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Bioremediation of hexavalent chromium from electroplating effluents by wild and mutant strains of *Bacillus amyloliquifaciens*

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Chromium, a heavy metal, is a major xenobiotic element found in electroplating effluents. It is a recalcitrant of concern as it is highly toxic and carcinogenic. It exists as Cr (VI) which is highly soluble and bioavailable. Various conventional treatment technologies like adsorption, ion exchange, filtration, reverse osmosis and electrodialysis have been used but they generate lot of sludge and residue. Hence, there is a need for an economical, faster and ecofriendly means of remediation for which bioremediation is preferred. In this study we attempted chromium removal by employing improved indigenous isolate *Bacillus amyloliquifaciens* sourced and identified from a previous study from chrome plating effluent collected from Peenya industrial area, Bengaluru, India. Strain improvement was achieved using physical mutagen (UV radiation) and chemical mutagens (ethidium bromide, ethyl methane sulphonate and acrylamide). A comparative study was carried out to screen the chromium remediation potential of wild type and mutants in chromium spiked sample and the electroplating effluent. The mutant generated using acrylamide (150 µg/mL) in comparison with wild type and other mutants was more capable of remediating chromium. At the end of 18 days, from the chrome electroplating effluent containing chromium (490 mg/100mL), the wildtype could remove 74% (362.6 mg/100 mL), UV radiation induced mutant removed 83% (406.7 mg / 100 mL) whereas acrylamide induced mutants removed 96.67% (470.4 mg/100 mL) of chromium. Enzyme assays confirmed involvement of enzymes at sub cellular level in chromium remediation.

Keywords: Chromium, Effluents, Heavy metal, Pollution, Remediation potential

The chromium as a representative heavy metal, possesses variety of industrial applications. It is used as a coating material on base metals in electroplating industry that gives luster, enhances longevity and attributes anticorrosion properties. In leather industries chromium is utilized for tanning leather, in wood preservatives and as an alloying agent in metallurgical industries. It exists in many oxidation states, Cr (VI) and Cr (III) being most stable forms. Cr (VI) is highly soluble, toxic and carcinogenic. Industrial effluents carrying hexavalent chromium, when released into the environment, seep into air, water and soil. Beyond permissible limits (for industrial effluents -2 ppm as per Environmental Protection Agency)[1], chromium is reported to cause various environmental and health hazards[2,3].

There are various physico-chemical treatment techniques like adsorption[4] ion exchange resins[5], electrodialysis[6], electrocoagulation[7], limestone treatment[8] etc. to remediate chromium from environment. But most of these techniques however are not economical and ecofriendly as they introduce secondary contamination. Thus, chromium bioremediation studies have an edge over other physicochemical effluent treatment procedures. Besides, bioremediation processes are both economical and eco-friendly[9,10].

Various microorganisms possess a natural ability to metabolise chromium thereby converting chromium from its toxic hexavalent form to non-toxic trivalent form[11]. Microbes that survive in effluents containing chromium have been detected, isolated and identified. This natural capability of indigenous microbes to resist and reduce chromium in environments laden with chromium is exploited for remediation. In normal and natural conditions, the bacterial strains show limited rate and extent of pollutant metal remediation. Therefore, it becomes essential to resort to strain improvement of the microbes[12,13]. This improvement can be facilitated by inducing mutations by physical as well as chemical means. In chromium remediation, chemical remediation appears to have fared better according to the reports available[14-16]. Random mutagenesis is still the most cost effective and short-term strain improvement procedure that is

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widely opted[17] Various mutagens can be used to induce mutagenesis like physical and chemical mutagens. Ultraviolet light, a form of physical mutagen[18] and chemical mutagens like acrylamide, ethyl methane sulphonate and ethidium bromide[19] were used for strain improvement process. The potential of chromium remediation by wild and mutated strains were validated using enzyme assays and electron microscopy. The hypothesis of the current work was facilitated chromium removal by the mutants than the wild type strains. In this study, we tried to optimize remediation of chromium by wild type and mutants of *Bacillus amyloliquifaciens* in chromium spiked samples under laboratory conditions as well as in electroplating effluents in the natural settings.

