Bioaccumulation of Lanthanum and use of a Model System to Develop a Method for the Biologically Mediated Removal of Lanthanum Nitrate from Aqueous Solution

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Summary: The aim of the present study was to develop a model system for the removal of heavy metals from aqueous solution using sodium phytate as a potential phosphate donor (Lanthanum nitrate was used as test metal). Biofilm of mixture of *Escherichia coli* and *Enterobacter cloacae* was formed on polyutherane foam cubes and packed in a glass column and challenged with lanthanum nitrate (1mM). The metal cation removed is accumulated in situ as insoluble metal phosphate on the surface of *E. coli* and *E. cloacae*. Removal of La^{3+} from aqueous solution by mixture of *E. coli* and *E. cloacae* was dependent on phosphate mediated phosphate release and the residence time in plug flow reactor (PFR) containing polyurethane immobilized cells in a stirred tank reactor (STR) and white precipitate of lanthanum phosphate was accumulated on immobilized cells of *E. coli* and *E. cloacae*. Analysis of accumulated white precipitate by scanning electron microscopy suggested formation of crystals of lanthanum phosphate.

Introduction

Increasing environmental legislation necessitating the treatment of wastewater has created a need for low cost, efficient remediation methods. The biological treatment of industrial effluent containing heavy metals has received attention for its advantages compared to traditional waste water treatment processes [1, 2]. Microorganisms can remove heavy metals from aqueous solution by various mechanisms, which, may or may not be related to the metabolic process of living cells [3].

An Alternative approach has focused on the uptake of metals through biosorption and bioprecipitation mechanisms [3]. Biosorption system fall into two main categories, those using non living biomass are prone to saturation at relatively low levels of metals and consequently require periodic desorption [2-4]. While those relent on live biomass usually function under physiologically permissive conditions and may fail due to metal toxicity.

In contrast bioprecipitation mechanisms utilizing enzymatically mediated metal accumulation process can accumulate metals to high load without saturation and can function in resting cells. The production of a cell bound atypical acid phosphatase enzyme by *Citrobacter. sp* has been documented [5-7] and previous studies have harnessed the release of inorganic phosphate from an inorganic phosphate donor molecule to the stoichiometric precipitation of metal cations as insoluble metal phosphate [6, 7]. One potential phosphate donor phytic acid (Inositol phosphate) a major plant phosphate component up to 80 % of the phosphate content of legumes and cereals has received attention with respect to removal of heavy metals, despite this compound comprising 50 % of the organic phosphate in soil [8] the role of phytic acid as a possible phosphate donor for metal precipitation in soil and subsurface has been postulated as a way of immobilizing toxic metals have suggested that there may be a role for phytic acid added to soils to facilitate in situ metal bioprecipitation [9]. The half life of phytate in synthetic ground water is 100-150 years but microorganisms can accelerate the rate of hydrolysis by several orders of magnitude [10].

Phosphatases are very abundant in nature these enzymes are virtually unable to hydrolyze phophomonoesters in phytic acid [11]. Phytases, a specific class of enzymes hydrolyzing phytic acid have been reported in number of bacteria [12]. It would be hoped that a survey of the possible use of this enzyme in bioremediation would balance the

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reaction rate versus the degree of dephosphorylation obtained. The use of organic phosphates as the phosphate donor for metal precipitation gained importance. Much waste water contains phosphate and harnessing of this phosphate waste to support heavy metal removal could be environmentally friendly solution to two waster problems at the same time. The present study was done to evaluate the role of calcium phytate hydrolysis by mix culture of *E. coli* and *E. cloacae* and the use of librated phosphate as a metal precipitant and lanthanum nitrate was used as a test metal.

Results and Discussion

The biological treatment of industrial effluent containing heavy metals has received increasing attention for its advantages compared to traditional wastewater treatment processes [1-2]. Microorganisms can remove heavy metals from aqueous solutions by various mechanisms, which may or may not be related to the metabolic process of living cells [4, 5]. Practical method of controlling environmental pollution relies on the use of settling tanks, mechanical separators, chemical separators, landfills and more recently bioreactors.

