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RESEARCH ARTICLE

Development of a Green Fluorescent Protein Based Bacterial Biosensor for Bioremediation

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ABSTRACT

Microorganisms are being used as specific and sensitivity sensing devices for measuring biologically relevant concentration of pollutants. These biosensors relay on analysis of gene expression by creating transcriptional fusions between a promoter of interest and a reporter gene, and the extent of reporter gene expression serves as a measure of the available concentration of a pollutant. A green fluorescent protein based biosensor was constructed and characterized for its potential to measure and reduce chromate (VI) in effluent and sludge. Plasmid from Pseudomonas putida strain carrying gene that encodes chromate reductase activities was cloned. A bacterial biosensor is developed that responds the Cr (VI) in the range of 10-100 µM by expressing green fluorescence protein. Cr responsive biosensor has been made by the green fluorescent protein (GFP), reporter gene under the control of the Chromate resistance regulatory element (Chr R) and its operator promoter derived from plasmid (9Kb) of P.putida and E.coli were used as a source of plasmid that carries the Chr R resistance operon. Escherichia coli DH5α was used as a host strain. P.putida and E.coli strains plasmids were used as a source of gfp gene, pBR 322 was used as cloning vector. . The induction method used here allows effluent or contaminated soil collected from various resources to be directly tested by using it to prepare the growth medium for the biosensor. The Chr R-GFP biosensor offers a simple and quick method for detection of available chromate in the polluted environment also effectively reduced toxic chromate as bioremediation.

Key Words: *Biosensor, green fluorescent protein P.putida, chromate reductase, pollutant*

INTRODUCTION

Environmental risk assessment is an essential tool in the investigation of polluted sites. The ability to monitor the bioavailability of a pollutant is essential, as it not only gives more accurate information regarding the risk that the contaminated site poses to human health, but also determines the effectiveness of potential bioremediation processes. Nowadays, increasing attention has been given to bioavailability assays that better predict the real exposure risks [Tecon and van der Meer 2008]. One such alternative is the use of biosensors which are highly selective and sensitive to a particular pollutant. Microbial biosensors have become one of the newest dimensions of molecular tools in environmental monitoring [Yagi 2007]. Microorganisms, due to their low cost, lifespan, and range of suitable pH and temperatures, have been widely employed as the biosensing elements in the construction of biosensors [Mulchandani and Rogers 1998].

In recent years, one of the most interesting areas utilizing biosensor technology is the detection of environmental pollutant bioavailability, bioremediation, and toxicity. These biosensors are constructed by fusing a pollutant-responsive promoter to a reporter gene coding for a protein that can be easily quantified, and such constructs can be located on plasmids or on the chromosome. The efficacy of such biosensors was demonstrated by Willardson *et al* [1998]. Bioluminescence has been very successful as a reporter for pollutant detection in part because of the sensitive instrumentation (fiber optic probes, integrated circuit chips) available for detecting light production and if the entire not required[Purohit 2003].

Human activities are adapted and interfered with natural cycles and produced a release to the aquatic and terrestrial systems. Living organisms require small doses of some essential

heavy metals and nonessential heavy metals which affect the surface water systems. Because heavy metals are relatively abundant in the Earth's crust and are often used in industrial processes and agriculture, consequently they become toxic to humans. These can make important alterations to the biochemical cycles of life. The heavy metals are transported by runoff water and contaminate water sources downstream from the industrial site. For life, all living (micro) organisms, plants, and animals depend on water, but because heavy metals can bind to the surface of microorganisms, they may be transported inside the cell [Srivastava and Majumder 2008].