Materials and Methods

The bacterial strain was mutated for improvising to facilitate effective remediation. Reduction of chromium by the bacterial cells were validated by enzyme assays and localization of chromium inside the bacterial cells was confirmed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

Revival of culture

Bacillus amyloliquifaciens, an indigenous bacteria isolated from electroplating effluent from Peenya industrial area, Bengaluru, India, (https://goo.gl/maps/mbN9KEQ2wrTqeu2MA), identified by 16 s rRNA sequencing and deposited as PES B-sequence at NCBI GENBANK No-JX112654 in a previous study[20] was used as wild type strain in the present study. The organism was revived on a sterile Luria-Bertini agar plate from the glycerol stocks stored at –80°C. The colonies revived were subjected to strain improvement using physical and chemical mutation techniques.

Strain improvement by physical mutation

UV radiation exerts its mutagenic effect by exciting the DNA molecules in thymine base pairs, inducing formation of pyrimidine dimers, thereby causing the damage[21-23].

UV survival curve

UV survival curve was plotted to understand the fraction of cells ability to absorb a specific dose of UV radiation and retain 50% viability. Overnight suspension culture of *Bacillus amyloliquifaciens* was taken in a sterile Petriplate to expose it to UV radiation. The culture was exposed to UV light such that the distance between the culture and UV light source (λ_{360}, germicidal 20W - Phillips) was 30 cm^3. Every 10 min, 1.0 mL of culture sample was drawn, serially diluted and 0.1 mL of 10^{-7} was plated on a sterile LB agar plates. The process was sequentially repeated at an interval of every 10 min till end of 120 min. Cells that were not exposed to UV radiation served as control. The plates were incubated overnight at 37ºC. By viable count technique, the colonies that appeared on overnight incubation were counted and a graph of time duration of UV exposure versus log CFU/mL was constructed. The time of exposure of colonies having more than 50% survival was noted[16].

Isolation of UV mutants

Log phase culture of *Bacillus amyloliquifaciens* (1.967×10^8 cells) prepared in LB broth was centrifuged at 6000 rpm for 15 min aseptically. The bacterial pellet was suspended in 10 mL of sterile saline water. This bacterial suspension was transferred into sterile Petriplates to expose it to UV radiation. The plate was placed under UV light ($\lambda_{360,}$ germicidal 20W-Phillips) at 30 cm distance. Based on the 50% survival viability check with reference to UV survival curve, the cells exposed to UV radiation for 80min (50%) onwards till 120 min were isolated[9]. From the suspension exposed to UV radiation, 0.1 mL of the culture was drawn and plated on sterile LB agar plates. The plates were incubated in an inverted fashion at 37ºC for 24 h.

Strain improvement by chemical mutation

Chemical mutagens are relatively stronger mutagens than the physical mutagens as they bring about a permanent change in the DNA structure. Acrylamide a form of chemical mutagen alkylates DNA forming adducts thereby bringing about the DNAdamage[16,17,25].

Isolation of chemical mutants

The chemical mutagens used for this study were ethidium bromide, ethyl methane sulphonate and acrylamide. 5 mL each of these mutagens of five different concentrations ranging from 50 to 250 µg/mL (50,100,150, 200 and 250 µg/mL) were pipetted into the sterile centrifuge tubes, containing 5 mL of overnight culture suspension of *Bacillus amyloliquifaciens*. Bacterial suspension not treated with chemical mutagens served as control. The tubes were incubated for 120 min at room temperature. The

tubes were centrifuged at 5000 rpm for 15 min to separate the cells from the chemical mutagen. The supernatant containing chemical mutagens was discarded and the cells were resuspended and given a wash in 10 mL of sterile saline water and recentrifuged. Three times, the tubes were recentrifuged to remove traces of chemical mutagen from the bacterial cells. The cells were finally washed and resuspended in 10 mL of sterile saline water. 0.1 mL of this suspension was drawn, plated on a sterile LB agar plate and incubated at 37ºC for 24 h[16].