The object of the present study was mainly based onto the development of indigenous technology for the removal of lanthanum nitrate from aqueous solution by evaluating the rate of calcium phytate hydrolysis and use of liberated phosphate as a metal precipitant. Lanthanum nitrate was used as test metal.

A total of 25 marine bacterial strains were analyzed for their ability to degrade calcium phytate and release free inorganic phosphate in the medium. Screening was done on both solid and liquid *tris* glucose minimal media. The bacterial strains showed hydrolysis of calcium phytate on solid medium formed clear zones of haloes around bacterial colonies (Fig. 1). CMG1001 and CMG1083 showed release of free phosphate as a result of calcium phytate hydrolysis. The partial sequences of CMG1055 showed highest similarity 99 % to *E. coli* (Genbank Acession DQ349232) and the partial sequence for CMG1065 showed 98 % similarity to *E. cloacae* Genbank Acession number (DQ349233). These strains were found to degrade phytic acid and release free inorganic phosphate. Amount of phosphate released was determined by high perfor-

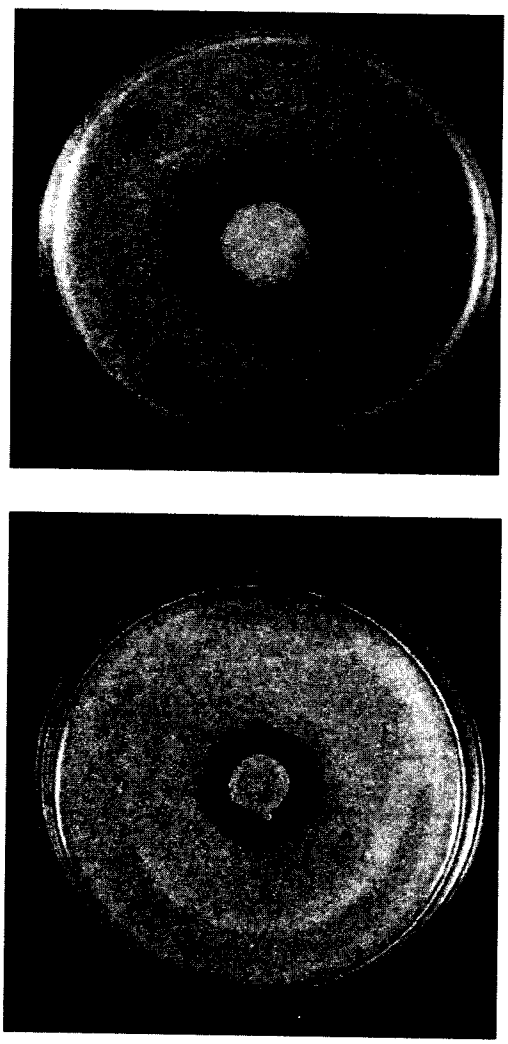

Fig. 1: Hydrolysis of Calcium Phytate
Key: (A) b y CMG1001 (B) by CMG1083 on *tris* glucose medium containing 10mM calcium phytate. The clear haloes, around the bacterial colonies, indicated the area where calcium phytate has been hydrolyzed.

mance liquid chromatography and spectrophotometer method (Fig. 2). *E. coli* released 3.5 mM phosphate where as *E. cloacae* released 2.5 mM phosphate (Fig. 2). Bioreactors having immobilized biomass have been successfully and therefore widely used in much bioremediation application. Bioremediation biomass has been immobilized in various materials such as alginate [12], beads [13] and sponges [14]. A number of different bioreactors design schemes evaluated