Genetically engineered microorganisms (GEMs) are being developed and assessed for their beneficial uses in agricultural pest control and bioremediation of toxic chemicals in the environment [Thompson *et al* 1998]. The bacteria are genetically engineered to respond to the presence of chemicals or physiological stresses by synthesizing a reporter protein, such as luciferase, β-galactosidase, or green fluorescent protein. In the present study, a novel type of bacterial biosensor was developed using recombinant DNA technology. The bacteria are genetically engineered to respond to the presence of chromium pollutant in tannery effluent through the synthesis of a reporter protein, such as green fluorescent protein (GFP). Present work was focused to describe the development and characterization of a GFP based biosensor for the detection of chromate (VI) in aqueous solution & to measure the chromate reduction efficiency in the process of bioremediation.

MATERIALS AND METHODS

To isolate Cr (VI) resistant microbial strains, soil samples were collectedfrom Ranipet, Tamilnadu lies about 120 kilometres southwest to Chennai. There are around 150 small-scale tanneries discharging their effluents into the Palar River that has been dumped five kilometres downstream. Additionally an estimated 220,000 tons of chromate sludge lies untreated in that area. Leaching of Cr (VI) from these wastes has resulted in contamination of the groundwater in Ranipet.

Microbial strains were examined its potential for metal screening to establish resistant against hexavalent chromium. The chromate reduction ability of two strains was measured by the following method (Park et al., 2000). Growth of strains & Chromate reduction was measure by biomass method & Chromate reductase assay respectively. Growth of cells was followed by A_{660} and Chromate reduction rated was quantified in growing cultures, as well as cell suspension. Residual chromate was measured by the diphenyl carbazide method (park *et al* 2002), after removing the cells by micro-centrifugation. *Pseudomonas putida* and *Escherichia coli* were selected for bioremediation of chromate (VI). *C*hromate reductase enzymes were purified from periplasmic extract of *P. Putida* and *E.coli* which has cultured in Luria – Bertani medium. The crude extract (periplasmic proteins) was passed on the sephadex G50 column chromatography to collect various fractions were monitored for the enzyme activity on the basis of protein concentration. The fractions corresponding to chromate reductase were pooled and precipitated by ammonium sulphate then used for next purification step applied to an anion-exchange chromatography column (1.7by 7.0cm) containing DEAE Sepharose CL-6B. Protein was eluted at a flow rate of 30ml /h. Selected fractions were analyzed by SDS-PAGE. The Chromate reductase activity of each fraction was measured analyzed based on protein concentration was measured by Lowry et al., (1951), using bovine serum albumin as the standard. Plasmid DNA isolation was done by alkaline lysis method of Sambrook et al., (1989) from *P.putida & E.coli* and plasmids were analyzed by agarose gel electrophoresis.

Chr R gene was amplified by PCR from the plasmid DNA isolated bacterial strains. Primers were designed on the basis of the nucleotide sequences of a Conserved hypothetical protein, in which the forward primer involves the conserved region in the 5' direction. Oligo Primers Analysis Software was used to avoid mismatch at the 3' end, avoid complementary sequences within a primer and between primers, the Tm values of the primers were calculated and GC contents were determine for good amplification in reaction. PCR reaction was carried out 35 cycles at 94°C for 1 mint/65°C for 45 sec /72°C for 1 mint for denaturation, annealing,

extension. PCR amplified product may be expected to (fragment) carrying the promoter/operator of the chromate and the regulatory R gene (ChrR genes/O/P) was amplified from plasmid (9Kb), forward primer and reverse primer.

Forward Primer: 5'- TGG ATC CTA TGA GCC AGG TGT ATT-3'
Reverse Primer: 5' – AAA GCT TTT TCA GAC CGC CCT GTT – 3'

Primer carrying *Hind III & EcoR I* sites respectively. The amplified PCR product was purified from 1.2% agarose and digested with *Hind III & EcoR I*. It was cloned into the *pBR322* plasmid just before the gfp gene. The entire ChrR/GFP region was then excised using *EcoR-I* and inserted into pBR322, the construct was transformated into *E.coli DH5α*. The recombinants were grown in LB plate containing 20µg/ml ampicillin as a selected clone was named ChrR-GFP biosensor.

