than $Ca^{2+}$. This could be the reason for preferential binding of strontium to live mycelia and/or to the isolated cell wall preparations of *N.crassa* though it is a non-essential element with no proven biological activity.

### *3.4 Mechanism of $Sr^{2+}$ accumulation by N.crassa*

Next it is our interest to check whether the accumulation of $Sr^{2+}$ by the fungus is active or passive. When alkali killed mycelial mats (3d) of *N.crassa* were incubated in the medium containing $Sr^{2+}$ (0, 1, 10, 50 and 100 mM) for 24 h there is about 50% reduction in $Sr^{2+}$ accumulation by alkali killed biomass of *N.crassa* and 40% reduction in mycelia treated with azide. This result clearly demonstrates that the $Sr^{2+}$ accumulation by *N.crassa* is an active energy dependent process (figure 5).

### *3.5 Distribution of $Sr^{2+}$ onto cell wall and intracellular fractions of N.crassa*

Since $Sr^{2+}$ uptake is metabolically active process, the distribution of $Sr^{2+}$ on cell wall and in tracellular regions were estimated. The data presented in the table show that there ~52% of total accumulated $Sr^{2+}$ was located on cell wall and the remaining was distributed in the

intracellular compartments *viz*, vacuoles (25%), mitochondria (3%), nuclei (5%) and cytosol 19%) indicatin that cell wall could be the major site of $Sr^{2+}$ accumulation. (Table 1).

### *3.6 Effect of inhibitors on $Sr^{2+}$ transport*

Next the inhibitors of calcium transport channels and proteins were used to study the transport of $Sr^{2+}$. The results of Table 2 show near complete inhibition of strontium transport by 100 µM concentration of calmodulin pathway inhibitors, calmidazolium (CaMZ) and trifluoperazine (TFP) by 96% and 83% respectively. Since vacuoles are the major storage site for calcium in *N. crassa* and the transport into vacuoles is dependent on IP3. A specific inhibitor of *N. crassa* V-type *ATPase*, concanamycin A inhibited more than 47% at 0.5 µM. Neomycin and Wortmanin, inhibitors of IP3 pathway inhibited $Sr^{2+}$ transspport by 94% and 99% respectively (Table-2).

### *3.7 Strontium transport in mutant strains of N. crassa*

In further experiments a mutant of *N. crassa* defective in vacuolar membrane *H+-ATPase* (*vma-5*) and calcineurin mutant (*Cnb- 1*) were used to study strontium transport. The data obtained (Table 3) clearly indicate that strontium accumulation inhibition percentages in these mutants were 17% and 65% in vma-5 and Cnb-1 respectively.

### *3.8 $Sr^{2+}$/$Ca^{2+}$ binding by N.crassa cell walls*

Similar experiments with isolated cell wall preparations of *N.crassa* resulted in the similar kind of observations. However, it was highly surprising to see that the cell walls were able to remove/bound similar quantities of both the metals $Sr^{2+}$ and $Ca^{2+}$ and the binding equilibrium reached a plateau by 1h (Data not shown).

Finally we were interested to study the ability of cell walls to displace $Ca^{2+}$ with $Sr^{2+}$ or vice-versa. When isolated cell walls (1g) were incubated in $Ca^{2+}$ containing medium for 24 h, washed and then incubated in $Sr^{2+}$ containing medium (0-50 mM) for 6h or vice-versa. The results presented in figure 6 indicate that both the metals have a strong ability to displace the other. However, this ability is little higher to strontium than calcium. This displacement could be due to the fact that both the metals possess similar atomic and chemical properties.

Further studies on isolation and characterization of calcium binding proteins from cell walls of *N.crassa* and the determination of $Ca^{2+}$/$Sr^{2+}$ binding sites with binding energies using in vitro and bioinformatics approach will provide clear picture about the strontium binding to the cell walls in significant quantities. Our present study for the first time high lights the active uptake and transport mechanisms of strontium in the filamentous fungus *Neurospora crasa*.

## 5. Figure Legends

**Figure 1:** Conidiospores of *N.crassa* were inoculated into 10 ml basal medium containing metal ion and incubated at 28 ± $1^0$ C for 3 d. The mycelia were harvested and washed thrice with glass-distilled water, pressed free of excess moisture with filter paper and dried over night in hot-air oven at 60-$80^0$ C. Growth was quantified by measuring dry weight of the mycelia. $I_{50}$ (50% growth inhibitory concentration) value for metal ion was derived from graphical plots of growth verses metal ion concentration.