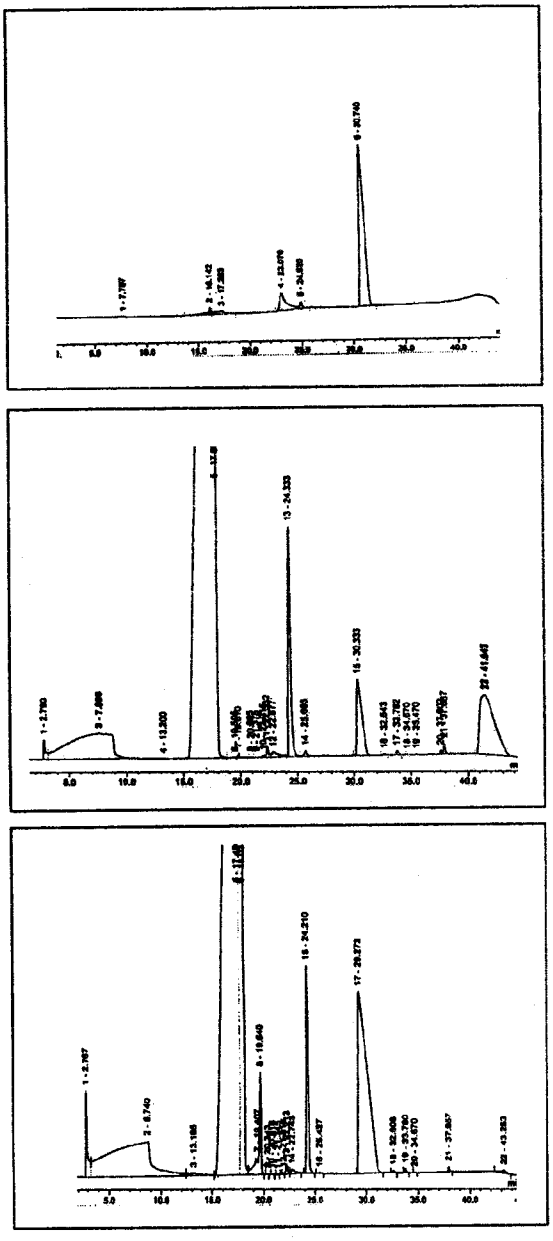

Fig. 2: Identification of free phosphate released as a result of hydrolysis of calcium phytate by CMG1001 and CMG1083 using HPLC.

Key; The peak that elute at 30 min represent phosphate A; standard of phosphate B; phosphate release by CMG1001 C; phospohate release by CMG1083.

experimentally for the purpose of designing a pragmatic solution for bioremediation. It was proposed to immobilize bacterial biomass on a support material and for this purpose an economical support material was proposed to be used.

To achieve the primary objective of this study a bioreactor (Fig. 3) was constructed containing the biomass of mix culture of *E. coli* and *E. cloacae*. The strains which showed release of highest amount of free phosphate from phytic acid for the bioremediation of lanthanum nitrate were selected for biofilm production on polyutherane foam cubes.

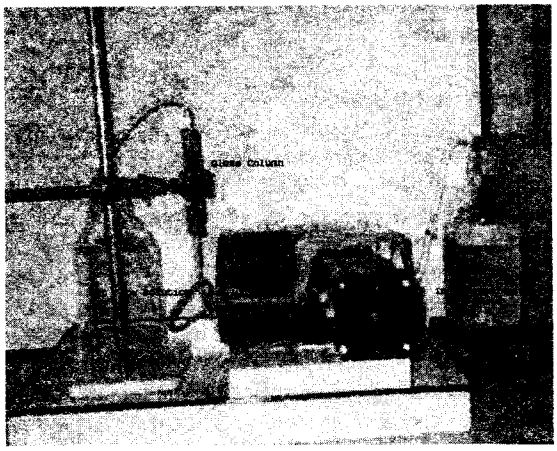

Fig. 3: Labscale bioreactor for the removal of lanthanum nitrate. Biofilm coated polyutherane cubes packed in a glass column; lanthanum nitrate solution passed through bottom of the glass column and discharged clean effluent from the other end.