The gene for green fluorescent protein (GFP) from *Aequoria victoria* (Chalfie 1994) is increasingly being used to construct whole cell biosensors (Ward et al., 1980) used extensively as a reporter for measuring biologically relevant concentrations of pollutants. GFP is an attractive marker system to monitor bacterial cells in the environment, detection of GFP requires irradiation by blue light or near ultraviolet (UV) light and does not require any exogenous substrate, complex medium or expensive equipment.

The details for development of GFP biosensor and measurement of chromate reduction given as below: The recombinant clone was inoculated in LB medium containing 20 g/ml ampicillin at 37°C for 24 hrs. The clone was streak on LB plates contain different concentration of chromate 10-100mg/L (25, 50, 60, 75,100mg/L) and plates were placed in UV radiation incubated at 37°C for 16 hrs. After treatment of UV radiation the clone was emitted the green fluorescence. The fluorescence was recorded by Fluorescence microscope. The intensity of fluorescence is depends upon concentration of chromate ion. The efficiency of chromate reduction was measured by the following method (Park et al., 2000).

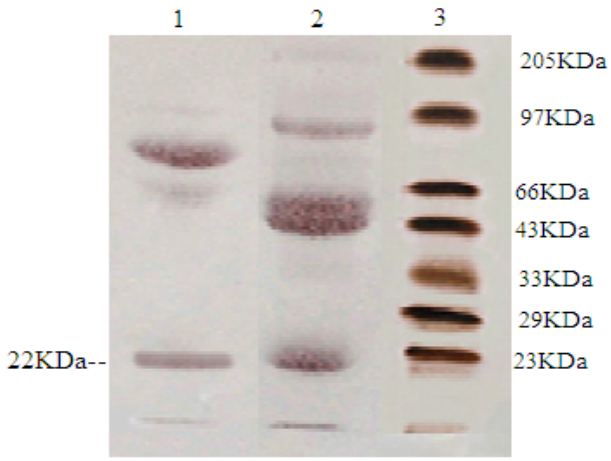
RESULTS AND DISCUSSION

More technically, a biosensor is a probe that integrates a biological component, such as a whole bacterium or a biological product (e.g., an enzyme or antibody) with an electronic component to yield a measurable signal. Biosensor can detect specific bacteria and concentration of hazardous chemicals. It can use biosensor to detect the presence of specific pollutant (Cr VI). GFP, a relatively new biomarker gene is the Green Fluorescent Protein (GFP) gene (gfg) originally isolated and cloned from the jellyfish *Aequorea victoria*.

The wild-type GFP protein auto fluoresces by emitting green light with a wavelength of 545 nm, upon excitation with UV light in the 395 nm ranges. Results explained development of bioengineered strains of bacterium *P.putida* and *E.coli* to become fluorescent green in the presence of toxic levels of chromate and biosensor was developed that responds the Cr (VI) in the range of 10-100 µM by expressing green fluorescence protein. Chromate responsive biosensor has been made by the green fluorescent protein (GFP), reporter gene under the control of the Chromate resistance regulatory element.

The isolated chromate reductase enzymes were identified according to their molecular weight by 12 % SDS-PAGE method. The 20µg chromate reductase enzymes were mixed with sample buffer and boiled at 70°C for 5 minutes. Then the chromate reductase enzymes extracts were in mixture of molecules were loaded under various fraction of preparation in the well along with protein marker. The molecular weight of isolated chromate reductase enzymes were identified by comparing with commercially purchased protein marker. The SDS-PAGE were shows the isolated chromate reductase enzyme (Figure 1) contain intense bands of ~22 kDa. The molecular weight of isolated chromate reductase enzyme was identified by comparing with commercially purchased protein marker.

