Microbial growth and degradation kinetics of perchlorate by *Proteus* sp. LMNCRE in a batch reactor system

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Abstract: The present work is a laboratory-scale study of perchlorate (ClO_4^-) degradation using *Proteus* sp. LMNCRE in a batch reactor. The average hourly value of perchlorate degradation rate in this system was 1.95 mg/L at optimum pH 7.5 and 0.25% NaCl salinity. The microbial growth and degradation kinetics have been studied to a variety of ClO_4^- levels (0 to 100 mg/L) under anoxic conditions. The biomass optical-density value of strain gradually increased from 0–0.37 during the operating period of 30h. The perchlorate level in which the bacterium could withstand for effective removal was found to be 75 ± 2.0 mg/L with zero order reaction coefficient (k) of 2.08 mg/L/h. The observed anoxic degradation rate of strain was 87.0 ± 0.6 % within 12 h of incubation period at 25 mg/L of initial perchlorate level. From the experimental findings, the uptake of perchlorate by the bacterium is suggested to obey a zero-order substrate-utilising kinetic model, allowed the growth rates constants to be determined.

Keywords: kinetic model; LMNCRE; perchlorate; *Proteus* sp. microbial growth; zero-order.

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1 Introduction

Perchlorate (ClO_4^-) is a highly water soluble anion and an anthropogenic environmental pollutant that poses threat to human health and other indigenous species throughout the environment. It is used in explosives, munitions, chemical industries, match industries, rocket fuels etc. Since the 1950s, it is estimated that more than 15.9 million kg of perchlorate has been discharged into the environment both by natural and manmade activities and its removal from the environment is of great importance (Motzer, 2001). The major health concern coupled with ClO_4^- is that even 4 µg/L of perchlorate that reaches to the body prevents iodine uptake by the thyroid gland in humans and animals (Urbansky and Schock, 1999). This has an adverse effect on human growth, brain development, and normal metabolism. It can also cause fatal bone marrow disorders and may lead to decreased learning capabilities in new-borns (Blount et al., 2009). Therefore, it is essential to remove or degrade perchlorate from the environment. Even though various physical and chemical methods are available for perchlorate removal, complete removal or degradation is not possible. At present biological treatment method of degradation has been identified as one of the best treatment technique for the removal perchlorate from contaminated sources (Srinivasan and Sorial, 2009).

The published studies on the ecotoxicity and the inhibitory effect of perchlorate on the bacterial species are limited. Considering the lack 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 the possible growth effect and its perchlorate removal kinetics mechanism over a concentration range of 0 to 100 mg/L perchlorate by using a potential perchlorate degrading bacteria, *Proteus* sp. LMNCRE in synthetic effluent. Bioremediation of ClO_4^- under anaerobic conditions offers an efficient technological solution as it can be eliminated in an eco-friendly manner. The present study demonstrates effective degradation of ClO_4^- under nitrogen

inert anaerobic, acetate fed conditions in a batch bioreactor system. Recent studies showed that specific microorganisms known as perchlorate reducing bacteria (PRB) do coupled mechanism that could reduce perchlorate under anaerobic conditions to the harmless chloride (Coates, 2003).

2 Materials and methods

2.1 Bacterium identification and characterisation

Perchlorate degrading bacterium *Proteus* sp. LMNCRE (GenBank accession no: JQ695940) originally isolated from an industrial effluent by enrichment culture technique by using Luria Bertani (LB) medium (Himedia Laboratories Ltd., Mumbai, India) is used in this study. Microbial screening studies reveal that, strain LMNCRE showed a reasonable perchlorate degradation activity in the growth medium compared to other strains isolated from industrial effluent, hence we have chosen this particular species. 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 N NaOH or 1.0 N HCl. After five successful transfers are done in a petri dish to achieve the required purity, the efficient bacterial isolates were screened for further studies in a batch reactor system (250 ml volume). The selected bacterial isolates were sub-cultured for further Gram staining and morphological studies. The biochemical characterisation and nitrate reducing property of isolate was tested by using the rapid identification kit (KB002 HiAssortedTM, HiMedia, Mumbai) by providing the necessary nutrients, acetate as a carbon source and perchlorate as the electron acceptor which activates the bacterial growth in the culture medium under stable environmental conditions.