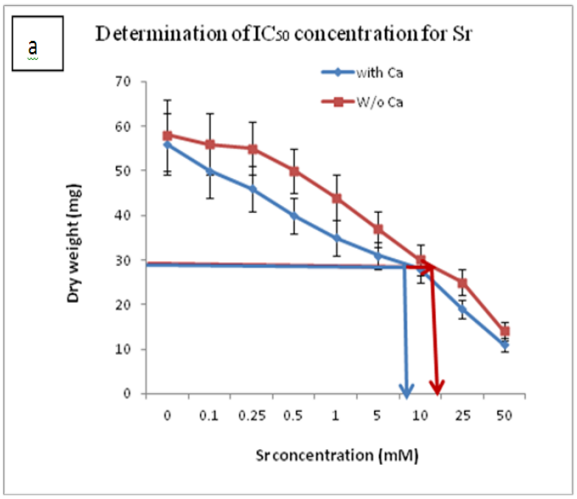
**Figure 2:** Conidiospores of *N.crassa* were inoculated into 10 ml basal medium containing strontium at IC-50 concentration (10 mM) and with varied calcium concentrations (0-50 mM) and incubated at 28 ± $1^0$ C for 3 d. The mycelia were harvested, washed thrice with glass-distilled water, dried over night in hot-air oven at 60-$80^0$ C. Growth was quantitated by measuring dry weight of the mycelia. Metal accumlated was estimated by AAS after subjecting the mycelia to wet acid digestion.

**Figure 3:** Conidiospores of *N.crassa* were inoculated into 10 ml basal medium containing calcium at IC-50 concentration (5 mM) and with varied strontium concentrations (0-50 mM) and incubated at 28 ± $1^0$ C for 3 d. The mycelia were harvested, washed thrice with glass-distilled water, dried over night in hot-air oven at 60-$80^0$ C. Growth was quantitated by measuring dry weight of the mycelia. Metal accumlated was estimated by AAS after subjecting the mycelia to wet acid digestion.

**Figure 4:** Conidiospores of *N.crassa* were inoculated into 10 ml basal medium containing varied concentrations (0-50 mM) of strontium & calcium and incubated at 28 ± $1^0$ C for 3 d. The mycelia were harvested, washed thrice with glass-distilled water, dried over night in hot-air oven at 60-$80^0$ C. Growth was quantitated by measuring dry weight of the mycelia. Metal accumlated was estimated by AAS after subjecting the mycelia to wet acid digestion.

**Figure 5:** Pre-grown mycelia mats of *N.crassa* were treated with 10 mM sodium azide for 1 hr or alkali killed biomass (mycelia boiled in 5% KOH for 5 min) of *N.crassa* (biosorbent) were incubated in medium with different concentrations of $Sr^{2+}$ solutions for 24 hrs and the metal taken up were determined after acid digestion process by AAS.

**Figure 6:** Isolated cell wall preparations of *N.crassa* (1g) were incubated in calcium containing medium for 24 hrs and then suspended in strontium containg medium for 6 hrs and vice versa. The calcium displaced and strontium bound ot strontium bound and calcium displaced by cell walls was estimated by AAS.