Fig. 1: Chromate reductase enzymes fraction on SDS PAGE

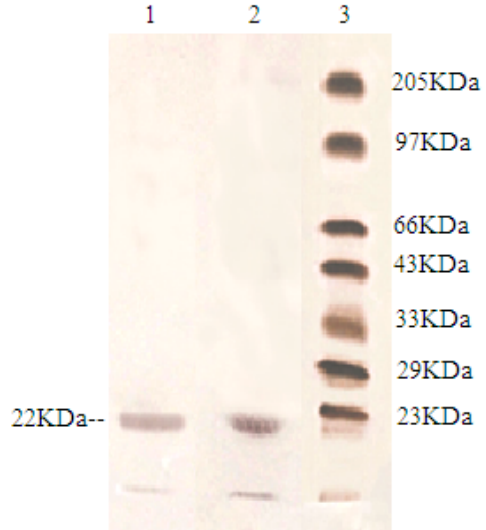


Legends: SDS-PAGE showed separated protein bands on gel prepared from the various step of preparation Chromate reductase enzymes fraction. The arrow head indicates the ~ 22 KDa protein bands whose intensity corresponds to chromate reductase activity.

Lane 1: *E. coli*, Lane 2: *P. putida*, Lane 3: Protein Marker

A novel enzyme prepared from bacterial periplasmic extract might be posses **c**hromate reductase activity used for purification process and resulted fraction separated on gel given in Figure 2, showed protein bands were separaed by SDS PAGE. It was evidence as visible single band on SDS PAGE from the purified sample has 22KDa protein separated from both bacterial strains as indicated intense band 22KDa. The particular protein fraction alone could be considered for further experiment to analyze chromate reduction potential. The protein concentration was determined by using bovine serum albumin as the standard.

Fig. 2: SDS-PAGE showed purified Chromate reductase

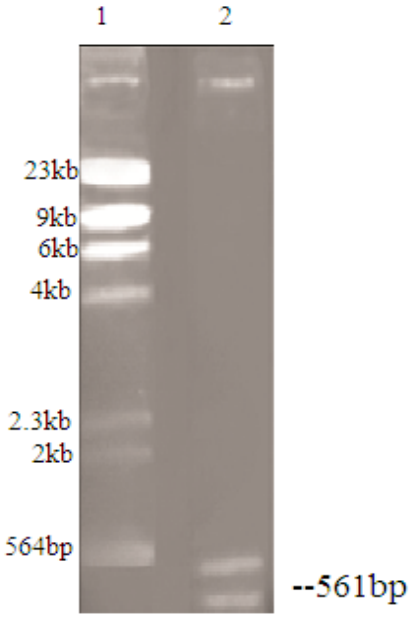


Legends: SDS-PAGE showed a single peak of chromate reductase enzyme band which has highest activity at the final stage of purification, the arrow indicates the ~ 22 KDa protein bands band of active fraction showed only a single band of silver stained on gel. Lane 1: Purified *P.putida* protein, Lane 2: Purified *E.coli* protein, Lane 3: Protein Marker

The plasmids isolated from *P.putida* & *E.coli* were not showed here. The results indicated that the size of the plasmids may be around 9Kb and 4Kb respectively. The gene encode for chromate resistance sequence was amplified by PCR (Figure 3). Char-R Construction of

vector for development of ChrR-GFP Biosensor based on bacterial gene from *P. putida* & *E.coli* strains were used for amplification of chromate reductase gene. *Escherichia coli DH5α* was used as host strain according to the reference by (Sambrook and Russell, 2001). Developed green fluorescence protein based device could be used for measurement pollutant in waste water and contaminated soil. Chr R-GFP biosensor grown in LB broth with 20µg/ml ampicillin of 37°C for overnight culture was used to streak LB plate containing different concentration of Cr (10-100 µM) in triplicate the plates were incubated at 37°C for 16 h before measuring fluorescence. Fluorescence was measured after various time and 16 h duration of exposure at 37°C, fluorescence was recorded using a fluorescence microscope with a GFP band pass filter set the image was recorded directly on a computer using AX10 vision & 2.1 software, version 4.3, the intensity of GFP fluorescence was estimated by measuring the gamma value of a selected fluorescing spot, the Chr R-GFP biosensor was tested for inducible expression of GFP on LB-Amp plates containing various concentration of Cr (VI). *DH5α* was used as an additional negative control. There was no GFP expression in both the control, fluorescence steadily increased in bacteria plated on inducing media containing up to 60 µM of chromate after which it observed as declined state. A chromate reductase responsive biosensor has been made by placing the green fluorescent protein (GFP) reporter gene under the control of the Chr R resistance regulatory element (Chr R) and its operator promoter derived from plasmid *P.putida* (9Kb). *P.putida* used as a source of plasmid that carries the Chr R resistance operon.

