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Kinetics of Perchlorate degradation by *Proteobacterium* ARJR SMBS

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Abstract : Microbial screening studies have shown that the strain *Proteobacterium* ARJR SMBS has a potential to degrade perchlorate. This microbe was isolated and the growth inhibitory kinetic studies were carried out on this strain ARJR SMBS over a perchlorate concentration range of 0 – 50 mg/L. Increasing perchlorate concentration affected the specific growth rate and degradation efficiency of the strain. Results show that 95.3% of perchlorate reduction was achieved with 9 h of incubation at an initial perchlorate concentration of 10 mg/L; whereas only 60.4% degraded at 50 mg/L perchlorate concentration. Dry weight of cells and colony forming unit analysis proved that there is growth retardation effect on this strain with respect to varying initial perchlorate concentration. Present data shows that specific growth rate of strain ARJR SMBS is highly dependent and inversely proportional to the perchlorate concentration. The study proves that the higher concentration of perchlorate exhibit mild toxicity effect toward this microbial community in the environment. A kinetic model was developed to predict the specific growth rate, cell density and perchlorate degradation rate based on the experimental data.
Key words: perchlorate, *Proteobacterium*, microbial degradation, growth rate, kinetic model.

INTRODUCTION

Perchlorate is an anthropogenic environmental pollutant with wide range of applications in explosives, chemical industries, munitions, match industries, rocket fuels etc. Since 1950s, it is estimated that more than 15.9 million kg of perchlorate has been discharged into the environment [1]. Studies have been proved that even 4 microgram per liter of perchlorate that reaches to human body prevents the iodine uptake by thyroid gland [2,3]. This causes adverse effect to human growth, normal metabolism, brain development and learning capability [4]. With this concern it is very essential to remove and/or degrade perchlorate and its related toxic effects from the environment. Even though various physical and chemical methods are available for perchlorate removal, but none of them are promising, due to the fact that complete removal or degradation is not possible. Currently biological treatment methods of degradation mechanism have been identified as the best treatment techniques for removing perchlorate from contaminated sources [3,5-7].

Published studies on the Ecotoxicity and/or inhibitory effect of perchlorate to bacterial species are limited, even though their characteristics have been reported in the literature [8]. Considering the paucity of prior literature reports on the effect of varying concentrations of perchlorate on potential perchlorate degrading bacterial species, the objective of the present work is to investigate and study the possible growth inhibitory effect and related kinetics of perchlorate over a concentration range (0-50 mg/L) on a potential perchlorate degrading bacteria, *Proteobacterium* ARJR SMBS. Recent studies proved that specialized microorganisms do coupled mechanism that can completely reduce perchlorate under anaerobic conditions to chloride [9-18].

MATERIALS AND METHODS

Sample preparation

Samples of varying concentrations (0, 10, 30 and 50 mg/L) of perchlorate were prepared and autoclaved. For better culture growth and enrichment of inoculum, inorganic mineral medium ($(NH_4)_2SO_4$, 225 mg; $MgSO_4.7H_2O$, 50 mg; K_2HPO_4, 225 mg; KH_2PO_4, 225 mg; $CaCO_3$, 5 mg; $FeSO_4.7H_2O$, 5 mg) and trace metal solution were provided as supplements. All reagent stock was prepared and autoclaved before the inoculation.

Bacterial strains

The bacterial studies were carried out with potential perchlorate degrading bacteria *Proteobacterium* ARJR SMBS (GenBank Accession No: HQ148164), which was isolated, identified and characterized through enrichment culture technique by using Luria Bertani (LB) medium (Himedia Laboratories Ltd., Mumbai, India). The same culture and nutrient medium was used and experiments were carried out in aseptic conditions. The medium pH was adjusted to 7.5 with 1.0 M NaOH or 1.0 M HCl and maintained throughout the study.

Growth curve study

Growth curve was carried out in a 250 ml batch reactor connected with air tightened rubber cork. Ports were provided on the rubber cork to allow syringe sampling and purging compressed nitrogen gas to make anoxic conditions inside the reactor. To study the growth rate of the isolates, each reactor bottles were inoculated with 1 ml cells from 24 h old mother culture which is estimated to be in the log phase of growth. Initial pH and salinity of the culture medium was adjusted and maintained at 7.5 and 0.25% respectively, which was found to be the optimum for the strain ARJR SMBS. The reactors were placed in an orbital shaker at 120 rpm. An optimum ratio of 1:2 of perchlorate to acetate has been used for the efficient perchlorate removal. The positive control of the experiment include reactors containing perchlorate, nutrient medium and without inoculum. The negative control of the experiment represent reactors containing nutrient medium, inoculum and without perchlorate. The negative control indicated the microbial growth profile in the absence of perchlorate (0 mg/L). Samples were withdrawn periodically (each 3 h) from batch reactors for bacterial growth measurement by taking optical density at 600 nm with the help of a UV-Visible spectrophotometer (AMERSHAM Biosciences, Ultraspec 1100 pro, Chennai, Tamil Nadu, India). The absorbance values for positive control were subtracted from the experimental ones which contain nutrient medium, inoculum and varying perchlorate concentrations. The experiments were carried out in triplicates and the mean value was reported.