One mL of overnight suspension cultures of the wild type, physical mutants and chemical mutants were separately inoculated into media spiked with chromium (100 mg/L). About 1.0 mL of media was drawn, centrifuged and supernatant was estimated for chromium every hour for 10 h by diphenyl carbazide (DPC) assay by using spectrophotometer[27,28]. The optimum chromium removing physical and chemical mutants were selected further to remediate chromium from effluent.

Bioremediation in electroplating effluent

The electroplating effluent from the industry was collected, filtered to remove debris and the amount of chromium present in effluent was analyzed by DPC assay. The wild type, physical and chemical mutants that reduced chromium optimally in spiked media were separately inoculated into 100 mL each of the filtered electroplating effluent sample. About 5% sucrose was provided as a carbon source for survival of microbes in the effluent. About 1.0 mL of the effluent was drawn every 24 h for14 days, centrifuged for 5 min at 3000 rpm at room temperature and supernatant was estimated for chromium by DPC assay[26-28].

Characterization of chromium reducing activity

To evaluate the involvement of enzymes in chromium reduction within the bacterial cells, the bacteria were subjected to resting cell assay, permeabilized cell assay and cell free extract assay. Overnight grown cultures of the wild type and the mutants at an alkaline pH 9 in minimal media were subjected to the enzyme assays. In all the three assays, cells that were heat killed served as control.

Resting cell assay

This assay was performed to screen the secretion of chromium reducing enzymes outside the cells by the resting cells[29]. The cells were centrifuged at 6000 rpm at 4ºC for 15 min. The pellet was rinsed in 10 mM Tris-HCl buffer of pH7 and resuspended in same buffer. To the bacterial suspension, Cr (VI) was added (20 mg/L) and bacterial suspension was incubated at 30ºC for 24 h. The chromium in the media was estimated at the end of 24 h[28].

Permeabilised cell assay

This assay was to confirm involvement of membrane bound enzymes in chromium reduction. Overnight cultures of wild type and mutants of *Bacillus amyloliquifaciens* were prepared in duplicates. The cells were pelleted by centrifugation as in resting cell assay. One set of pellets were suspended in 1% toluene and the other set were suspended in 2% triton-X and vortexed gently to permeabilise cells and Cr (VI) (20 mg/L) was added. Small aliquots of the bacteria-detergent suspension were withdrawn, centrifuged and the supernatant was subjected to DPC assay to estimate chromium during the time of introducing chromium and at the end of 24 h[30].

Cell free extract assay

This assay was to screen chromium reducing enzymes in cytosolic extract[32]. The overnight cultures were centrifuged and washed twice with 20 mL of 10 mM Tris-HCl of pH 7. The suspended cells were placed on an ice bath and subjected to sonication for 10 min (20×30s) (M/s Jhonson plastonic) to disrupt the cells. The homogenate obtained by sonication was spun at 8000 rpm for 30 min at 4ºC to obtain the cytosolic fraction and pellet fraction. The supernatant or the cytosolic fraction was subjected to filter sterilization and the filtrate was subjected to DPC assay to estimate chromium. The chromium was also estimated in the pellet fraction[31].

Scanning electron microscopy

Overnight cultures of wild type and mutants of *Bacillus amyloliquifaciens* grown with and without chromium were individually centrifuged at 6000 rpm for 10 min at 4ºC. The pellets were treated with 0.3% glutaraldehyde in sterile phosphate buffer (pH 6.9). The smear was prepared on the slide with the bacteria-fixative suspension and was air dried. The smear was dehydrated in sequential grades of alcohol (50%,70%,80% and 100%). Since the bacterial surfaces are nonconducting, gold particles were sputtered on it using an Ion sputter (Hitachi ion sputter MC-1000). The coated films were subjected for examination under Scanning Electron Microscope (TESCAN -Vega 3) at a high magnification using an accelerated voltage of 10-15KV[33-35].

Transmission electron microscopy (TEM)

TEM was performed in bacterial cells (both which were grown in presence as well as in absence of chromium) fixed in glutaraldehyde (3%) buffered with sodium cacodylate buffer (pH 7.2) Fixation was done at 18 h at 4 ºC. After several washes the bacterial cells were fixed for 2 h in osmium tetroxide (1%) in same buffer. The specimen was dehydrated in sequential grades of alcohol and embedded in epoxy resin. Ultrathin sections of 10 nm were cut using ultramicrotome (M/S Leica) and sections were stained with uranyl acetate and Reynolds solution (Sodium citrate and lead nitrate). The sections were mounted on TEM (Technai -T12 spirit) and images were photographed[36,37].