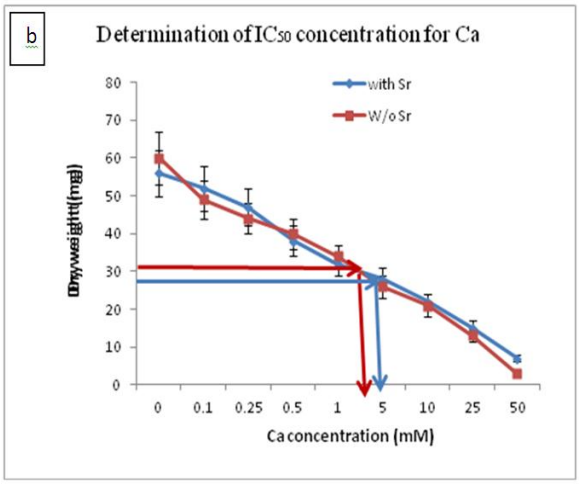




**Figure 1: Determination of IC-50 concentration for (a) $Sr^{2+}$ and (b) $Ca^{2+}$**

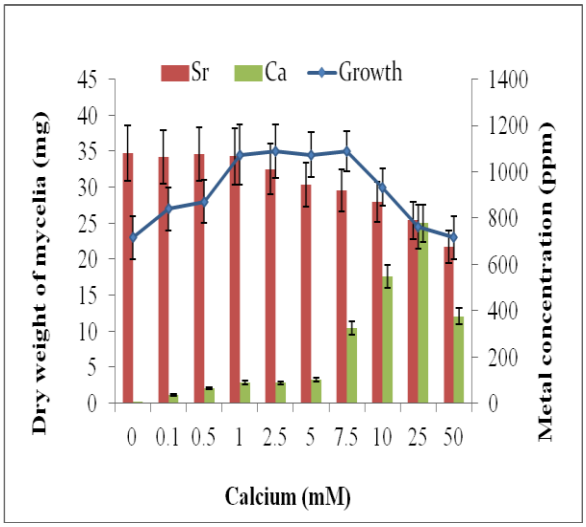



**Figure 2: Growth and metal uptake by *N.crassa* with constant $Sr^{2+}$ concentration (10 mM= $IC_{50}$) and varied $Ca^{2+}$ concentrations**

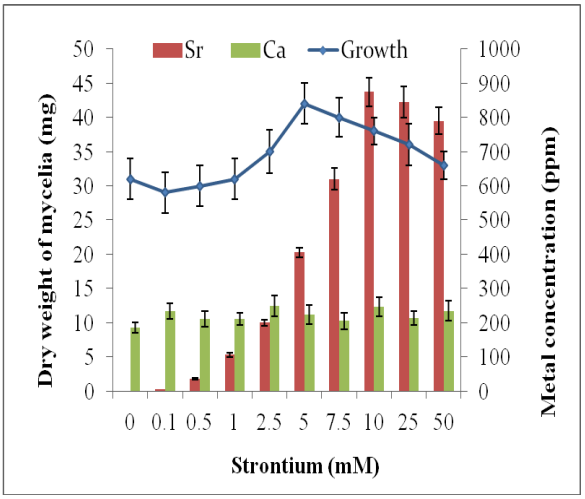



**Figure 3: Growth and metal uptake by *N.crassa* with constant $Ca^{2+}$ concentration (5mM= $IC_{50}$) and varied $Sr^{2+}$ concentrations.**