Colony forming analysis

Samples were withdrawn periodically from all batch reactors and serially diluted (using 0.85%NaCl solution) to 10^{-6} dilution. 0.1 ml of each diluted sample was used for spread plating on Nutrient Agar medium to study colony forming units per millilitre (CFU/ml). Subsequently plates were incubated at 37°C for 24 h and colonies formed on agar pates were countered by using a colony counter (SERVELL, India). Due to the formation of large number of colonies on agar plates, 10^{-8} dilution was used for degradation at an interval of 21 and 24 hours. The experiments were carried out in triplicates and the mean value was reported.

Measurement of dry weight of cells

Samples were taken periodically from all batch reactors for dry weight of cell measurements. Initial weight of eppendorf tube was measured as weight before centrifugation (WBC). Subsequently samples were collected aseptically and cells harvested by centrifugation (REMI, ILE Co., Chennai) at 10,000 rpm for 15 min at 4ºC. Followed by cell pellet was dispersed in deionized water, washed three times and dried in hot air oven (SEREVELL, India) at 80°C until constant weight. Subsequently the final weight of eppendorf tube was measured as weight after centrifugation (WAC) and dry weight of cells were calculated by using the following formulae:

Dry weight of cells = [(A – B)/ C] x 1000 g/L

Where, A= Weight after centrifugation (WAC)

B= Weight before centrifugation (WBC)

C= Volume of sample taken

The dry weight measurement values for positive controls were subtracted from the experimental ones which contain nutrient medium, inoculum and respective perchlorate concentrations. The dry weight of cells was weighed to determine the growth of strain ARJR SMBS. The experiments were carried out in triplicates and the mean value reported.

Perchlorate Quantification

A standard operating procedure (SOP) was developed using Ion selective electrode (ISE) to analyze perchlorate in water samples [19-21]. Samples were withdrawn periodically from batch reactors for perchlorate quantification with the help of ion analyzer (Thermo Fisher Scientific Orion Star and Star Plus Series, ORION, USA), perchlorate ion selective electrode (ISE-93 series) (Thermo Fisher Scientific, Cat.No.938101, ORION, USA) and a double junction reference electrode (Thermo Fisher Scientific, Cat.No.900200, ORION, USA) [19]. For samples with

a pH above 10, adjust the reference electrode filing solution pH with NaOH to match the sample pH and for samples with pH below 2, adjust filling solution pH with HNO_3 to match the sample pH. Initial pH (adjusted with 1 M NaOH or 1 M HCl) and salinity of the culture medium was adjusted and maintained to be 7.5 and 0.25% respectively, which was found to be the optimum for strain ARJR SMBS (data not shown). Before sample analysis, the calibration of the ion analyzer was carried out by using perchlorate standards (0.1, 1.0, 10 and 100 mg/L) added with perchlorate ionic strength adjuster (ISA, Cat.No.930711, ORION, USA), which act as a constant background ionic strength for samples and standards [19]. Sample measurements were carried out with a direct calibration technique with slope value in between -54 to -60 mV which provides the best means for checking the electrode operation [19]. The degradation values for positive controls were subtracted from the experimental values. The experiments were carried out at least in triplicate to ensure reproducibility. All data were subject to average analysis and expressed as mean ± SD.

RESULTS AND DISCUSSION

Growth curve of the strain

Figure 1 shows the effect of degradation of perchlorate by *proteobacterium* at every 3 hour interval for the reactors with initial concentration of 0, 10, 30 and 50 ppm perchlorate. At a given interval the reactor with higher concentration of perchlorate shows growth retardation. As shown in figure, the growth curve of bacteria was characterized as initial lag phase (0 - 6 h), followed by exponential phase (6 – 24 h) and finally reaches to stationary-death (≥ 27 h) phase. Results show that the lag phase of strain ARJR SMBS was increasing reasonably for 50 ppm perchlorate concentration. This is due to the slow adaptation of bacteria to the new environment and the inhibitory effect of higher perchlorate concentration on the growth of the organism. The microbial growth curve shows that the acclimatization period of strain ARJR SMBS for varying perchlorate concentrations is not same even though the initial MLSS was equal. Generally, each microorganism has a specific resistance at a specific growth condition.