Fig. 3: Agarose gel showed PCR amplified product



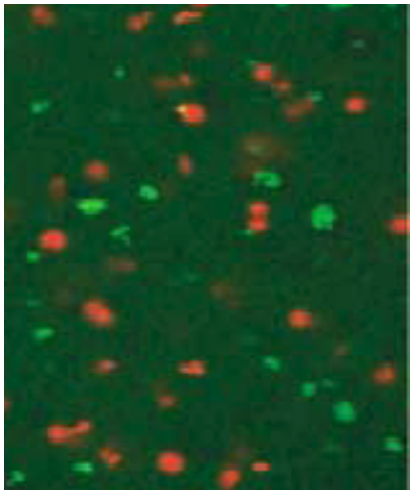
Legends: 1.2% Agarose gel showed PCR product (amplicon). The gfg cassette was then excised and sub cloned between the BamH1 and EcoR1 sites of pBR322 to create a clone contain the chromate reductase gene. The size of the amplicon was ~561 bp shown along with standard marker in the figure 3. Lane 1: λ DNA (Hind III Digest) Marker; Lane 2: PCR amplified product.

Most of the regulatory element used in the construction of metal specific biosensors originates from bacteria that possess natural and precisely regulated heavy metal resistance operons. Most researchers have fused luciferase as the reporter protein and various strains of *E.coli* as the host. The Chr R-GFP biosensor is based on the Chr R locus located on the *P.putida* plasmid DNA. A regulatory protein, Chr R controls the transcription of Chr R structural genes by bending to the operator in the absence of Cr (VI) in the external source in the presence of chromate concentration that triggers the GFP-biosensor, and can be

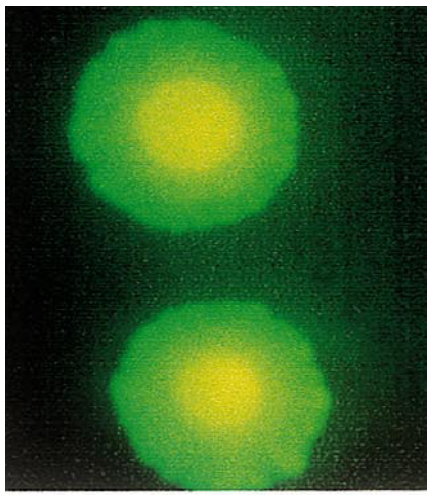
measured on plates in broth culture in terms of fluorescence intensity. Figure 4 a&b showed the expression of cloned *gfp* gene produces green fluorescence**.** GFP marked *Psedomonas putida* cells resulted from a mutated GFP protein which was more fluorescent,more soluble and more evenly distributed throughout the cytoplasm than the wild-type .The GFP can be used as a reporter for the visualization of gene expression and protein subcellular localization also showed that the GFP can be introduced into cells and intact organelles within cells, the GFP is an attractive marker system to monitor bacterial cells in the environment. In this study, GFP was expressed in cell free *In Vitro* translation system and the system might be used as a high quality method to express different proteins which are toxic for the expressing organism or are difficult to be obtained in pure form the cell. GFP has biosensor has advantages associated with its ability to use fluorescence without the need of exogenous substrates, the ability to use fluorescence microscopy to monitor induction and stability of the protein.