Biofilm of *E. coli* and *E. cloacae* was formed on polyutherane foam cubes. *E. coli* did not produce good biofilm on polyutherane foam cubes whereas *E. cloacae* formed good biofilm so it was decided to form biofilm by mixing both *E. coli* and *E. cloacae*. The immobilized cells of *E. coli and E. cloacae* were packed in a glass column and challenged with a inflow solution contains 1 mM $LaNo^3$ and 2 mM citrate buffer and 5 mM calcium phytate. The biofilm immobilized on polyutherane foam cubes was packed in a bioreactor (Fig. 3) having glass column and challenged with a solution of lanthanum nitrate (1mM) after 24 hours of contact, white precipitate of lanthanum phosphate was observed on polyutherane foams. It was found that efficiency of lanthanum nitrate removal was 70 % in contrast with control column containing polyutherane foam cubes devoid of biofilm, whereas after

saturation it showed less removal (Fig. 4). It has been reported by Nott [15] that the reaction rate in packed bed bioreactor where fluid heterogeneous reaction occur is based on the mass of solid catalyst and polyutherane foam, which contains the highest amount of immobilized biomass. Macaskie *et al.,* [16] reported that metal biocrystallization process depends on various factors e.g. the phosphatase activity, the solubility product of the metal phosphate the nature and concentration of the complexing legends present, and the pH of the solution. In this study to suppress metal cation hydrolysis the pH of the challenge solution was kept 6 in citrate buffer. Lanthanum content of out flow solution was measured after different time intervals. Maximum removal of lanthanum from challenging solution was observed after 24 hrs (Fig. 4). Scanning electron microscopy of white precipitate formed in the column of bioreactor showed presence of crystals of lanthanum phosphate (Fig. 5). In conclusion bioreactor constructed using immobilized mix culture of phytate hydrolysing strains can remove 70 % lanthanum nitrate from aqueous solution by forming precipitates of lanthanum phosphate.

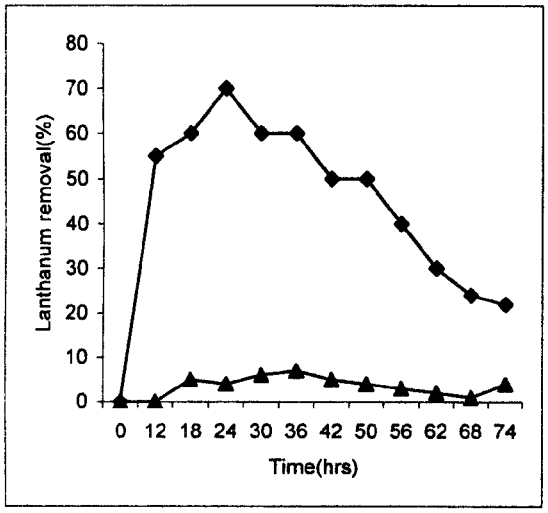

Fig. 4: Removal of Lanthanum nitrate through lab scale bioreactor.

Key; ▲ Polyutherane foam cubes without biofilm, ■ Polyutherane foam cubes with biofilm.

Experimental

All the reagents were of analytical grade. All glassware was immersed overnight in 2 % decon 90 (Decon Laboratories Ltd) and washed with distilled water 3 times and dried. All the experiments were done using at least two independent batches and all the results were reproducible. The reagents used were stannous chloride, sodium molybdate, lanthanum nitrate, arsenazo111, calium phytate

Organisms and Culture Conditions

Marine bacteria were isolated from different kinds of fish, crustaceans, sponges, seaweeds, shells, bivalve and water samples of Sindh and Baluchistan coast of Pakistan using marine agar 2216. Morphologically distinct colonies were preserved in marine broth 2216 containing 20 % glycerol at -70 °C. The bacteria were given CMG code and slanted on the respective media before using in the experiment. Selected strains were identified by 16S rRNA gene sequence analysis.

Screening of Phytate Hydrolyzing Strains

Twenty five bacterial strains isolated from marine samples were screened for their phytate hydrolyzing ability. These strains were grown onto phytase screening plates containing 1.5 % d-glucose, 0.5 % calcium phytate, 0.5 % NH_4Cl, 0.055 % $MgSO_4$, 0.05 % KCl and 1.5 % agar adjusted to pH 7.0 and incubated at 37 ° C for 24 h. The colonies with clear zones around were selected.

