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Biomonitoring of Heavy Metal Pollution by Bioluminescent Bacterial Biosensors

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Abstract

Objectives: The current study focuses on the isolation of bioluminescent bacteria from the gut of Sea urchin and using its luminescence property as a potential biosensor for detecting chromium toxicity in water. **Methodology:** Bioluminescent bacteria, JAAKP J2 was isolated from the gut of *Pseudoboletia indiana* species of Sea urchin collected from Pondicherry university beach. The morphological, biochemical, enzyme profiling and molecular characterization through 16S sequencing were performed to identify the strain. Growth kinetic assays and spectrophotometric analysis under chromium stress condition were performed on bioluminescent strains for luminescence inhibition studies. The bioluminescent bacteria was then immobilized in nutrient agar cubesand used as a biosensor for the detection of hexavalent chromium concentration in water samples. **Findings:** The morphological, biochemical and molecular characterization revealed that the isolate JAAKP J2 was closely related to *Vibrio campbellii*. Industrially important enzymes like Protease, Lipase, Agarase, Cellulase, Xylanase and Gelatinase were also screened in this study. The developed biosensor using bioluminescence property of the isolate was able to detect the level of chromium toxicity in water samplesat concentration not more than 9 mg L^{-1}. Further we have elucidated a possible hypothetical pathway for reduction in luminescence property due to ROS (Reactive oxygen species) caused by hexavalent chromium toxicity in bacteria. **Novelty:** The study is first of its kind in isolation of bioluminescent strain from sea urchin and using it as a biosensor for heavy metal detection in water. Our study also throws lights on futuristic approach of detecting other heavy metals like lead, zinc, cadmium, mercury in water samples by bacterial luminescence.

Keywords: Bioluminescent Bacteria, Biosensor, Chromium Toxicity, Enzyme Profiling, Sea Urchin

1. Introduction

Marine ecosystem forms the largest aquatic system in the world enclosing more than 70 percent of the planet. With regards to wide bacterial diversity and novelty among bacterial species this ecosystem is very promising. Most marine bacteria are halophiles which thrive in extreme salt conditions in a much broader way than other life forms[1]. Vibrios are generally salt loving with different species opting for different concentration of salt requirements[2].

Bioluminescence is a natural phenomenon of production of light by living organismsthat involves chemical reactions. The purpose ofproducing bioluminescence varies from organism to organism, generally as a defense mechanism, predation or mating etc[3]. Bioluminescent bacteria are commonly found in ocean water,there they generally prevails in the digestive tracts of marine fishes, persist as parasites in crustaceans and insects or as light organ symbionts in teleost fishes and squids[4]. The major organisms capable of exhibitingbioluminescence comprises of four bacterial genera: Vibrio, Photobacterium, Shewanella, and Photorhabdus[5]. *V. fischeri*, *V. harveyi*, *V. logei*, *Photobacterium phosphoreum*, *P. leiognathietc* are few among these genera[6]. The members of Vibrionaceae are well documented for understanding the underlying mechanism of bacterial bioluminescence[7].

In a bioluminescence reaction, aliphatic aldehyde substrate, O_2 and reduced riboflavin mononucleotide ($FMNH_2$) are converted into corresponding aliphatic acid, H_2O and FMN with concomitant production of light at wavelength of 490nm[8].

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + \text{light (490 nm)}$$

This mechanism is catalyzed by luciferase enzyme in the presence of O_2. The enzyme luciferase is a heterodimeric molecule of 77 KDa. The genes coding for luciferase enzyme gets activated when $FMNH_2$ reacts with O_2. As a result, it produces a compound that forms a highly stable complex with aldehyde and decays slowly by dissipating light energy due to the substrate oxidation[3]. A set of 8 lux genes (lux ICDABEG + transcriptional terminator) responsible for coding the proteins for bioluminescence comprise the rightward operon. This encodes the enzyme which is required for the synthesis of the lux I gene (autoinducer) and the alpha (lux A) and beta (lux B) subunits of the enzyme luciferase. The lux C, D and E genes encode the enzymes participating in the formation of the aliphatic aldehyde, luxG does not directly participate in luminescence. It increases the capacity of the cell to synthesize FMN. LuxR gene encodes the transcriptional regulatory protein which constitutes the leftward operon[9]. Bioluminescence in bacteria can also be regulated by the process of auto induction or Quorum Sensing (QS) which is the ability of the bacteria to regulate gene expression in response to cell density.

