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Chromate Reduction by *Allochromatium* sp. Isolated from the Coastal Area of Visakhapatnam

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ABSTRACT

A phototrophic purple bacterium *Allochromatium* sp. strain GSKRLMBKU–01 was used in this study for the reduction of hexavalent chromium. This phototrophic bacterium was isolated from samples collected from the coastal area of Visakhapatnam, India. Both the cells (free (FC) and alginate entrapped immobilized (IC)) are used for the reduction of chromate. Among them, chromate reduction was increased using immobilized cells. Immobilized cells entrapped in sodium alginate reduced the chromate up to 33 ± 3.0 µM on the 8th day of incubation by *Allochromatium* sp., incubated in presence of light (2000 lx) under the strictly anaerobic conditions, while a chromate reduction up to 26 ± 0.20 µM was recorded by FC of *Allochromatium* sp. Chromate reduction can be recorded even up to the 20th day by both FC and IC. An incubation period of 8 days was found to be optimum for its growth and chromate reduction. The maximum growth in terms of dry cell weight (DCW) of FC is recorded up to 1.7 ± 0.20 g.L^{-1} and IC is 2.0 ± 0.30 g.L^{-1}. The growth was recorded even on complete chromate reduction. The final pH of the FC was recorded at pH 8.5 ± 0.10, while the final pH of 8.6 ± 0.20 was recorded for the IC of *Allochromatium* sp. in the growth medium. The obtained results were mentioned in terms of mean and standard deviation which are statistically significant at P ≤ 0.001 level. The detoxification of chromium in the large-scale systems by employing a purple phototrophic bacteria *Allochromatium* sp., is proposed.

INTRODUCTION

Microorganisms have to cope and adapt to the various stressors of natural or anthropogenic origin in their environment (Mariann Kis et al. 2015). Scientists are assigned with developing useful applications for environmental protection, including protecting the biodiversity of aquatic habitats (Koblizek et al. 2005, Falkowski et al. 2007, Hojerova et al. 2011, Medova et al. 2011, Ferrera et al. 2011, Douglas et al. 2016) and monitoring and remediating pollutants (Borsetti et al. 2009, Kushalatha et al. 2010, Idi et al. 2015) in the environment. One of the most important factors affecting toxicity can be the metal ions produced by contamination. Low concentrations of important metal ions play an essential part in the natural development of microorganisms: They act as catalysts for several biochemical reactions, transition metals such as iron, copper, and nickel participate in redox processes, stabilize magnesium and zinc, various enzymes and DNA by electrostatic forces, and iron, magnesium, nickel, and cobalt are part of complex molecules with diverse functions (Mariann Kis et al. 2015, Nies 1992). Among heavy metals, the second most common metal is chromium which is considered a serious environmental inorganic pollutant widely spread in nature. This is very important in the treatment of different industrial wastes contaminated with metal (Williams & Silver1984). A huge number of industrial applications such as metallurgical and refractory are associated with chromium. The oxyanions of Cr(VI) are widely distributed in leather treating processes, metal plate removal, rust prevention, water cooling tower treatment, color manufacture, masking, and wood preservation (Shanker et al. 2005). The metal toxicity will depend upon its state of oxidation, whereas the compounds of Cr(VI) are dissolved in water and move in nature. Although, the insoluble chromium hydroxides of Cr(III) are slightly acidic and alkaline (Cieslak-Golonka 1995). The hexavalent chromium species are carcinogenic, teratogenic, and mutagenic in nature and are highly toxic to all life forms. The CrO_4^{2-} tends to cross the cell membrane through external anion transport systems (channels of SO_4^{2-} and HPO_4^{2-}) and reacts with nucleic acids as well as alternative cell constituents that affect oxidation and damage the cells (Bagchi et al. 2002, Rajyalaxmi et al. 2019). Previously the removal of chromium-polluted wastes are executed by conventional methods which were more expensive and required higher energy and a larger number of chemicals. To overcome these current problems, the employment of biological systems and their uses in the

biodegradation of pollutants has gained much consideration in the past few years (Gadd 2000). In the reduction of toxicity, various microorganisms are exposed to Cr(VI) by altering the elements of the genes into a dangerous and subtle trivalent type (Daulton et al. 2002).