2.2 Synthetic effluent preparation and growth media

The media was prepared using ultrapure water (Milli-Q system; Millipore Corp., New Bedford, MA) and research-grade chemicals were used in this study. Samples of varying concentrations (0, 25, 50, 75 and 100 mg/L) of ClO_4^- were prepared and autoclaved for better culture growth and enrichment of inoculum, inorganic mineral medium ($(NH_4)_2SO_4$, 225 mg; $MgSO_4 \cdot 7H_2O$, 50 mg; K_2HPO_4, 225 mg; KH_2PO_4, 225 mg; $CaCO_3$, 5 mg; $FeSO_4 \cdot 7H_2O$, 5 mg) and trace metal solution was added as a supplement in the synthetic effluent. At most care was taken while preparing the reagents stock which were autoclaved before the inoculation. The acetate was used as a carbon source in this study. Therefore, the acetate acts as the electron donor and perchlorate acts as the electron acceptor. The pH of the reaction medium was adjusted to 7.5 using 1 N NaOH mixed with a nutrient broth at 37°C.

2.3 Batch bioreactor system setup and operation

A laboratory scale batch reactor with a total volume of 250 ml is used in this study (Anoop Raj and Muruganandam, 2012). The reactor system was connected with air tightened rubber cork and the necessary ports were provided on the rubber cork to allow syringe sampling and purging compressed nitrogen gas. The bioreactor system contains,

medium components, other than the catalytic agents, dissolved nitrogen etc. that function cooperatively with *Proteus* sp. LMNCRE. The pH in batch reactor was adjusted using 1 N HCl or 1 N NaOH solution. The entire content in each bottle was deaerated by sparging the compressed nitrogen from a nitrogen cylinder for nearly 5 minutes to make it anoxic in nature and incubated under the room temperature. The samples were collected from each reactor at regular intervals (every 3 h); vacuum filtered and then the perchlorate quantity was measured by perchlorate ion specific electrodes. The experiment was repeated in triplicates and the mean value reported.

2.4 Growth curve study and colony forming analysis

To study the growth rate of the isolates, each reactor bottle was inoculated with 1 ml cells from 21 h old mother culture which was estimated to be in the log phase of growth. The initial pH and salinity of the culture medium was adjusted and maintained at 7.5 and 0.25% respectively. The reactors were placed in an orbital shaker at 80 rpm. An optimum ratio of 1:2 of perchlorate to acetate was used for the efficient perchlorate removal. To nullify the experimental errors, two controls were used in this study. The 'control-A' of the experiment included the reactors containing perchlorate, nutrient medium and without inoculum. The 'control-B' of the experiment represented batch reactors containing the nutrient medium, inoculum without the perchlorate. The 'control-B' indicated the microbial growth profile in the absence of perchlorate (0 mg/L). Samples were withdrawn periodically (every 3 h) from the batch reactors for bacterial growth measurement by taking the 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 'control-A' were subtracted from the experimental reactor bottles which contained the nutrient medium, inoculum and varying perchlorate concentrations. The experiments were carried out in triplicates and the mean value was reported.

In parallel, 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 the nutrient Agar medium to study the colony forming units per millilitre (CFU/ml). Subsequently the plates were incubated at 37°C for 24 h and colonies formed on agar plates were countered upon by using a colony counter (SERVELL, India). The experiments were carried out in triplicates and the mean value was reported.

2.5 Perchlorate quantification

A standard operating procedure was developed using the ion selective electrode (ISE) to analyse perchlorate in water samples (Anoop Raj and Muruganandam, 2013). Samples were withdrawn periodically from batch reactors for perchlorate quantification with the help of the ion analyser, perchlorate ion selective electrode (ISE-93 series) and a double junction reference electrode (Thermo Fisher Scientific, ORION, USA). Before the sample analysis, the calibration of the ion analyser was carried out by using the perchlorate standards (0.1, 1.0, 10 and 100 mg/L) added with the perchlorate ionic strength adjuster, which act as a constant background ionic strength for samples and standards (Anoop Raj and Muruganandam, 2013). The degradation values for 'control-A' were subtracted from the experimental values to overcome the possible loss of biomass

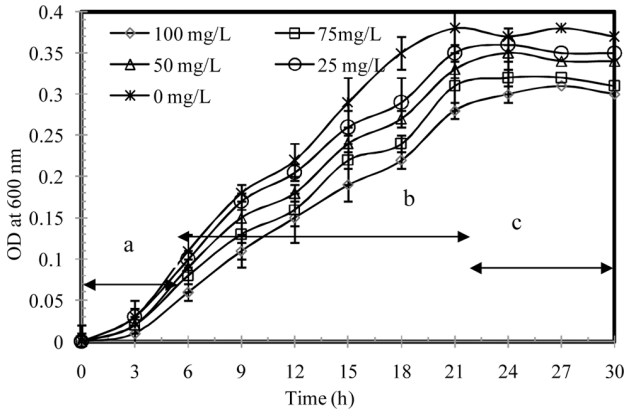
concentration and the sample volume, we have conducted the batch experiments with 12 to 15 replicas for each sample of perchlorate concentration study. The above experimental procedure would nullify the false results which may arise due to the changes in the sample volume during the ISE based analysis. All data were subject to average analysis and were expressed as mean ± SD. In each set of the repeated experiments, standard deviations and standard errors showed 95% confidence interval.