Phytate Hydrolysis in Liquid Culture

Bacterial strains, which exhibited the largest zones of clearing on the above agar surfaces, were selected for studies on P-release in liquid culture as a result of hydrolysis of calcium phytate. Bacterial strains were grown in 125 mL liquid tris glucose minimal medium supplemented with 10 mM calcium phytate in 250-ml Erlenmeyer flasks on a shaking incubator at (125 rpm) at 30 °C for 20 days.

Estimation of Inorganic Phosphate by Spectrophotometer Method

A 10 mL aliquot was aseptically removed from above flasks at different intervals of time (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days) and centrifuged at 5000 g for 15 min. The supernatant was filtered through a sterilized 0.45 μM millipore filter and assessed for free phosphate content in the filtrate using spectrophotometeric method of Murphy and Riley [17]. Distilled water (0.95mL) and 0.6mL

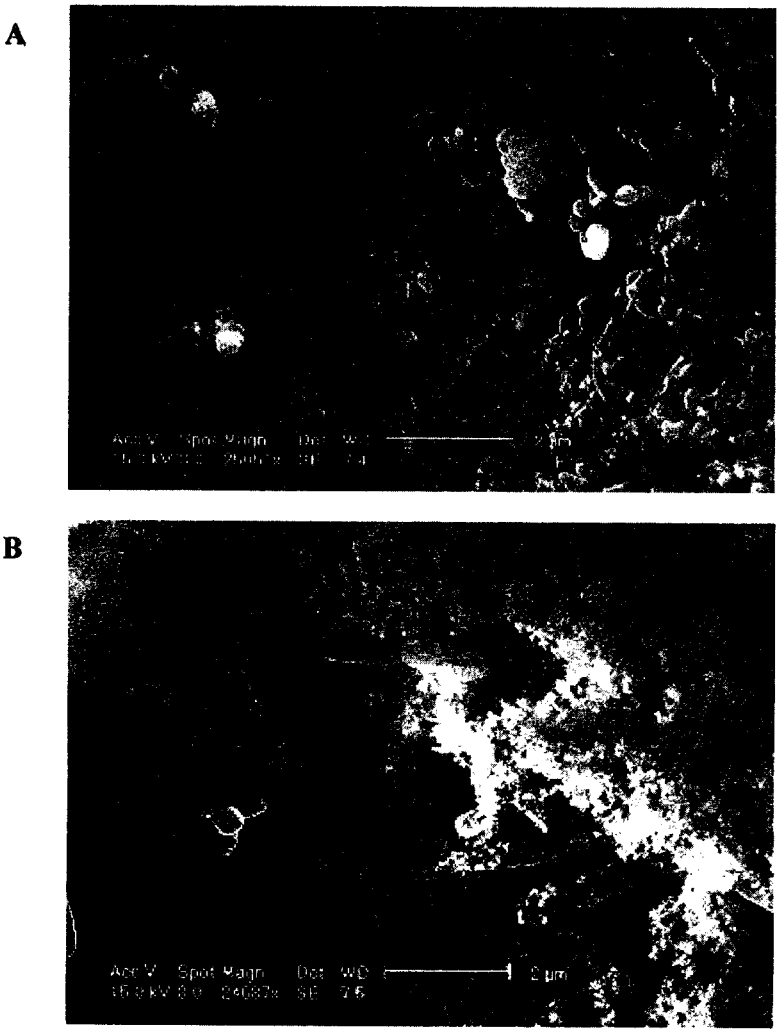

Fig. 5: Scanning electron microscope of precipitate formed in a glass column of bioreactor showing crystals of lanthanum phosphate. A; control column precipitates not formed B; precipiatate formed in glass column.

of a solution of (2.5 % w/v) sodium molybdate was added into test tube containing 0.05mL of sample (up to 2mM PO_4^{3-}). The phosphate in the solution was visualized by adding 4ml $SnCl_2$ (fresh solution 0.25mL stock solution was made monthly by dissolving 15 g SnCl2 in 2.5mL concentrated HCl with the estimation of blue complex at 720nm (Phamacia LKB Novaspec. 11spectrophotometer), with reference to a standard curve, similarly prepared.