Because of their unique nature, these phototrophic bacteria are a great fit for their application in biotechnological industries as they get energy from solar radiation and are highly resistant to several heavy metals. These bacteria have strong capabilities in decomposing as well as transforming several organic compounds, which are highly resistant to toxicants such as phenols, cyanides, chlorine, and salts involved in synthesizing cell materials (Zhou et al. 2015). He et al. (2017) reported the combination of nanoparticles and microbes for the treatment of wastewater using phototrophic bacteria. *Rsp. rubrum* and *Rhodobacter sphaeroides* were used for the bioaccumulation and biosorption of cadmium (Watanabe et al. 2003, Smiejan et al. 2003). The high concentrations of chromium, cobalt, molybdenum, as well as nickel, were also removed by using *Rhodobacter sphaeroides* (Buccolieri et al. 2006). The *Rba. capsulatus* was found to be cadmium (Cd) tolerant (Gad El-Rab et al. 2006). The bioaccumulation of cobalt and nickel as well as oxyanions reduction as selenite and tellurite was also reported (Borsetti et al. 2003, Italiano et al. 2009). The reduction and oxidation of metals using hydrogenases produced from *Thiocapsa roseopersiciana* were studied by Zadvorny et al. (2006). The chromate reductases produced by bacteria and some phototrophic bacteria were used for the detoxification of toxic chromate to less toxic Cr(III) form (Philip et al. 1998; Ramchander et al. 2011, Rajyalaxmi et al. 2019). Panwichian et al. (2012) have undertaken detailed studies on the remediation of heavy metals and treatment of water collected from contaminated shrimp ponds using purple non-sulfur bacteria (PNSB). A purple phototrophic bacteria such as *Allochromatium* sp., isolated from a marine source was used for phosphate solubilization (Rajyalaxmi et al. 2015). Takaiekhozani and Rezania (2017) have excellently reviewed heavy metals removal from polluted water by employing photosynthetic bacteria. These phototrophic purple bacteria are not only used in bioremediation processes but are also used in the production of other high-value-added products (Sasikala et al. 1995a, Ramchander et al. 2010a,b, Rajyalaxmi et al. 2018, Rajyalaxmi et al. 2021). Ramchander et al. (2011) and Rajyalaxmi et al. (2019) investigated the reduction of chromate by employing *Rhodobacter capsulatus* KU002 and *Rhodobacter* sp. strain GSKRLMBKU-02. Very few reports are published on anoxygenic phototrophic bacteria. The results of this investigation carried out on phototrophic purple bacteria such as *Allochromatium* sp. intended to show its possibilities in the field of remediation of chromium in the presence of light under anaerobic conditions. This is the first report on chromate reduction by this bacterium.

MATERIALS AND METHODS

Chemicals

0.25% Diphenyl-carbazide (DPC) solution, 0.05 µM potassium dichromate ($K_2Cr_2O_7$), 3% sodium nitride (NaN_3) solution, 1N H_2SO_4, 0.01 M $KMnO_4$ solution, Distilled water, $MgSO_4{\cdot}7H_2O$, $KH_2PO_4{\cdot}7H_2O$, NaCl, NH_4Cl, 0.4; $CaCl_2.2H_2O$, Sodium acetate, Ferric citrate, Yeast extract, ZnCl, $MnCl_2.4H_2O$, H_3BO_3, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, $CuCl_2.2H_2O$, $NaMO_4.2H_2O$, Hydrochloric acid (25%v/v) and Cyanocobalamin (Vitamin B_{12}). All the above-mentioned chemicals used for this study are purchased from Hi-Media and Sigma Aldrich (Mumbai, India).

Basal Medium (Modified Biebl and Pfennig's Media)

The media employed for this study is taken from the previous literature reported by Rajyalaxmi *et al*. (2018). The media contains (in g.1000mL^{-1}) KH_2PO_4, 1.0; NH_4Cl, 1.0; $MgCl_2.5H_2O$, 0.5; Yeast extract, 0.1; Organic carbon (sodium acetate), 1.0; H_2S, 0.25; Sodium bicarbonate, 3.0; Yeast extract, 0.1; double distilled H_2O, 1000 mL; Trace element solution (modified SL4 (Pfennig & Lippert 1966)): ($MnCl_2$, 100; $CoCl_2$, 190; H_3BO_3, 300; $NiCl_2.6H_2O$, 24; $CuCl_2.2H_2O$, 0.2; $NaMO_4.2H_2O$, 10; $ZnCl_2$, 70; Ethylenediaminetetraacetate-Na(Na_2-EDTA), 3.0) (mg.L^{-1}), 1.0 mL and the mixture of vitamin solution contains (Biotin, 10; Calcium pantothenate, 10; Pyridoxal HCl, 10; PABA, 20; Thiamine dichloride, 30; vitamin B12, 5 and Niacinamide, 35) (mg.L^{-1}), 1.0 mL. The initial pH of the basal medium was maintained at pH 8.0.