3 Results and discussion

3.1 Microbial characterisation and growth curve of strain LMNCRE

The strain LMNCRE colonies grown on the petri dish within the range of 1.2 to 1.6 mm size were filamentous, lobate and with time they tend to rapidly spread on the surface of the agar with pale green colour. The colony morphology and the biochemical characterisation showed that, strain LMNCRE is Gram negative, rod shaped and a nitrate reducer. The biomass optical-density value of the strain gradually increased from 0 to 0.37 during the operating period of 30 hours. Overall, the observed OD values were very less which indicates the growth of organism which was not efficient. It speculates that the lower OD values may be due to strain LMNCRE exhibited in different metabolic characteristics within the medium, which warrants a further scientific study. Figure 1 shows the microbial growth profile of *Proteus* sp. LMNCRE at varying initial concentration of perchlorate (0, 25, 50, 75 and 100 mg/L). At a given interval the reactor with a higher concentration of perchlorate shows growth retardation.

Figure 1 Growth curve of strain LMNCRE at varying perchlorate (0, 25, 50, 75 and 100 mg/L) concentrations



Note: Error bar represent the means and standard deviation of three independent reactors. a - Lag Phase, b - exponential phase, c - stationary death phase

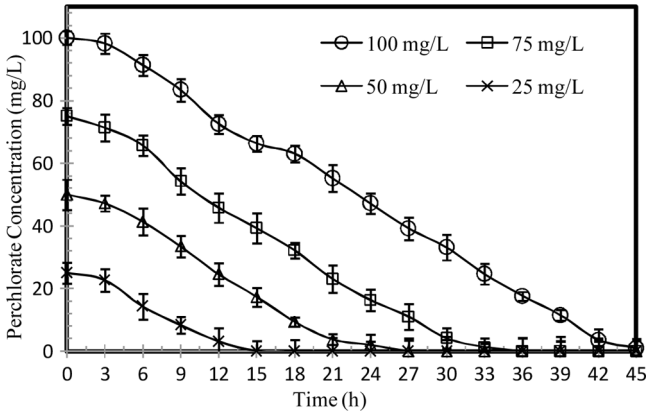
As shown in the figure, the growth curve of bacteria was characterised as an initial lag phase (0–6 h), followed by an exponential phase (6–21 h) and finally reaches to stationary-death (≥ 21 h) phase. Results show that the lag phase of strain LMNCRE was increasing reasonably in the reactors treated with 75 mg/L of initial perchlorate

concentration. This is may be due to the slow adaptation of bacteria to the reactor's environment and the inhibitory effect of the perchlorate level in the medium. The microbial growth curve shows that the acclimatisation period of strain LMNCRE for varying perchlorate concentrations is not same even though the initial MLSS injected into the reactors were same. The above growth pattern of the strain LMNCRE reveals that, the microbial growth and system efficiency was primarily dependent upon the inlet perchlorate concentration.

3.2 Perchlorate degradation study and CFU analysis

Degradation curve infers that the three different stages of degradation included an initial slow rate followed by a rapid stage and finally a degradation at a much slower rate. The initial slow rate due to the slow adaptation of bacteria to the varying perchlorate concentration and then further in the second stage the microbial consortia was able to withstand under the stress conditions and it shows a linear degradation rate, subsequently it reaches a point at which the maximum degradation occurs and further the degradation efficiency would be constant and/or reduced, is represented as the third stage as depicted in Figure 2. Results showed that 99.8% of perchlorate reduction was observed within 15 h of incubation for an initial perchlorate level of 25 mg/L in the media. Whereas there was a considerable reduction in perchlorate removal (33.7%) were observed at 100 mg/L perchlorate concentration with zero-order reaction coefficient (k) of 2.2 mg/L/h. However the degradation efficiency was moderate at 50 mg/L concentration as shown in Figure 2. Overall, the average hourly value of perchlorate degradation rate in the reactor system was 1.95 mg/L at optimum pH 7.5 and 0.25% NaCl salinity. However, the perchlorate level in which the bacterium could withstand for effective removal was found to be 75 ± 2.0 mg/L with zero order reaction coefficient (k) of 2.08 mg/L/h. The observed anoxic degradation rate of strain was 87.0 ± 0.6 % within 12 h of incubation period at 25 mg/L of initial perchlorate level.

Figure 2 Perchlorate degradation curve at different initial pechlorate (100, 75, 50 and 25 mg/L) concentrations



Note: Error bar represent the means and standard deviation of three independent reactors.

The number of colonies formed on petri plates after the incubation period was measured by a colony counter and the Log (CFU/ml) was calculated as shown in Table 1. Results show that as the perchlorate concentration increases and the number of colonies formed on the agar plates decreased continuously.