Statistical analysis

The experiments were conducted in triplicates. For each experiment, the mean ± standard error was reported. Standard error is the measure of the statistical accuracy of an estimate which is equal to standard deviation derived from the sample. It is a process of estimation of standard deviation of theoretical distribution of a large population of such estimates.

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}}$$

SE $\bar{x}$ = Standard error of mean; s=Standard deviation of mean; and n= Number of observations of the sample.

Results

Revival of culture

Pure culture (Creamy white colonies) of *Bacillus amyloliquifaciens* appeared on the LB agar plate on overnight incubation at 37ºC.

Strain improvement by physical mutation

UV survival curve

Bacillus amyloliquifaciens strain (PES B – NCBI GENBANK number - JX112654) was improved using physical and chemical mutagens separately. Also, the involvement of enzymes in chromium reduction in wild type and mutants were evaluated through assays for which the results are recorded. From the UV survival curve (Fig. 1), the mutants exposed to radiations for 80 min onwards were chosen from the culture plates as they had more than 50 % survival rate**.**

Isolation of UV mutants

The mutants isolated based on viability check by UV survival curve were screened for optimal reduction of chromium. Compared to all the physical mutants, the mutants that were exposed to UV radiations for 80 min removed chromium effectively up to 99% (99 mg) in 10 h from the media spiked with chromium. The isolated UV mutants from chromium removal efficiency of various UV mutants are depicted in (Fig. 2A).

Strain improvement by chemical mutation

Isolation of chemical mutants

The mutants obtained on treatment with various concentrations of the tested mutagens were inoculated separately in LB broth spiked with chromium at a concentration of 100 mg/L at room temperature to screen the chromium remediation potential. Among the different chemical mutants tested, acrylamide demonstrated better degree of chromium removal efficiency. Chromium removal efficiency of the chemical mutants generated using various concentration of acrylamide is depicted in (Fig. 2B) by which it is relevant to note that the chemical mutant generated using acrylamide of concentration 150 µg/mL is more effective in removing 100% chromium in 8 h in spiked sample.

Bioremediation in electroplating effluent

The wild type and the optimally chromium reducing UV mutant (mutant exposed to UV radiation for 80 min) and chemical mutant (mutant exposed to 150 µg/mL of acrylamide) when inoculated in effluent containing chromium at a concentration of 490 mg/100 mL, remediated chromium to different extents. At the end of 19 days, wild type removed 74% (362.6 mg of Cr/100 mL of effluent) the physical mutant remediated chromium to an extent of 83% (406.7 mg of Cr/100 mL of effluent) and chemical mutant removed 96% (470.4 mg of Cr/100 mL of effluent) of chromium indicating that chemical mutant

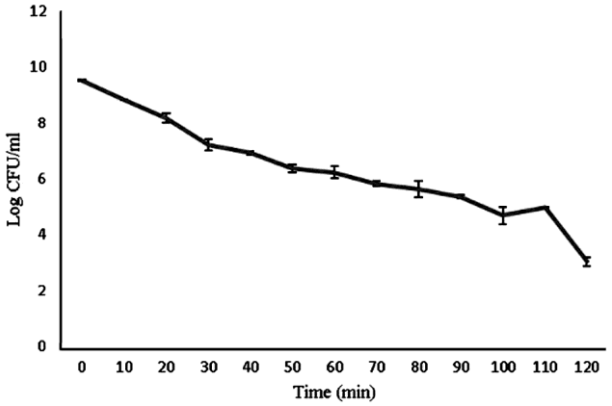

Fig. 1 — UV survival curve of *Bacillus amyloliquifaciens*

is relatively has better chromium removal efficiency than the wild type and the UV mutant (Fig. 2C)

Characterisation of chromium reducing activity

Enzyme assays of wild type and the mutants of *Bacillus amyloliquifaciens* are tabulated. As indicated inTable1, in case of resting cells, chemically mutated cells have removed chromium more efficiently than the physical mutants and the wildtype. In permeablised cell assay, triton-X permeabilised the cells more than toluene and it is evident by the less chromium removal by triton-X permeabilized cells than toluene-permeabilised cells. In cell free extract assay the pellet fraction has shown higher chromium removal efficiency than the cytosolic extract in case of wild type as well as in mutants.