Hexavalent chromium is a highly toxic compound found it polluted aquatic systems. They are potent genotoxic carcinogens[10,11] derived mainly from chromium trioxide and various salts of chromate and dichromate[10]. Adverse health effects associated with the exposure of hexavalent chromium includes eye irritation and damage, perforated ear drums, occupational asthma, respiratory cancer, skin irritation, discoloration of the teeth, gastrointestinal bleeding, hemolysis, and pulmonary dysfunction[12]. The bioluminescent assays in combination with other bio-tests or chemical analysis can be used effectively to monitor the level of this toxic compounds in the aquatic ecosystem. Application of bioluminescent assays to detect toxic pollutants like hexavalent chromium in water, enable to monitor pollutants in a rapid, cheap and simple way. In toxicity assays, bioluminescent bacteria can be used for either in short term, based on the change in light intensity or in long term by examining the change in their growth rate and viability[13]. Bio engineering of the genes which are responsible for bioluminescence also have potent environmental applications[14]. As there is an increasing risk caused by large number of organic and inorganic toxic substances in the environment, particularly in aquatic systems, the need for an effective economic tools like biosensors to detect these pollutants is essential. The current study shows an inverse relationship with increase in the concentration of chromium in water with a decrease in the bioluminescenceof isolate JAAKP J2. This makes the isolate JAAKP J2 as an effective and promising tool as a biosensor for the detection of hexavalent chromium in water.

2. Materials and Methods

2.1 Sampling

Pseudoboletia indiana species of Sea urchin was collected from Pondicherry university beach (12°00′48.2″N 79°51′38.9″E) through net catchment on 14th of December, 2018. The temperature of the location was between 28°C−30°C at the time of sampling. The samples were collected in a sterile container, well-sealed and transported to the laboratory in ice cold condition at 4°C for further processing.

2.2 Isolation of Bioluminescent Bacteria

The Sea urchin samples were surface sterilized and dissected dorsoventrally with the help of surgical scissors and needles. On breaking down the exoskeleton of sea urchin, the intestinal parts of the organism were collected and homogenized using mortar and pestles. 1ml of the homogenized sample was taken, serially diluted and pour plated on Luria-Bertani (LB) Agar (HI Media Laboratories, Mumbai, India) supplemented with 50% marine water (SWC Media). In order to isolate bioluminescent vibrio colonies, Thiosulfate-Citrate-Bile-Salts-sucrose (TCBS) agar (HI Media Laboratories, Mumbai, India) plates were used[15]. The plates were incubated at 37˚C for 24 hours.

2.3 Characterization of the Isolate

After incubation, colonies were picked based on their intensity of luminesce in SWC media and unique yellow and green colonies with luminesce property on TCBS plate. Conventional bacteriological methods of screening the isolates were followed: grams staining, methyl-red and voges-proskauer tests, mannitol motility test, triple sugar iron tests, cytochrome oxidase activity tests, nitrate reduction tests and urea reductase tests[16]. Utilization of carbohydrates as the sole carbon source with production of acids were tested using sugars: D-Glucose, Sucrose, Lactose, Galactose, D-Fructose, Xylose, Dextrose, Arabinose, Raffinose and Mannitol.Amino acid reduction test were also performed for Cysteine, Tyrosine, Histidine and Arginine.

2.4 Preparation of Cell Free Supernatant and Enzyme Characterization

For enzymatic activity assay, the isolate was mass produced at 28°C for 4 days at 220 rp min Luria-Bertani (LB) broth (HI Media Laboratories, Mumbai, India). The bacterial culture was then centrifuged at 10,000 rpm for 15 min to extract the extracellular proteins in the supernatant[17]. The cell free bacterial supernatant was checked for the production of enzymes like Amylase, Xylanase, Carrageenase, Cellulase, Gelatinase, Pectinase, Lipase, Agarase, Protease and Urease, in order to identify a general enzyme profile of the organism. The screening was performed on LB agar plates supplemented with suitable substrates of certain concentration and observed for zone of clearance (Table 1).