Dry weight of cells

The cell dry weight analysis as in **Figure 2** shows that at higher perchlorate concentration (30-50 mg/L) the microbial biomass is less and found to be mildly inhibitory for the strain ARJR SMBS. The maximum dry weight of the cell was at zero concentration of perchlorate and decreases with increase in values from 10-50 mg/L. At the end of the growth phase (21 hour) the average cell dry weight was 10.05 g/L for 50 ppm concentration and is constant. For zero concentration the cell dry weight is 15 g/L. Result showed that high concentration of perchlorate (>30 mg/L) had some inhibitory effect on cell growth. When the cells entering into log phase on the 6th and 8th hour of incubation the dry weight content was relatively increased (3.05 – 5.15 g/L). This would be the best time to inject perchlorate for the effective degradation process. The reaction mechanism of perchlorate degradation of cells is closely related with the cell cycle of bacteria.

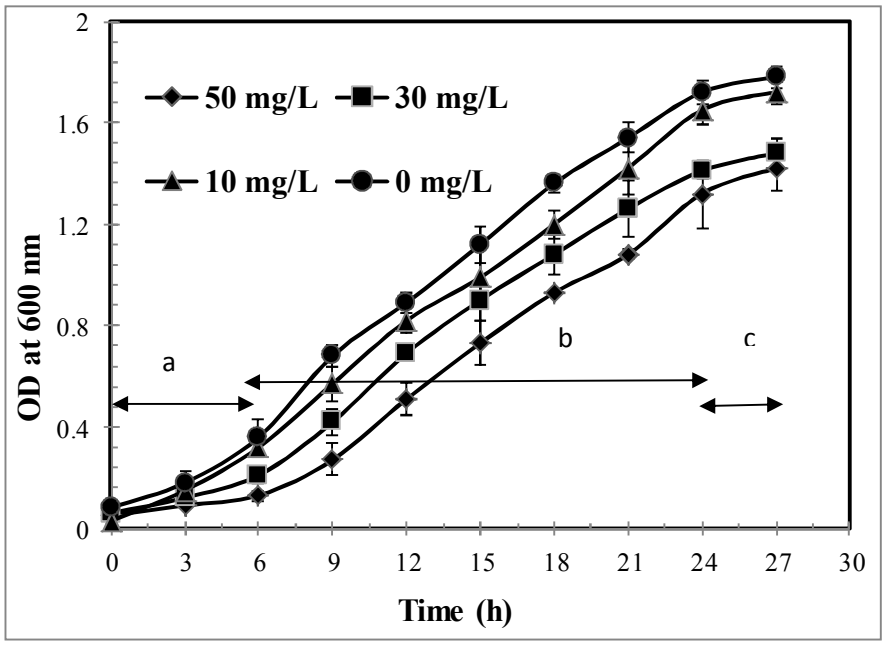

Figure 1: Growth Curve of strain ARJR SMBS at varying perchlorate (0, 10, 30 and 50 mg/L) concentrations. Error bar represent the means and standard deviation of three independent reactors. a – Lag Phase, b – exponential phase, c – stationary death phase

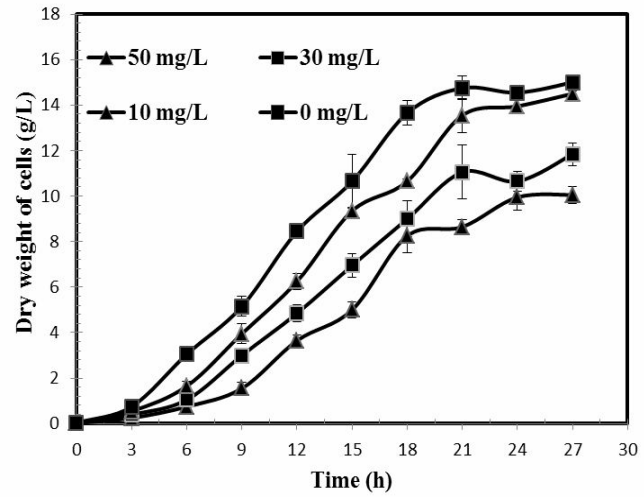

Figure 2: Dry weight of cell measurement of strain ARJR SMBS at varying perchlorate (0, 10, 30 and 50 mg/L) concentrations. Error bar represent ± one standard deviation for three replicate reactors