Fig. 4 a: The micrograph shows both strains in alive on gel matrix
b: GFP-marked *Pseudomonas putida* colonies on agar plate

4a:



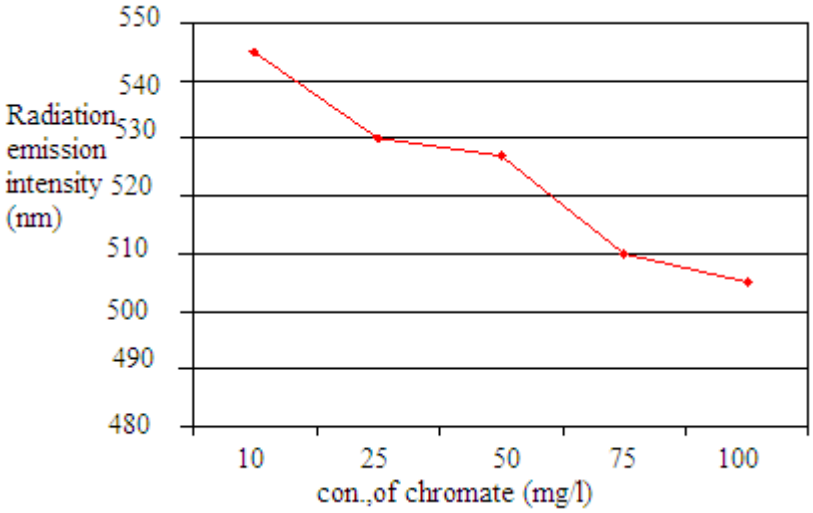
4b:



Legend 4a: A confocal micrograph of two strains, each containing a different fluorescent protein gene (GFPuv, green fluorescence, or DsRed2, red fluorescence) fused to the recA promoter. The strains were coimmobilized in a 160 mm thick sol-gel film Images were recorded 17 hours after induction. The micrograph shows that both strains were alive and inducible inside the sol-gel matrix. 4b: Two large green fluorescent colonies of GFP-marked *Pseudomonas putida* cells grown on TSB agar, mutant *gfp* colonies were viewed and photographed. GFP-plus filter set (excitation filter 480 nm; beam-splitting mirror, 505 nm; barrier filter, 510 nm).

Biosensors have been used to predict the bioavailability of heavy metals in effluent and polluted water. Biosensor efficiency of chromate reduction were confirmed on comparison of its ability with wild strain was grown in 400µM concentration of chromate to observe growth on the basis of concentration of media provided for its culture of *P.putida* & *E.coli* strain. The graphical representation was shown in the figure 5, which was used for identification of chromate in tannery effluent or aqueous solution. The selection of the promoter portion of the biosensor construct is dependent on the target molecule being monitored. A selected promoter sequence is normally placed at the 5′-region of the reporter system where it can be switched on in the presence of the target pollutant, thus turning on the expression of the reporter. The key factors when choosing promoters are sensitivity and specificity. Promoters often respond to groups of compounds rather than to a specific compound, and may also behave differently in different microorganisms.

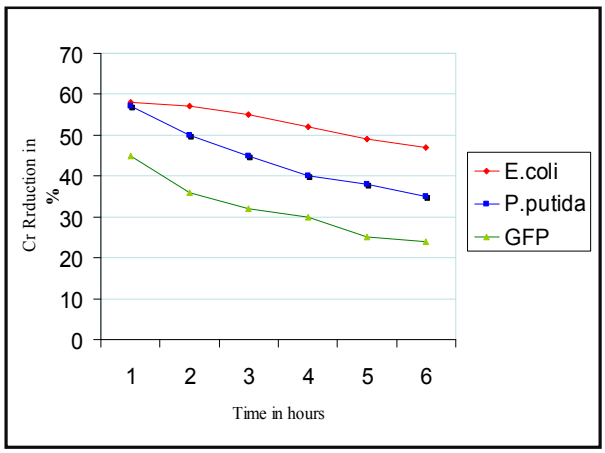
Fig. 5: Fluorescence microscope measurement



Legend: The graph was developed by using the various concentration of chromate and the fluorescence readings were used for, to identify the unknown concentration of chromate present in the sample.