Table 1. Enzyme profiling of the isolates

Enzymes	Basal Medium	Substrate Name	Substrate Concentration	Temp	Time	Indicator Stain
Amylase	Luria-Bertani	Starch	1%	28°C	72 h	Grams iodine
Xylanase	Luria-Bertani	Xylan	0.25 %	30 °C.	72 h	Congo red
Carrageenase	Luria-Bertani	Carragenan	0.5 %	30 °C	48 h	Congo red
Cellulase	Luria-Bertani	Cellulose	1%	30 °C	48 h	Congo red
Gelatinase	Luria-Bertani	Gelatin	1%	28°C	48 h	Congo red
Pectinase	Luria-Bertani	Pectin	0.5 %	30 °C	72 h	Iodine solution
Lipase	Luria-Bertani	Olive oil	10 drops	30 °C	72 h	Iodine solution
Agarase	Luria-Bertani	Agarose	1%	28°C	48 h	Congo red
Protease	Luria-Bertani	Skim Milk	1%	30 °C	48 h	Grams iodine
Collagenase	Luria-Bertani	Type 4 collagen	0.5 %	30 °C	48 h	Grams iodine

2.5 Hemolytic Activity

Sheep blood agar plate (HiMedia Laboratories, Mumbai, India) was used to monitor the bacterial isolate for their ability to producebloodhemolysis, which is a preliminary test for pathogenicity. The bacterial culture was seeded on the blood agar plates and was incubated at 37°C overnight to observe results for alpha-, beta- and gamma-hemolytic activity[18].

2.6 Molecular Characterization of the Isolate

Molecular characterization of the isolate was done by 16S rRNA sequencing. Bacterial strains were characterized using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')[19]. DNA extracts from cells and the 16S rRNA sequence was determined by fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on an ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic analyzer, Korea). The aligned sequences were computed using ClustalW software and sequence homologies were determined using BLASTn search to create an evolutionary distance matrix.

2.7 Growth Kinetics Study- Untreated

Growth kinetics studies of the obtained isolates were carried out. Overnight culture was prepared by inoculating pure colony into 50 ml SWC media and was incubated at 37°C in a shaker at 120 rpm. Absorbance was measured

at 620 nm for regular intervals of one hour till OD reaches 0.5. For growth kinetics study, 2% of culture was inoculated into 100 ml SWC media prepared in 250 ml side arm Erlenmeyer flask and was incubated at 37˚C in a shaker at 120 rpm. The absorbance was measured at 620 nm for every hour till the culture broth reached the decline phase. A standard growth kinetics graph was plotted by taking absorbance on Y -axis and time interval on X –axis[20].

2.8 Growth Kinetics Study under Stress Conditions - Treated

Hexavalent chromium stock of 10,000 mg/L was prepared using $K_2Cr_2O_7$ to study the effect of Cr (VI) on the expression of bioluminescent in the isolated bacteria. Of which 0, 2, 4, 6, 8, 10 and 12 ppm Cr (VI) were inoculated into Erlenmeyer flask containing SWC broth with 0.5 OD bacterial culture. The broth was incubated at room temperature in a shaker at 120 rpm. Absorbance was measured at 620nm at regular intervals of one hour till decline phase was attained. A standard graph was plotted for the obtained values.

2.9 Spectrophotometric Analysis of Luminescence under Stress Condition

Overnight bacterial culture was prepared prior to the experiment. 20 µl of 0.1 OD of overnight culture was inoculated in 130 µl of SWC media in a microtiter plate (96 well plates- black bottom) as control. Remaining test wells were also inoculated with 20 µl of 0.1 OD of overnight culture with SWC media bearing 2, 4, 6, 8, 10 and 12 ppm of Cr (VI) to study luminescence under stressed conditions. The plates were then incubated at 37˚C in shaker at 120 rpm. The Quantitative analysis (OD at 460 nm) was performed to estimate the intensity of luminescence with normal and stressed conditions between 0–12 hrs using iMark Microplate Absorbance Reader (Bio-Rad, California, USA).