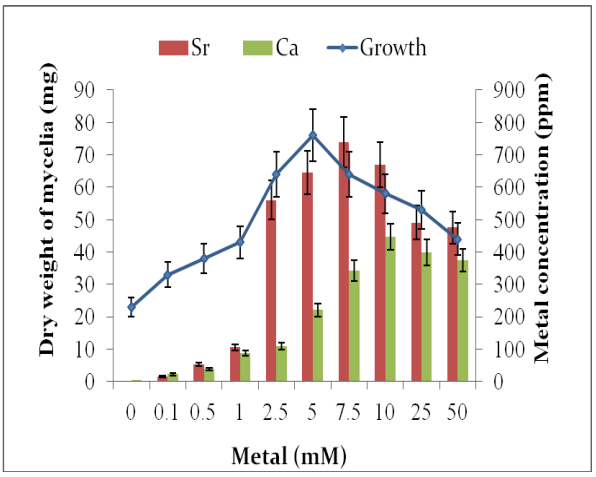



**Figure 4: Growth and Metal uptake by *N.crassa* with equal concentrations of $Sr^{2+}$ and $Ca^{2+}$**

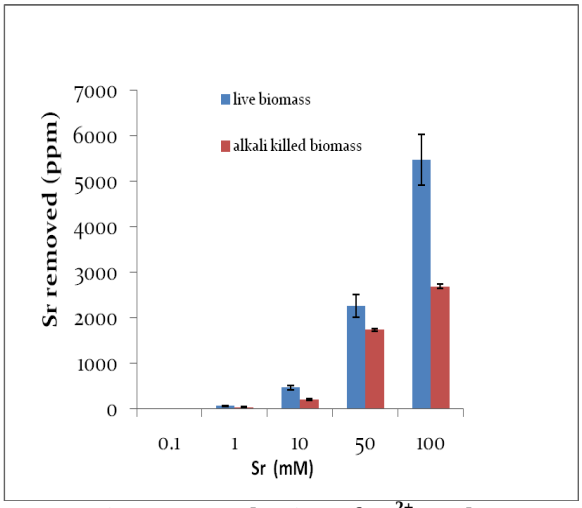



**Figure 5: Mechanism of $Sr^{2+}$ uptake**

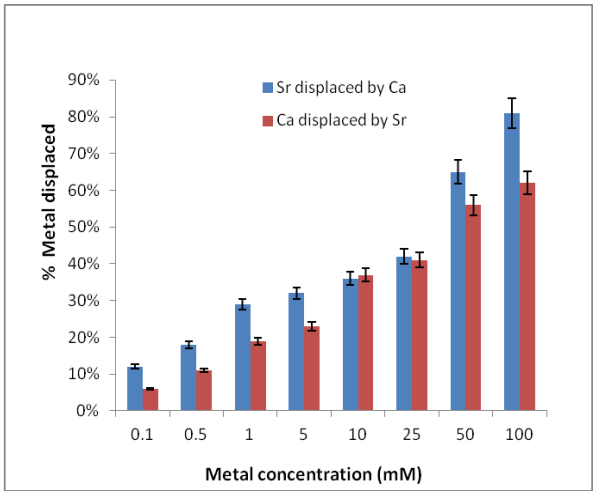



**Figure 6: Metal displacements in *N.Crassa* cell wall preparations**

**Table.1: Sub-cellular compartmentation of $Sr^{2+}$ in *N.crassa.***

| Cell fraction | $Sr^{2+}$ accumulated ppm | % located |
|---|---|---|
| Cell wall | 470 ± 29 | 52 |
| Vacuolar | 227 ± 18 | 25 |
| Nucleus | 46 ± 7 | 5 |
| mitochondria | 28 ± 4 | 3 |
| cytosolic | 170 ± 19 | 19 |

Pre grown mycelia mats of *N.crassa* were incubated in medium containing strontium for 24 h and the mycelia were subjected to subcellular organelle isolation and the strontium in each sample was estimated by AAS.

**Table.2: Determination of $Sr^{2+}$ transport mechanism in *N.crassa* (IP3-inhibitor, CaM-inhibitor)**

| Inhibitor | Concentration (µM) | Sr accumulated (µg/ml) | % inhibition |
|---|---|---|---|
| Control | 0 | 521 ± 48 | 0 |
| Concanamycin A | 0.50 | 274 ± 31 | 47.4 |
| Neomycin | 0.1 | 32 ± 4 | 93.9 |
| Wortmanin | 20 | 7.5 ± 0.9 | 98.6 |
| Calmidazolium | 100 | 20 ± 3 | 96.2 |
| Trifluoperazine | 100 | 84 ± 9 | 83.9 |

Mycelial mats (3 d) were incubated in medium containing 10 mM Strontium with specific for 24 hr and then suspended in 10 ml of EDTA (10 mM) to remove cell wall bound metals and strontium taken up by mycelium (intracellular) was estimated by AAS.

**Table.3: Determination of $Sr^{2+}$ transport in *N.crassa* mutants (*Vma-1 and CNB-1*)**

| Organism | $Sr^{2+}$ bound (µg/ml) | |
|---|---|---|
| | Cell wall | Intracellular |
| *N. crassa (wild)* | 456 ± 41 | 511 ± 43 |
| *vma-5 (vacuolar ATPase mut)* | 487 ± 50 | 17 ± 2 |
| *Cnb-1 (Calcineurin mut)* | 445 ± 40 | 65 ± 8 |

Mycelial mats (3 d) of different strains were suspended in 10 ml of strontium solution (10 mM) for 24 hrs and the Sr taken up (cell wall bound and intracellular) by the mycelia were determined by AAS followed by acid digestion.

**ACKNOWLEDGEMENTS**

The authors (JNLL) would like to thank SERB, NewDelhi (SR. No LS-129/2010) for financial assistance.