Green fluorescence protein was absorbing UV radiation that means excitation at 350nM for same in all concentration, but variation was observed in emission of radiation only. GFP has responded variously according to the induction of chromate ranging at 10-100 µM. The variation in the fluorescence beyond 10-100 µM was related to drop in copy number of the biosensor plasmid in the cells. The recombinant strain was used to check the reduction of chromate in presence of 400µM concentration as initial step in the experiments. Initial concentration can be considered as 100%, from this concentration how much amount of chromate reduced has given in the figure 6. Recombinant strain more efficiently reduced chromate (55%) than other two wild type strains *P.putida* (45%) and *E.coli* (32%) for 3 hour time interval. The chromate reduction potential was high in the GFP-recombinant biosensor when compared to respective wild types.

Fig. 6: The recombinant strain efficiency of chromate reduction



Legend: The recombinant strain (▲) was reducing chromate 55% effectively, but the *P.putida* (■)strain reduces 45% and *E.coli* (♦) 32% for 3 hour time interval. The chromate reduction potential was high in the GFP-recombinant biosensor when compared to respective wild types. The present construct device has effectively involved in chromate reduction potential as well as useful to detect chromate in polluted samples.

The Chr R-GFP biosensor is based on the Chr R locus located on the *P.putida* plasmid DNA. A regulatory protein, Chr R controls the transcription of Chr R structural genes by bending to the operator in the absence of Cr (VI) in the external source. The efficiency of chromate reduction was measured and it was confirmed as evidence for monitor of chromate and its reduction in any environmental polluted area when compare to wild strains. The chromate reduction capacity of *P.putida, E.coli* and Chr R-GFP recombinant strains were measured by fluorescence microscope for the intensity of radiation emitted from the strains Figure 6. This would enable direct use of samples collected from natural sources to make the media for growing the biosensor and simple subsequent estimation of the chromate in the sample. The genetic material that senses Cr6+ indicates the regulatory protein gene (Chr R) along with promoter /operator (Chr O/P) of the chromate resistance operon from plasmid *pBR322* also controls the GFP reporter gene expression. *E.coli DH5α* is the host organism. The GFP recombinant clone was grown in chromate 400µM concentration and it reduce chromate concentration when compare to the wild strains. The clone was effectively eliminating metal (Cr) toxicity.

CONCLUSION

In this work, bioengineered strains of the bacterium *P.putida* and *E.coli* to became fluorescent green in the presence of toxic levels of chromateA PCR fragment (561bp) carrying the promoter/operator of the chromate and the regulatory R gene (ChrR genes/O/P) was amplified from plasmid (9Kb), forward primer (5' TGG ATC CAT TGA GCC AGG TGT ATT 3') and reverse primer (5' AAA GCT TTT TCA GAC CGC CCT GTT 3'). Primers carried *Hind III & EcoR I* sites respectively.The amplified PCR product was purified from 1.2% agarose gel and digested with *Hind III & EcoR I.* It was cloned into the *pBR322* plasmid just before the gfp gene. The entire Chr R/GFP region was then excised using *EcoR I* and inserted into *pBR322*. The construct was transformed into *E.coli DH5α*. The recombinants were grown in LB plate containing 100µg/ml ampicillin as a selected clone was named ChrR-GFP biosensor. The biosensor is based on detection of light emitted by a specifically engineered microorganism that is involved in bioremediation. This type of biosensors are useful for monitoring efforts to clean up industrial spills because these light emitting bacteria can 'report' containing the progress of biodegradation. The GFP is a useful marker in environmental microorganisms, allowing new research that will increase our understanding of microorganisms in the environment.

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