2.10 Development of Biosensor

20 ml of stationary phase culture was taken and added to 40˚C molten agar,mixed well and poured into a petri plate for solidification. Small cubes of approximately 1×1 cm were made and individually packed in small highly perforated polythene pouches. Thesepouches were added to flasks containing distilled water supplemented with 50% marine water with various concentration of Cr (VI) ranging from 0 to 12 mgL^{-1}. The flasks were incubatedat room temperature and the disappearance of bioluminescence was noted after 1 hrs.

3. Result and Discussion

3.1 Bacterial Identification and Biochemical Characterization

Mixed colonies of both gram negative and gram positive strains were obtained from the gut of sea urchin [Figure 1]. Bioluminescent strains were isolated based on their unique luminescence property in SWC and TCBS media. Yellow colored colonies with luminesce property on TCBS plates further confirmed to be members of vibrionaceae family. The bioluminescent colonies were further passaged until pure colonies were formed. The final pure colonies were translucent, non- pigment producing with round margins and rode shaped. Colonies were predominantly viable in SWC media. The colonies were observed in day light and as well as in darkness for luminescence using professional camera (Camera model- Nikon D7000, Exposure time- 5 s, ISO speed: ISO-1000, Exposure bias +0.3 step, Focal length- 55 mm, Max aperture- 4.5) [Figure 2].

Figure 1. Dissecting intestinal parts of *Pseudoboletia Indiana* for isolation of bioluminescent bacteria.

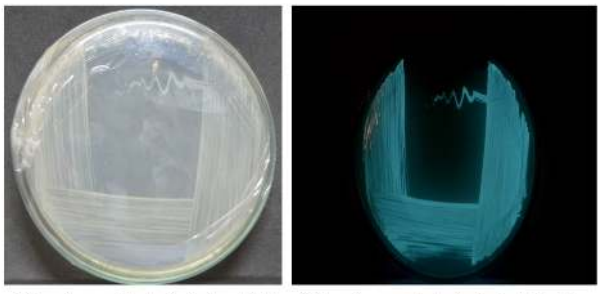

Figure 2. Observation of bacterial Bioluminescence in day and dark light.

The intensity of bioluminescence was also measured using lux meter for the selection of potential luminescent strain for further inhibition studies. Strains showing lux meter reading of 0.7 lux was selected [Figure 3].

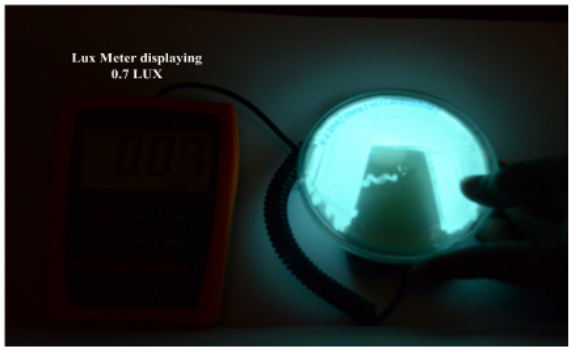

Figure 3. Determination of Lux using a Lux Meter.

Table 2. Biological profiling of the isolates

Experiments		Results
Biochemical Characterization		
1	Gram Staining	Gram Negative
2	Catalase	+
3	Oxidase	+
4	MR	–
5	VP	–
6	Indole	–
7	Citrate	+
8	Urease	–
9	TSI	+
10	H2S	–
11	Nitrate reduction	–
12	Temperature Stability	25 – 38°C
13	pH stability	6 – 8
14	TCBS	Yellow colonies
15	Pigmentation	Nil
Carbohydrate fermentation test		
1	D- Glucose	+
2	Sucrose	+
3	Lactose	+
4	Galactose	+
5	Maltose	+
6	Xylose	–
7	Fructose	+
8	Arabinose	–
9	Raffinose	–
10	Mannitol	+
Amino acid decarboxylase test		
1	Cysteine	–
2	Tyrosine	+
3	Histidine	–
4	Arginine	–
Enzyme Profiling		
1	Protease	+
2	Amylase	–
3	Lipase	+
4	Carragenase	–
5	Agarase	+
6	Pectinase	–
7	Cellulase	+
8	Xylanase	+
9	Gelatinase	+
10	Collagenase	–

"+" represents positive results and "–" represents negative results

Biochemical profiling for assimilation of sugars as a sole carbon source revealed the ability of the vibrio strain to ferment majority of sugars used in the studies except xylose, arabinose and raffinose. Amino acid decarboxylase test also revealed their ability to reduce Tyrosine (Table 2). It is also noted that vibrio are capable of fermenting sugars without visible gas production[21] which exactly correlated with our results.