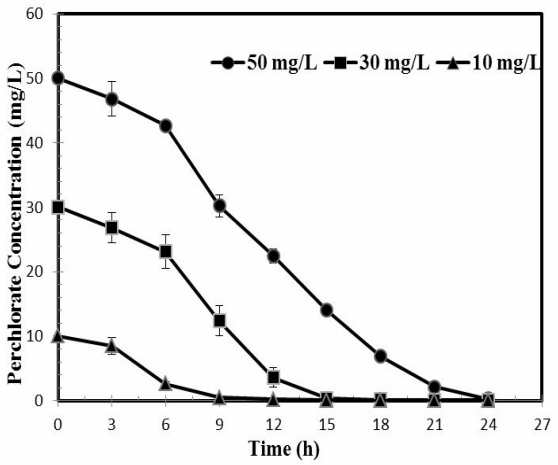

Figure 3: Perchlorate degradation curve at different initial pechlorate (50, 30 and 10 mg/L) concentartions. Error bar represent the means and standard deviation of three independent reactors.

Perchlorate degradation study

Degradation curve infers that three different stages of degradation include an initial slow rate followed by a rapid stage and finally degradation at a much slower rate. The initial slow rate due to the slow adaptation of consortia to the varying perchlorate concentration and then further in the second stage microbial consortia was able to withstand under stress conditions and it shows a linear degradation rate, subsequently it reaches to a point at which maximum degradation occurs and further degradation efficiency would be constant and/or reduced, represented as the third stage. Around 95.3% perchlorate reduction was achieved within 9 h of incubation with an initial perchlorate concentration of 10 mg/L; whereas only 60.4% perchlorate degraded at 50 mg/L concentration causes growth retardation effect on bacteria. However the degradation efficiency was moderate at 30 mg/L concentration as shown in Figure 3.

CFU study

The number of colonies formed on petri plates after the incubation period was measured by a colony counter and Log (CFU/ml) was calculated as shown in Table 1. The number of colonies formed at 0^{th} hour of incubation of strain ARJR SMBS was found to be $1x10^7$, $5x10^7$, $5x10^7$ and $11x10^7$ for 50, 30, 10 and 0 mg/L of perchlorate concentrations respectively. Results shows that as the perchlorate concentartion increases the number of colonies formed on agar plates decreased continiousily.

Table 1: Colony Forming Units per mililiter analysis of strain ARJR SMBS.

Time (h)	A	B	C	D
0	7	7.7	7.7	8
3	7.9	8.1	8.3	8.4
6	8.2	8.3	8.5	8.5
9	8.3	8.5	8.6	8.6
12	8.5	8.6	8.7	8.7
15	8.6	8.7	8.8	8.8
18	8.7	8.8	8.9	8.9
21	8.7	8.9	9.1	9.1

Bacterial growth kinetic study in the presence of varying concentrations of perchlorate

The dynamic growth rate of a bacterial species in the logarithmic phase is represented by the following equation:

$$\ln N = \ln N_0 + \mu t \qquad \text{.....(1)}$$

Where N_0 is the initial cell count, N is the bacterial cell count at time t and μ is the growth rate constant for the bacteria. From the growth curve as shown in Figure 1, the logarithmic phase was identified between 6 and 24 hours of incubation at different concentrations of perchlorate. The growth data in this time interval was plotted as shown in **Figure 4** from which the values of growth rate (μ) corresponding to various doses of perchlorate (x) were calculated. The values are found to be μ_1 = 0.0333 for 10 mg/L, μ_2=0.0369 for 30 mg/L and μ_3=0.0393 for 50 mg/L respectively.

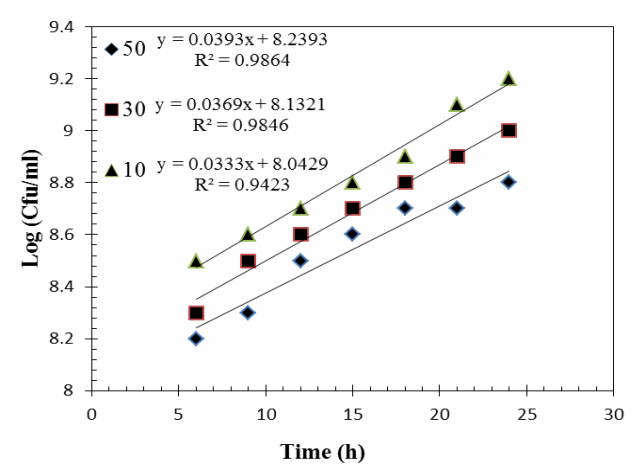

Figure 4: Cell growth rate of strain ARJR SMBS at varying concentration of perchlorate (50, 30, 10 and 0 mg/L). The following μ values μ_1=0.0333 for 10 mg/L, μ_2=0.0369 for 30 mg/L and μ_3=0.0393 for 50 mg/L were obtained. Cfu, Colony forming units.