**4. REFERENCES**

1. G.M. Gadd. *Microbiology* ., 2010; 156: 609 – 643.
2. M.N. Hughes, R.K. Poole. *J. Gen. Microbiol*.,, 1991; 137: 725-734.
3. J.M.Tobin, D.G. Cooper, R.J. Neufeld. *Appl. Environ. Microbiol*., 1984; 47: 821-824.
4. N.N. Greenwood, A. Earnshaw. Chemistry of the elements 2nd edition. Pergamon Press, Oxford., 1984.
5. J. Naveena Lavanya Latha, K. Rashmi, P. Maruthi Mohan. *Can. J. Microbiol.*, 2005; 51: 1-6.
6. G.W.F.H. Borst-Pauwels. *Biochim. Biophys. Acta*., 1981; 650: 88-127.
7. A.V. Simon, J.M. Tobin. *Appl. Environ. Microbiol.*, 1992; 58(12): 3883–3889.
8. G.M. Gadd. *The growing fungus.* Edited by Gow NAR, Gadd GM. London: Chapman & Hall., 183-210 (1992).
9. D.S. Bush. *Ann. Rev. Plant. Physiol. Plant Mol. Biol.*, 1995; 46: 95–122.
10. A. Trewavas, N. Read, A.K. Campbell, M. Knight. *Biochem. Soc. Trans*., 1996; 24: 971–974.
11. A.A.R. Webb, M. R. McAinsh, J.E. Taylor, A.M. Hetherington. *Adv. Bot. Res.*, 1996; 22: 45–96..
12. O.H. Petersen, C.C.H. Petersen, H. Kasai. *Ann. Rev. Physiol*., 1994; 56: 297–319.
13. M.D. Bootman, M.J. Berridge. *Cell*., 1995; 83: 675–678.
14. R. Dabbagh, H. Ghafourian, A. Baghvand, G.R. Nabi, H. Riahi, et al., *J. Radioanal. Nucl. Chem.*, 2007; 272: 53–59.
15. N. Cohen Shoel, D. Ilzycer, I. Gllath. *Water Air Soil Pollut*., 2002; 195: 195–205.

16. S. Ghorbanzadeh Mashkani, P. Tajer Mohammad Ghazvini. *Bioresour. Technol*., 2009; 100: 1915–1921.
17. N. Ngwenya, E.M.N. Chirwa. *Miner. Eng.,* 2010; 23, 463–470.
18. D. Chakraborty, S. Maji, A. Bandyopadhyay, S. Basu. *Bioresour. Technol*., 2007; 98: 2949-2952.
19. P. Pohl, W. Schimmack. *J. Appl. Phycol.,* 2006; 18: 135-143.
20. G. Vezzoli, I. Baragetti, S. Zerbi, A. Caumo., et al., *Clin. Chem*., 1998; 44: 586-90.
21. R.H. Wasserman. *Clin. Chem*., 1998; 44(3): 437-439.
22. E.J. Bowman, R. Kendle, B.J. Bowman. *J. Biol. Chem*., 2000; 275: 167-76.
23. P. Maruthi Mohan, K.S. Sastry. *Biochem. J*., 1983; 212: 205-11.
24. L.S. Sajani, P. Maruthi Mohan. *Biometals.*, 1998; 10(3): 175-83.
25. K. Rashmi, J. Naveena Lavanya Latha, P. Maruthi Mohan. *J. Coastal Life Med*., 2014; 2(12): 953-961.
26. J.C. Schmit, C.M. Edson, S. Brody. *J. Bacteriol.*, 1975; 122(3): 1062-1070.
27. G.O. Kothe, S.J. Free. *Fungal Genet. Biol.,* 1998; 23: 248-58.
28. J.J.R. Da Silva, R.J.P. Williams. The biological chemistry of the elements. Oxford: Oxford University Press, 1991; 478-81.
29. M. Anupama, T. Vinay Kumar, J. Naveena Lavanya Latha. *Int. J. Biol. Phar. Res*., 2014; 5(1): 22-26.
30. R.H. Wasserman, E.M.Romney, M.W. Skougsted, R. Siever. *Geochemistry and the environment* edited by Cannon HL, Hopps HC. *National Academy of Sciences Press*, 1977; 2: 73-87.
31. T.N. Sowjanya, P. Maruthi Mohan. *J. Basic Microbiol*., 2009; 49: 371-376.
32. C.S. Bauer, C. Plieth, B. Bethmann, O. Popescu. et al., *Plant Physiol.*, 1998; 117: 545-557.