3.2 Molecular Identification through 16S Sequencing

Molecular identification through 16S rRNA gene sequences revealed the strain as *Vibrio campbellii* (NCBI accession no : MN49473.1) [Figure 4].

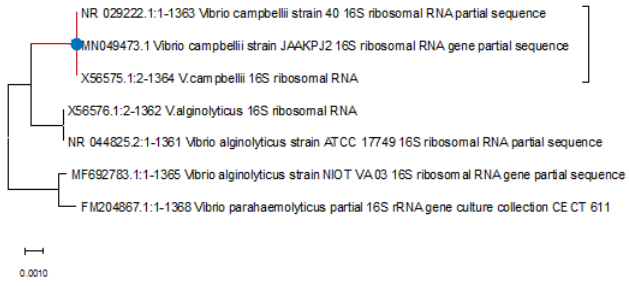

Figure 4. Molecular identification of the strain.

3.3 Enzyme Profiling of the Isolate

Enzyme profiling of the isolate revealed them as potential strain for producing enzymes like Protease, Lipase, Agarase, Cellulase, Xylanase and Gelatinase and it has many industrial applications [Figure 5]. Bioluminescent vibrio bacterium capable of producing lipase enzymes have been previously reported[22]. But strains of *Vibrio campbellii* capable of producing different enzymes remains not much explored. Hemolytic activity of our strain revealed that the strain was not capable of producing any haemolysins when compared with that of previous studies conducted by[23]. This could possibly reveal that *Vibrio campbellii* is an opportunistic pathogen.

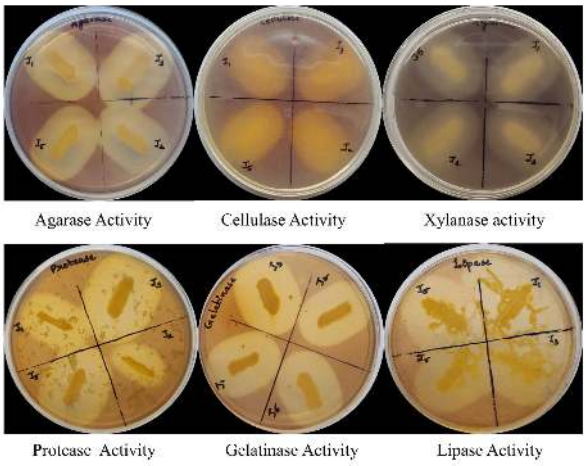

Figure 5. Enzyme profiling of the strain.

3.4 Growth Kinetic Studies

3.4.1 Untreated

Growth kinetic study conducted with overnight culture of 0.5 OD for a period of 12 h at a fixed interval of 1 hour shows a clear lag, log, stationary and decline phase [Figure 6]. Bioluminescence was observed when the bacteria growth phase reached 5 h and approximately retained its luminescence till 10th h. The decline in the luminescence was observed from the decline phase which started from 11th hr. This led to the understanding that bioluminescence of this particular strain remains active during the stationary phase of the growth kinetics and reduces with the decline phase.

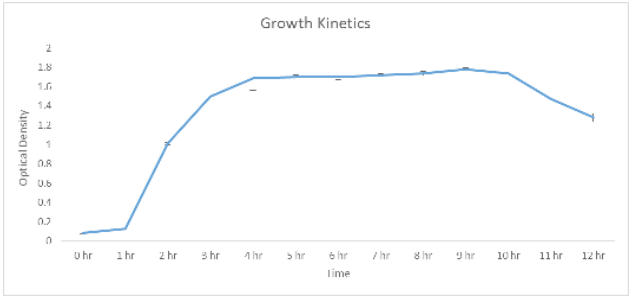

Figure 6. Growth kinetics of the isolate.