Isolation and Identification of *Allochromatium* sp.

Allochromatium sp. strain GSKRLMBKU-01 used in this present investigation was isolated by enrichment techniques (Pfennig & Truper 1992, Rajyalaxmi et al. 2015). Bacteria thus isolated were identified morphologically (Staley et al., 1994) and further confirmed molecularly by 16S rRNA sequencing analysis (Rajyalaxmi et al. 2019).

Immobilization

The method used for the immobilization was adopted from the method suggested by Johnsen and Flink (1986) and Rajyalaxmi et al. (2019). Freshly cultured cells of *Allochromatium* sp. were separated by centrifugation at 5000 rpm for 10 minutes at 4°C. The obtained cells were washed and resuspended in sterile 0.3 % saline and fixed with an alginate cell trap by immobilization. A 3.0 % solution of sodium

alginate was prepared by dissolving 3.0 g of sodium alginate in 100 mL of boiling distilled water and kept for autoclaving for 15 minutes at 121°C. On cooling, the cell and alginate suspension were mixed and stirred thoroughly for 10 minutes to form a homogenous mixture. Drop this mixture into an ice-cold solution of 0.2 M $CaCl_2$ with a sterile syringe from the height of 5 cm and be subject to curing at 4°C for about 4 h. These cured beads were washed with sterile distilled H_2O about 4 times and are stored in the refrigerator by preserving in the 0.9% solution of sodium chloride (Rajyalaxmi et al. 2019).

Estimation of Chromium

The Uv-Vis spectrophotometric method was suggested for chromium determination using diphenyl–carbazide (DPC) (Greenberg et al. 1992, Rajyalaxmi et al. 2019). The reddish-violet complex is observed when chromium (VI) reacts with diphenyl-carbazide. This reaction is very sensitive and selective for chromium. To a 15 mL sample solution, add 1.0 mL of 1 N sulphuric acid (H_2SO_4) and a solution prepared with 0.5 mL of 0.01 M potassium permanganate ($KMnO_4$) and heat the solution for 40 minutes in boiling water. A 4% sodium azide (NaN_3) solution was added and kept for warming for 3 minutes at 60°C to reduce the excess amount of $KMnO_4$. After cooling in ice water, add 2.0 mL of the solution prepared with 0.25% diphenyl-carbazide and make up the volume to 25 mL using distilled H_2O. Allow the reaction mixture for about 20 minutes and measure the absorbance at 540 nm against a blank prepared using only the reagent. Different concentrations of potassium dichromate ($K_2Cr_2O_7$) are used for the preparation of the standard graph (Rajyalaxmi et al. 2019).

Estimation of Biomass

The UV-Vis spectrophotometer (turbidimetric analysis) is used to determine the growth of the *Allochromatium* sp. by reading the optical density at 660 nm. The biomass of the cells was also determined in terms of the dry weight, for this, aluminum cups were prepared and weighed before and after the experimental procedure (Rajyalaxmi et al. 2019). The culture suspension was transferred into the cups and dried at 80°C. After cooling at room temperature, the aluminum cups were weighed in a single pan balance, whereas the uninoculated media was used as blank.

Statistical Analysis

The obtained results were subjected to statistical analysis to record the mean and SD by using GraphPad Prism.InStat Version 6 (GraphPad Software Inc., USA) and subjected to analysis of variance (ANOVA) to test the significance level of the treatment at P □ 0.05 (Rajyalaxmi et al. 2019).

RESULTS AND DISCUSSION

Isolation and Identification

Allochromatium sp. strain GSKRLMBKU-01 was isolated from the water sampled from the coastal area of Visakhapatnam, India using modified Biebl and Pfennig's media (Rajyalaxmi et al. 2018). The cells were motile, Gram – ve, appear as ovoid to rod-shaped, 2.0 to 5.0 µm in size, multiplied by binary fission and they form single or paired cells. This bacterium appears in brown because of the presence of bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series i.e., spirilloxanthin as the main carotenoid. Further, growing on the agar plates they form smooth round as well as slimy colonies. After 3-5 days of its incubation at a temperature of 30±2°C in the presence of light (2000 lx) under strict anaerobic conditions, the light brown colored cells were transformed to dark brown in liquid media. This phototrophic bacteria grows photoorganoheterotrophically in the presence of light (2000lx) by utilizing acetate (0.1% w/v) under anaerobic conditions.