Estimation of Inorganic Phosphate by HPLC

Presence of free inorganic phosphate released as result of hydrolysis of calcium phytate at different intervals of time was confirmed by HPLC using (AS11-HC/AG11-HC) column. An aliquot of 10-ml aliquot was aseptically removed from each flask and centrifuged at 5000 g for 15 min. The supernatant was filtered through a sterilized 0.45 μM millipore filter and assessed for phosphate content in

the filtrate and packed in a column and allowed to run for 45 minutes using organic acids auto sampler PY 4 program. The operating conditions consisted of 0.1 % H_3PO_4 as the mobile phase, a constant flow rate of 0.5mlmin^{-1}, and sample injection of 20 μL. The phosphate content were quantitatively determined by comparing the retention times and peak areas of chromatograms with those of standards.

Selection of Bacterial Strains for Immobilization Experiment

The bacterial strains were screened for hydrolysis of calcium phytate on solid media. The strains, which produced largest zone of halo around bacterial colonies, were selected to study, soluble phosphate release in liquid media. CMG1001 and CMG1083 showed best results and were selected for further studies.

Support Material and Biofilm Production

Polyutherane foam cubes were selected as support material for biofilm production. A mix culture of *E. coli* (CMG1001) and *E. cloacae* (CMG1083) were grown as biofilm on polyutherane foam cubes. The strains were grown in 250 ml liquid *tris* glucose minimal medium supplemented with 10mM calcium phytate in 500-ml Erlenmeyer flasks containing 20 polyutherane foam cubes on a shaking incubator at (125 rpm) at 30 °C for 6 days.

Metal Uptake by Polyutherane Immobilized Cells in a Plug Flow Reactor (Pfr)

Mixture of cells of CMG1001 and CMG1083 were immobilized on polyutherane foam cubes (1 cm^3). These foams were cut in to cubes (0.5 cm^3, 1.0 cm^3) and then washed in isotonic saline solution to remove excess and loosely bound biomass and were packed in a glass column (height 9 cm, width 1.5 cm). The column of polyutherane-immobilized cells was challenged with inflow solution (10 mM phytic acid, 1 mM lanthanum nitrate and 2 mM citrate buffer). This inflow solution was pumped upward *via* an external periplastic pum (Waston-Marlow 505U) at a constant flow rate (7 mL/h) and was allowed to run continuously for 120 h. As precipitation of respective metals could occur at various pH values, the influent pH was kept at neutral value. Metal removal by the immobilized cells was determined by assay of the residual metal in the reactor outflow solution and comparison with the metal content of the inflow solution.

Assay of Heavy Metal

Lanthanum content of inflow and outflow solutions was determined by lanthanum assay using Arsenazo111. Column inflow and outflow solution (0.03mL) were transferred into test tube containing 1.97mL of solution of ammonium acetate. Metal in the solution was visualized by adding 0.1mL of 0.15 % Aresenazo111 (2, 2`-[1,8 dihydroxy-3, 6-disulfo-2, 7-naphthalene-bis (azo) dibenzenearsonic acid, (0.038g was dissolved in 25mL distilled water for 1 h then filtered to remove residual solid) to each tube with estimation of the purple complex at 660nm) (phamacia LKB Novaspec11 spectrophotometer) with reference to a standard curve similarly prepared.

Scanning Electron Microscopy

SEM (Scanning electron microscopy) of biofilm saturated with lanthanum phosphate was done. The precipitate of lanthanum phosphate was taken out from column and were centrifuged at 5000g for 15 min and slides were prepared and examined at a voltage of 15-25 KV using a JEOL JSM-35 SEM

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