Scanning electron microscopy

The SEM micrograph of *Bacillus amyloliquifaciens* grown in presence of chromium (Fig. 3A) revealed chromium peak in Energy dispersive X-ray analysis (EDAX) indicating the uptake of chromium by the bacteria (Fig. 3B). The microbes grown in absence of chromium (Fig. 4A) failed to display the chromium peak in EDAX (Fig. 4B).

Transmission Electron Microscopy

Bacillus amyloliquifaciens cells grown in absence of chromium displayed a smooth membrane (Fig. 5A) while those cells which were exposed to chromium, exhibited chromium accumulation in cytoplasm. The cell membranes were observed to be irregular (Fig. 5B) The cells exposed to chromium clearly revealed morphological changes in exposed cells and revealed electron dense inclusions within cytoplasm (Fig. 5C).

Statistical analysis

The mean remediation potential of Wild type strain = 73.8% $\pm$0.96 (SD; 0.96, SE; 0.554); Physical mutant = 82.52% $\pm$ 0.45 (SD ;0.45, SE; 0.260); and Chemical mutant = 96.22% $\pm$ 0.38 (SD;0.38, SE; 0.219).

Discussion

The results from the previous study has indicated that the indigenous isolate identified as *Bacillus amyloliquifaciens* has reduced the amount of hexavalent chromium in electroplating effluent. Microbial remediation of chromium has been reported by several authors. According to a recent study, it has been established that bioremediation is a promising and cost-effective alternative compared to that of physical and chemical methods[38,39].

It is also evident from the results that the mutated bacterial strains have remediated chromium more effectively than the wild type strain. Among the wild type and the mutants, the chemical mutant has demonstrated optimal efficiency for the removal of

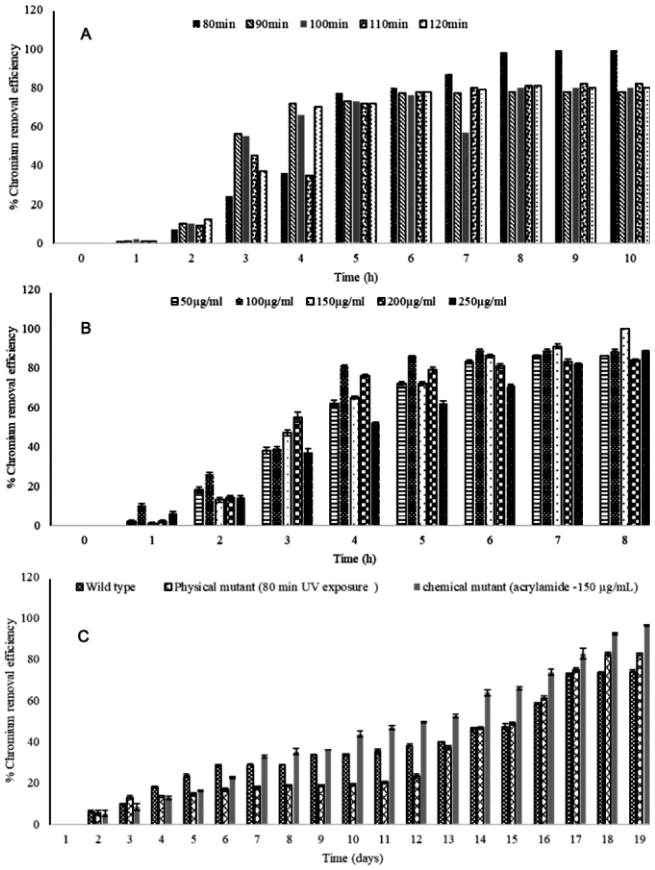

Fig. 2 — Chromium removal efficiency of (A) physical (UV) mutant; (B) chemical (acrylamide) mutants; and (C) *Bacillus amyloliquifaciens* in electroplating effluent.