Thus the morphologically identified *Allochromatium* sp. was further confirmed molecularly by 16S rRNA sequencing analysis (Rajyalaxmi et al. 2015). The obtained sequence was analyzed at the NCBI (Bethesda, MD) (http://www.nbi.nlm.nih.gov/BLAST) (Rajyalaxmi et al. 2019) to find out the closed homology by means of a BLAST algorithm and also deposited in the National Centre for Biotechnology Information based on obtained evolutionary analysis. Fig. 1 illustrates that isolated and identified strain GSKRLMBKU-01 of *Allochromatium* species differed hereditarily from other species because of the source from where it is isolated. The obtained sequence data showed that a new isolate was observed and branched separately, however, it is grouped with other members of *Allochromatium* which were varied with the genera of other anoxygenic phototrophic bacteria (Tamura et al. 2011). The gene sequence obtained from 16S rRNA gene sequence analysis of an *Allochromatium* sp. strain GSRLMBKU-01 was deposited at the NCBI database with GenBank Accession Number HF677171.1 (Rajyalaxmi et al. 2015).

Estimation of Chromium

The Uv-Vis spectrophotometric method with diphenyl-carbazide (DPC) is used for the determination of chromate reduction at different incubation time intervals. In the present study both the FC and IC of *Allochromatium* sp. were used for the chromate reduction. The concentration of 0.05 mM potassium dichromate is added to the 15 mL screw-capped glass tubes containing growth medium which are incubated at a temperature of 30 ± 2°C for 4 to 20 days in the presence

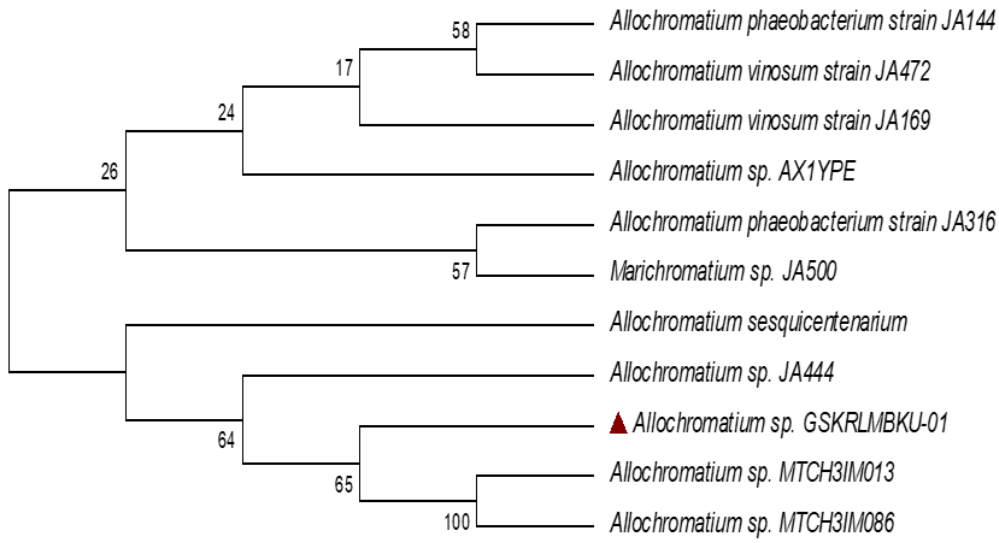

Fig. 1: Phylogenetic tree showing the genetic diversity of *Allochromatium* sp., based on partial 16s rRNA gene sequences of isolate and NCBI reference strains, obtained by the neighbor-joining method using the MEGA 5.0 software (Tamura et al. 2011).

of light (2000lx) and strict anaerobic conditions are maintained throughout the investigations. The immobilization of bacterial cells increased the percentage of reduction of chromate (Fig. 2). From Table 1 and Table 2, it is evident that the reduction of chromate was initiated from the 4th day by FC (10.0 ± 2.0 µM) and IC (14.0 ± 1.0 µM) of *Allochromatium* sp., and it was even recorded on the 20th day. These investigations carried out are similar to the study reported by Rajyalaxmi et al. (2011) and Rajyalaxmi et al. (2019) where they recorded the reduction of chromate even on the 20th day. The benefit of this investigation might be that the reduction of chromate was observed for a persistent period (up to 20 days).