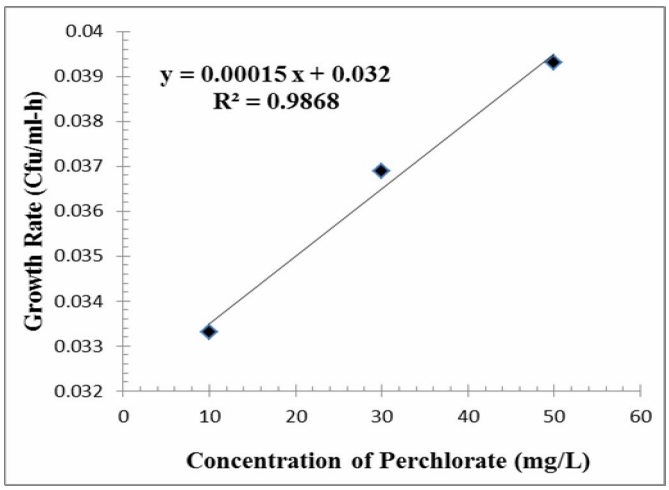

Figure 5: Dynamic cell growth rate (μ) of *Proteo - bacterium* ARJR SMBS Vs perchlorate (x) concentrations (10, 30 and 50 mg/L).

A linear relationship between this μ and x was obtained as shown in Figure 5:

$$\mu = \alpha x + b \qquad \text{……..(2)}$$

This correlation infers that value of α signifies the growth rate on the concentration of the perchlorate. The growth rate at 50 ppm is comparatively higher when compared to 30 and 10 ppm concentrations, within the log phase. The μ is positively correlated with concentration of the perchlorate. As perchlorate concentration increases the cell growth rate increases continuously and is given by the relation with ± 2.5% deviation as,

$$\mu = 1.5 \times 10^{-4} x + 0.032 \qquad \text{…….(3)}$$

The observed relationship between growth rate (μ) and perchlorate concentration (x) proves that the perchlorate concentration and biomass level are the key factors in the sensitivity of bacteria in the biodegradation mechanism. From the analysis of the batch study it is profound that the parameters like acetate concentration, pH, salinity and anoxic environment are also the important factors that decide the perchlorate degradation mechanism by the strain ARJRSMBS. Even though the microbial growth retardation was observed by increasing concentration of perchlorate as shown in Figure 1, the bacterial growth rate (μ) shows positive correlation with perchlorate concentration (x) (Figure 5). This is due to the inhibitory effect of perchlorate on the growth mechanism of bacteria which is predominant at higher concentrations of perchlorate with respect to a fixed amount of initial inoculum injected in all batch reactors. The lack of enzyme production (perchlorate reductase and chlorite dismutase) with respect to increase in perchlorate concentration in the culture medium causes a negative impact on microbial degradation. The mechanism of action involves protons (H^+) from the aqueous solution combined with oxygen deducted from perchlorate anion (ClO_4^-) to form water with the help of perchlorate reductase and subsequently by the action of chlorate dismutase further reduced and accumulated as chloride [22]. The biochemical characterization results shows that strain ARJR SMBS have nitrate reducing capacity. This is another reason that if nitrate was present in the culture medium that would leads to act as a protonophore. The inhibitory effect of nitrate in perchlorate reduction is due to the toxic effect of accumulating nitrite (as a protonophore) in the medium rather than competition with nitrate act as electron donor [23-25].

CONCLUSION

The laboratory scale study was performed to find out the effect due to varying perchlorate concentrations on a potential important microorganism *Proteobacterium* ARJR SMBS isolated through enrichment culture technique from a perchlorate contaminated site. The hypothesis in this study proves that perchlorate at the tested concentrations inhibit growth of the test microorganism under defined experimental conditions. The strain has mild growth-inhibitory effect at perchlorate concentration in the range 10-50 mg/L and it is evident that the time required for perchlorate

degradation varies with initial perchlorate concentration.

The environmental behaviour may be more complex in nature depending upon physicochemical interactions of the medium components or presence of microbial consortia and other environmental factors effecting the growth of bacteria. The growth inhibitory effects is significant at high concentrations (>30 mg/L). This concentration-dependent negative effect on strain may be overcome by slow adaptation to stress environmental conditions and involvement of new metabolic pathways in the microbial degradation mechanism. The above findings on this bacterium are potentially useful for the development of perchlorate remediation systems.

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