Table 1 — Enzyme assay of wild type and mutants of *Bacillus amyloliquifaciens*

Enzyme assay	Time (h)	Centrifugation /chemical treatment	Cr removed (mg/mL of broth) wild type		Physical mutant		Chemical mutant	
			Ctrl	Exp	Ctrl	Exp	Ctrl	Exp
Resting cell assay	0	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	24	Nil	Nil	3.5	Nil	Nil	Nil	9.6
Permeabilised assay	0	Triton X (2%)	Nil	Nil	Nil	Nil	Nil	Nil
	24		2.2	5.0	10	4.7	Nil	5.2
	0	Toluene (1%)	Nil	Nil	Nil	Nil	Nil	Nil
	24			10.0		6.02		1.94
Cell free extract assay	0	Supernatant	Nil	Nil	Nil	Nil	Nil	Nil
	24		Nil	0.9	Nil	Nil	Nil	Nil
	0	Pellet	Nil	Nil	Nil	Nil	Nil	Nil
	24		Nil	2.3	Nil	1.7	Nil	3.6

[Cr, Chromium; Ctrl, Control; and Exp, Experimental]

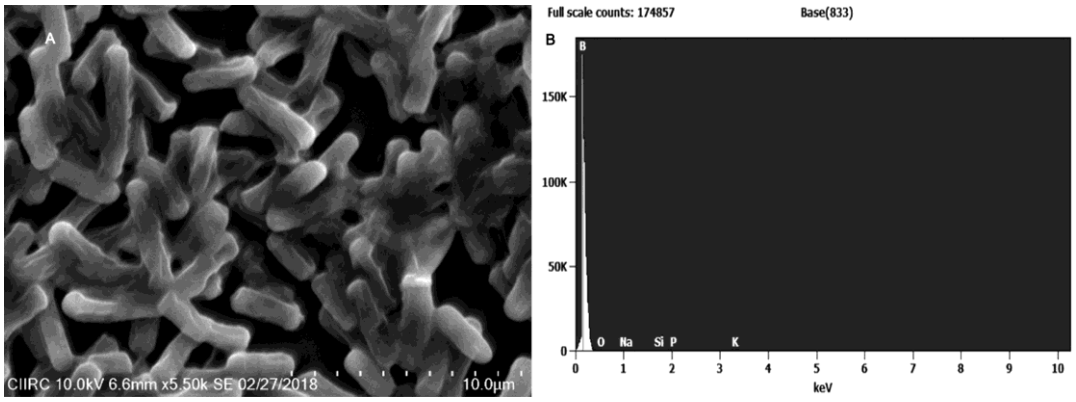

Fig. 3 — (A) SEM micrograph; (B) EDAX of *Bacillus amyloliquifaciens* cultured in absence of chromium

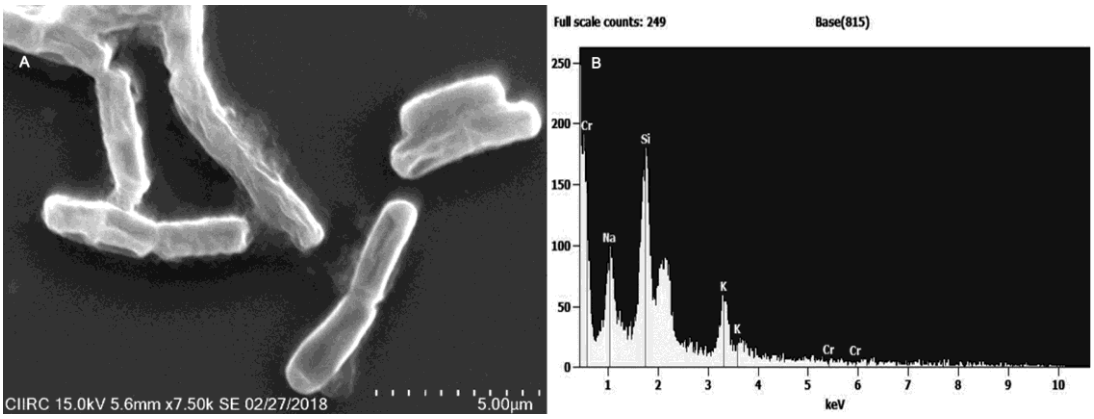

Fig. 4 — (A) SEM micrographs; (B) EDAX of *Bacillus amyloliquifaciens* cultured in presence of chromium