The chromate up to 33.0 ± 3.0 µM is reduced by alginate entrapped cells of *Allochromatium* sp. (Table 2), whereas chromate up to 26.0 ± 2.0 µM is reduced by FC of *Allochromatium* sp. (Table 1). Eight days of incubation proved to be optimum for the reduction of chromate by both the immobilized and free cells. The chromate reduction was increased up to the 8th day and it gradually decreased on a further increase of the incubation period (Fig. 2). Similarly, Rajyalaxmi et al. (2019) reported the maximum chromate reduction of up to 40 µM on the eighth day of the incubation by alginate entrapped cells of *Rhodobacter* sp. Nepple et al. (2000) examined the growth conditions of *Rba. sphaeroides* and found that it can grow anaerobically even in the presence of chromate up to 46 µM and also converted Cr (VI) to trivalent Cr (III). He also recorded the chromate reduction up to 20 µM anaerobically in the presence of light by using some phototropic bacteria (*Rhodobacter capsulatus, Rhodospirillum rubrum, Rcy. tenuis,* and

Table 1: The Growth, pH changes, and chromate reduction recorded at different incubation days by free cells of *Allochromatium* sp.

Days of incubation	Growth in optical density	Dry cell weight [g.L^{-1}]	Final pH	Chromate reduction by Free cells (µM)
4	0.3 ± 0.10	0.5 ± 0.06	8.2 ± 0.06	10 ± 2.0
6	1.0 ± 0.20	1.2 ± 0.20	8.4 ± 0.15	20 ± 1.5
8	1.4 ± 0.25	1.7 ± 0.20	8.5 ± 0.10	26 ± 2.0
10	1.2 ± 0.15	1.5 ± 0.30	8.6 ± 0.20	24 ± 1.0
12	1.0 ± 0.10	1.3 ± 0.10	8.8 ± 0.20	21 ± 3.0
16	0.7 ±0.10	1.0 ± 0.15	9.2 ± 0.25	15 ± 2.0
20	0.4 ± 0.10	0.6 ± 0.10	9.5 ± 0.10	8 ± 1.0

This experiment was carried out in triplicates and the obtained results were noted in terms of mean and SD which are analyzed for ANOVA and the obtained results are statistically significant at the level of $P < 0.001$.

Rhodobacter blasticus). Ramchander et al. (2011) reported the immobilized cells of *Rba. capsulatus* showed enhanced growth and reduction of chromate up to 28 µM within 12 to 16 days of its incubation period.

The UV-Vis spectrophotometer is used to determine the growth of *Allochromatium* sp. by reading optical density at 660 nm and the growth of the bacterium was estimated in terms of the DCW (Italiano et al. 2012). The maximum growth in optical density of free cells was recorded as 1.4 ± 0.25, whereas DCW (dry cell weight) was recorded up to 1.7 ± 0.20 $g.L^{-1}$ (Fig. 2). The 8^{th} day is considered as the optimum incubation period for maximum growth and chromate reduction. On the other hand, the immobilized cells of the bacterium showed maximum growth in O.D as 1.8 ± 0.20, while the DCW was recorded as 2.2 ± 0.30 $g.L^{-1}$. Similarly, Rajyalaxmi et al. (2019), reported the maximum growth of both FC and IC of *Rhodobacter* sp. in terms of optical density (1.5 to 1.8) and dry cell weight (1.8 to 2.2) was observed on the 8^{th} day respectively. Italiano et al. (2012) reported the total reduction of 0.2 mM concentration of chromate within 3 to 4 days in a growth medium containing *Rhodobacter sphaeroides*. A very long lag phase is observed in their investigations for the reduction of a very small concentration of CrO_4^{2-} which takes place very.

Table 2: Growth, pH changes, and chromate reduction recorded at different incubation days by sodium alginate immobilized cells of *Allochromatium* sp.

Das of incubation	Growth in optical density	Dry cell weight ($g.L^{-1}$)	Final pH	Chromate reduction by Immobilized cells (µM)
4	0.6 ± 0.10	0.8 ± 0.10	8.3 ± 0.10	14 ± 1.0
6	1.3 ± 0.30	1.7 ± 0.20	8.5 ± 0.30	25 ± 2.0
8	1.8 ± 0.20	2.2 ± 0.30	8.6 ± 0.20	33 ± 3.0
10	1.5 ± 0.10	2.0 ± 0.20	8.8 ± 0.20	30 ± 2.0
12	1.4 ± 0.20	1.8 ± 0.20	9.0 ± 0.30	26 ± 3.0
16	0.9 ± 0.20	1.2 ± 0.10	9.5 ± 0.10	18 ± 2.0
20	0.6 ± 0.15	0.8 ± 0.20	9.8 ± 0.20	12 ± 2.0

This experiment was carried out in triplicates and the obtained results were noted in terms of mean and standard deviations which are analyzed for ANOVA and the obtained results are statistically significant at the level of $P < 0.001$.