3.4.2 Treated

Growth kinetic study conducted with overnight culture of 0.5 OD treated with various concentration of Cr (VI) (2, 4, 6, 8, 10 and 12 mg L^{-1}) for a period of 12 h at a fixed interval of 1 hour revealed a reduction in the stationary phase duration as well as the time taken to reach stationary phase as the concentration of Cr (VI) increases [Figure 7]. The decrease in the stationary phase further led to the decrease in luminescence intensity as confirmed by Spectrophotometric analysis.

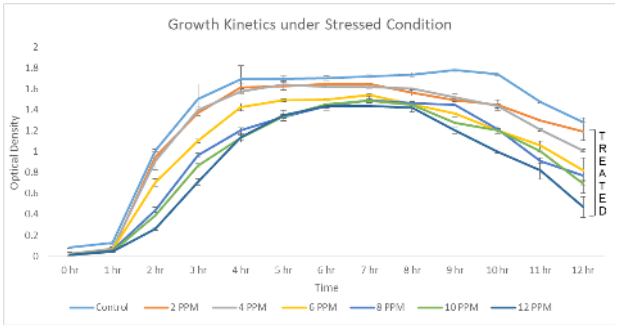

Figure 7. Growth kinetics study under stress conditions.

3.5 Determination of Luminescence

Spectrophotometric analysis of luminescence depicted the decrease in the intensity of bioluminescence with increase in the chromium concentration from 2 ppm to 12 ppm [Figure 8]. The results from spectrophotometric analysis further revealed that, as chromium concentration increases it hampered the prolonged luminescence of the strain unveiling the possibility of developing a new biosensor for monitoring chromium toxicity in water samples. Luminescence visible to naked eyes persisted in the culture up to 8 ppm concentration of chromium in the medium.

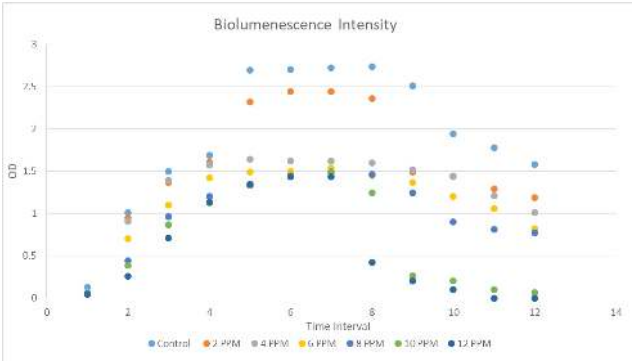

Figure 8. Comparing Optical density vs Luminescence in particular time interval.

3.6 Development of Bioluminescence Biosensor

Our prototype biosensor consisting of bacteria immobilized in nutrient agar in perforated polythene pouches showed a decreasing trend in the intensity of luminescence as the concentration of Cr increased from 0 to 12 mg L^{-1} in the medium. It could be dueto the decreased expression of bioluminescence. Complete absence of bioluminescence was observed from 9 mg L^{-1} of Cr (VI) concentration [Figure 9].