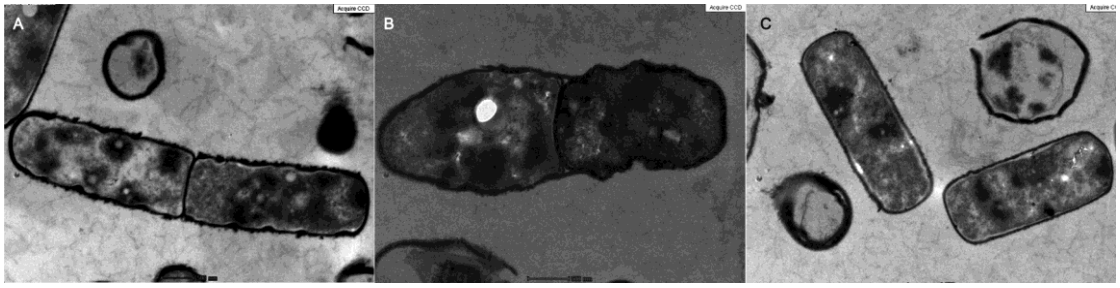

Fig. 5 — TEM micrograph of *Bacillus amyloliquifaciens* (A) grown in the absence of chromium; (B) with irregular membrane grown in presence of chromium; and (C) showing dense cytoplasmic granular inclusion of chromium

chromium from the spiked as well as effluent chromium laden sample. This is well supported by another study conducted, which confirms that improvised strains are metabolically robust and efficient with respect to bioremediation[10,39]. According to a published report, random mutagenesis is still the most cost-effective way to generate mutants[15,32].

A study has reported that UV induced mutated *Bacilli* was successful in removal of chromium from polluted water facilitated[19]. Further, usage of mutagen acrylamide at a concentration of 150 µg/L, exhibited better chromium removal efficiency compared to naturally existing wildtype strain in effluent water. The results of the present study are also corroborated by another study which also demonstrated that chemical mutagens showed better mutation rates as well as extent of remediation with respect to *Brevibacillus* spp.[16]. Thus, it was resorted to employ the chemical mutant acrylamide for strain improvement in the present study.

The results of the enzyme assay in case of resting cells, shows that the chemical mutant has remediated chromium more effectively than the wild type and the

physical mutant. In case of permeabilized assay overall considering the assay of the wild type, physical mutant and chemical mutant, toluene was shown to be a better solubiliser than triton-X. In cell free extract assay, the remediation of chromium with respect to chemical mutant was maximum in pellet form than remediation achieved from the supernatant. The reduction of chromium is attributable to two characteristics namely membrane binding and the innate capability of quality and quantity of enzyme secretion. Thus, it is the constitution of the membrane that augments the reaction of enzyme with chromium, facilitating reduction. The results of permeabilized assay are in tune with the fact established that triton-X is a protein solubilizer[40] and toluene is a lipid solubiliser[41]. A similar inference has been reported by another study with respect to chromium remediation by *Bacillus subtilis*[25].

Conclusion

The current study was accomplished to improvise the indigenous isolate *Bacillus amyloliquifaciens* using random mutations to facilitate the chromium bioremediation process. The imminent remediation potential by improvised strains of the microbe *Bacillus amyloliquifaciens* at a sub cellular level is studied with respect to chromium electroplating effluent which reveals the role of enzymes in remediation of chromium. The study reveals that the mutants generated using chemical mutagens exhibit better chromium bioremediation potential compared to wild type. Strain improvement by random mutagenesis can be a promising technique to facilitate chromium bioremediation process in laboratory set up. However, in natural settings, further research needs to be carried out to facilitate bioremediation *in situ*.

Conflict of Interests

The authors declare no conflicts of interests.

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