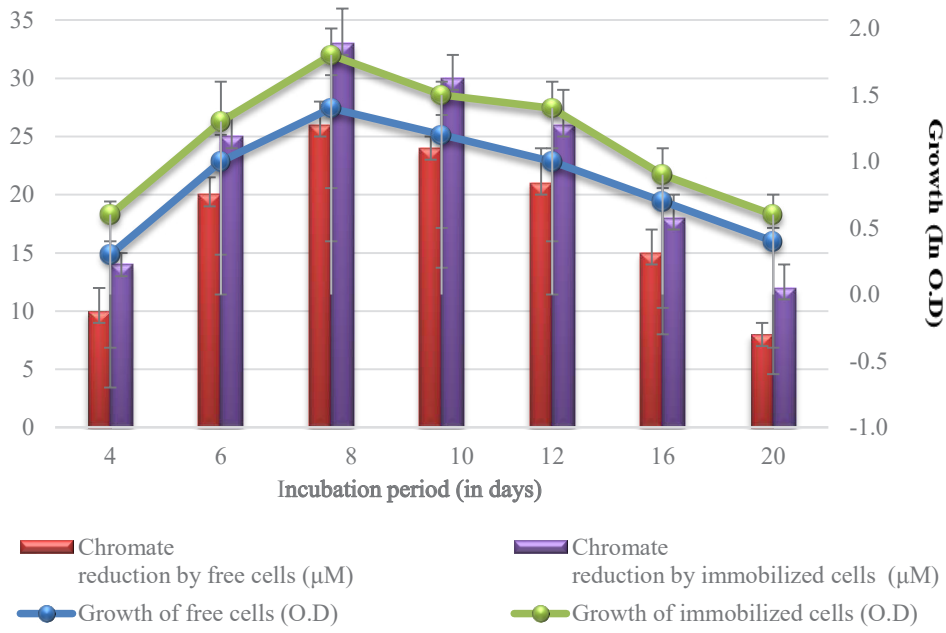

Fig. 2: Graph shows the growth in optical density and chromate reduction by free and immobilized cells of *Allochromatium* sp., in the presence of light under strictly anaerobic conditions. The obtained results are analyzed for ANOVA which are showing statistically significant at $P < 0.001$ level. The error bars in the graph indicate the mean and ± standard error noted from the triplicate samples tested in the laboratory.

The initial pH of the growth medium is changed to the alkaline side on the 8th day, which was recorded in the range of 8.5 ± 0.10 to 8.6 ± 0.20 by free cells and alginate entrapped immobilized cells of *Allochromatium* sp. on 8th day. On the other hand, the final pH was changed to alkaline in the range of 9.5 ± 0.10 to 9.8 ± 0.20 at the end of the incubation period. These investigations are identical to the investigation carried out by Ramchander *et al.* (2011), wherever he recorded the final pH at pH 8.2 (alkaline) in the growth medium containing *Rhodobacter capsulatus* KU002). Rajyalaxmi et al. (2019) studied the maximum reduction of chromate at pH 7.6 ± 0.15 by *Rhodobacter* sp., whereas Nepple et al. (2000) studied the maximum reduction of chromate at neutral pH 7.0 and temperature 30°C. During this investigation, the optimal growth conditions are directly proportional to the optimal chromate reduction by these bacteria. The statistical analysis was performed on the results obtained and the ANOVA of the obtained results revealed that the significance of the experiment is at P □ 0.001 level. A positive relationship has been recorded between the growth of the *Allochromatium* sp. as well as chromate reduction at different incubation periods.

CONCLUSION

The present investigations on chromate reduction by *Allochromatium* sp. provide the information that this bacterium is found to be very efficient in reducing chromate and to date, there is no information is available on chromate reduction by *Allochromatium* sp., hence this investigation is considered the first report on *Allochromatium* sp. However, more studies are required to expose the determination mechanism involved in chromate reduction by *Allochromatium* sp. in comparison with other anoxygenic phototrophic bacteria involved in chromate reduction. Bioremediation using *Allochromatium* sp. is cost-effective as well as environmentally best for treating the different water sources contaminated with different heavy metals.

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