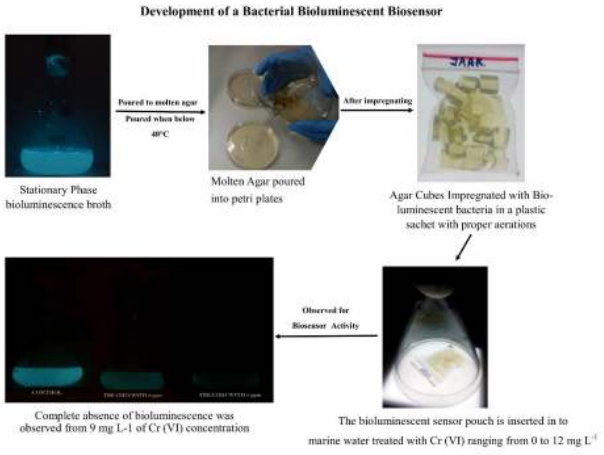

Figure 9. Development of a bacterial biosensor

3.7 Hypothetical Pathway

Hexavalent Cr is a potent carcinogen and mutagen. Cr (VI) causes apoptosis as measured by mitochondrial damage, DNA fragmentation (DNA strand breaks and dG hydroxylation) and it generates harmful reactive oxygen species (ROS) resulting from cellular reduction of Cr (VI)[24]. But the exact effects of ROS on the Quorum sensing signaling molecules have not been extensively studied[25]. While previous studies have revealed that the Cr (V) intermediates are strongly responsible for Cr (VI)-induced DNA damage with recent suggestions that hydroxyl (-OH) radicals may also play a vital role[26]. Glutathione reductase and NADPH within the cell increases the Cr (VI) induced generation of ROS. Inside the cell Cr (VI) is reduced to Cr (V) by certain flavoenzymes like glutathione reductase. Molecular oxygen is reduced to O2- and produces H_2O_2 by dismutation reaction happens. The Cr (V) formed reacts with H_2O_2 to generate OH free radical by a fenton -like reaction[27,28]. Thus ROS is generated during the electron reduction of Cr (VI) which possibly leads to DNA damage leading in reduction of bioluminescence with increasing concentration of chromium [Figure 10].

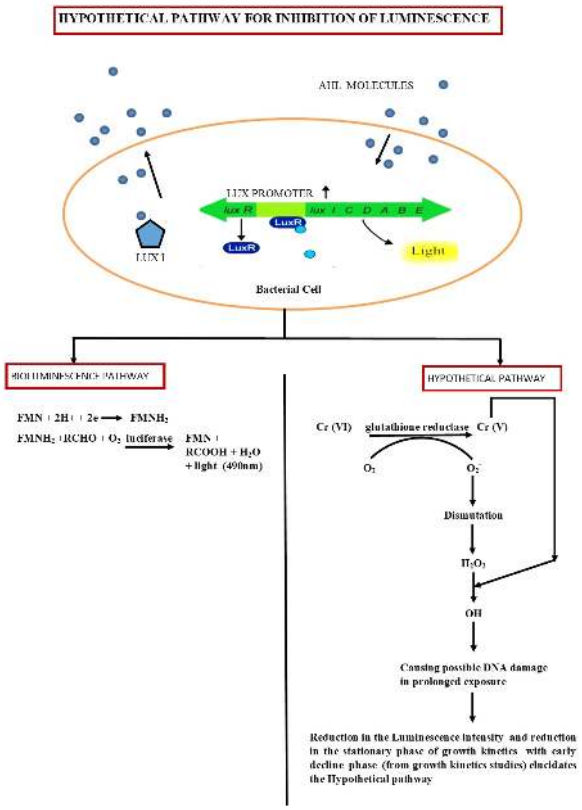

Figure 10. Hypothetical pathway for luminescence inhibition.

4. Conclusion

The heavy metal chromium is extensively being used in various industrial processes and is generally discharged into the natural water sources. It is a potential threat to the environment and public health as it is non-biodegradable

and environmentally persistent. Detection of such chromium contamination in natural water sourcesthrough biological activities of bacteria would be a cost effective futuristic approach. Through our growth kinetic studies it is evident that *Vibrio campbellii* is capable of producing luminescence at the stationary phase of its growth cycle. Taking into consideration of this vital observation, we studied luminescence activity of *Vibrio campbellii* under different stress levels of chromium concentration in the culture medium and found that bioluminescence started diminishing as the concentration of Cr (VI) increased and no luminescence was exhibited after 9 mg L^{-1} of Cr concentration. It was also observed that, as chromium concentration increased, the growth phase declined accordingly with a limited stationary growth phase. This ideology has been used in developing a bacterial biosensor to detect the concentration level of chromium in water. This study could be further extended to the detection of heavy metals like Co(II), Ni(II), Ag(I), Fe(II) Cu(II), Cd(II), Pb(II), Zn(II) in a futuristic approach. Overall, bioluminescent bacteria can effectively be used as a tool in detecting chemical pollutants in water in a very cost effective and eco-friendly manner.

5. Acknowledgement

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