Table 1 Colony forming units per mililiter analysis of strain LMNCRE

Time (h)	*A*	*B*	*C*	*D*	*E*
0	7	7.2	7.2	7.3	7.3
3	7.2	7.3	7.5	7.6	7.7
6	7.4	7.6	8.0	8.4	8.5
9	7.6	7.9	8.4	8.8	9.0
12	7.8	8.1	8.8	9.3	9.5
15	7.9	8.3	9.0	9.6	9.8
18	8.0	8.4	9.2	9.9	10.3
21	8.2	8.5	9.4	10.2	10.5
24	8.3	8.5	9.5	10.3	10.6
27	8.4	8.6	9.6	10.3	10.7
30	8.4	8.6	9.6	10.4	10.7

Note: A,B,C, D and E represents Log (CFU/ml) of strain LMNCRE at 100, 75, 50, 25 and 0 mg/L perchlorate concentration respectively.

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

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

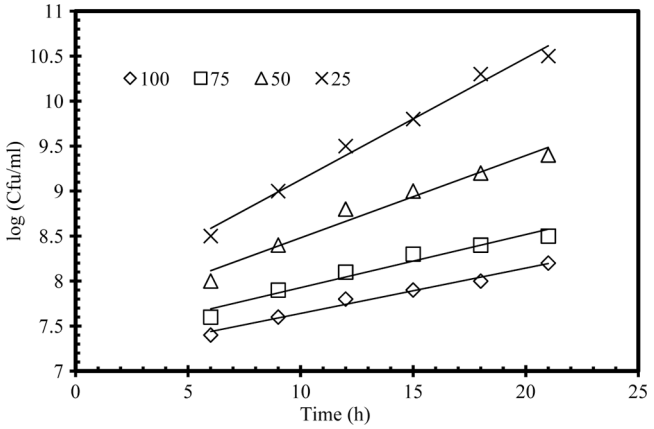
$$\ln N = \ln N_0 + \mu t \tag{1}$$

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 (Sadiq et al., 2009), from the growth curve as shown in Figure 1, the logarithmic phase was identified between 6 and 21 hours of incubation at different concentrations of perchlorate. The growth data in this time interval was plotted as shown in Figure 3 from which the values of growth rate (μ) corresponding to various doses of perchlorate (x) were calculated. From the experimental findings, the uptake of perchlorate by the bacterium is suggested to obey a zero-order substrate-utilising kinetic model, allowed the growth rate constants to be determined. The values are found to be $\mu_1 = 0.0505\ h^{-1}$ for 100 mg/L, $\mu_2 = 0.059\ h^{-1}$ for 75 mg/L $\mu_3 = 0.0914\ h^{-1}$ for 50 mg/L $\mu_4 = 0.1352\ h^{-1}$ for 25 mg/L respectively.

A kinetic relationship was developed to predict the microbial growth rate (μ), biomass concentration and perchlorate level (x) based on the experimental values. A linear relationship between μ and x was obtained as shown in Figure 4:

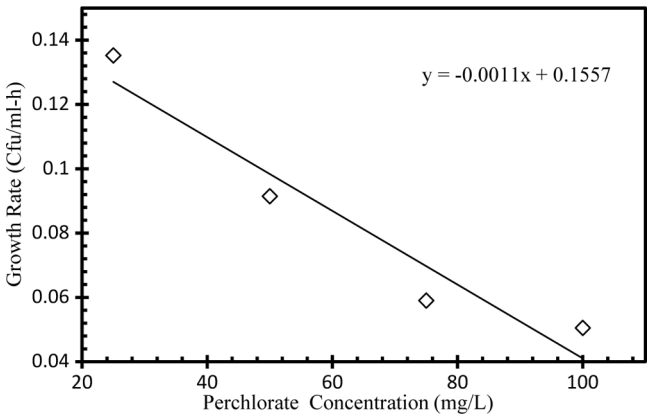
$$\mu = \alpha x + b \tag{2}$$

Figure 3 Cell growth rate of strain LMNCRE at varying concentration of perchlorate (100, 75, 50 and 25 mg/L)



Note: The following μ values $\mu_1 = 0.0505$ h^{-1} for 100 mg/L $\mu_2 = 0.059$ h^{-1} for 75 mg/L $\mu_3 = 0.0914$ h^{-1} for 50 mg/L and $\mu_4 = 0.1352$ h^{-1} for 25 mg/L were obtained.

Figure 4 Dynamic cell growth rate (μ) of *Proteus* sp. LMNCRE vs perchlorate (x) concentrations (100, 75, 50 and 25 mg/L)



This correlation infers that value of 'α' signifies the growth rate on the concentration of the perchlorate (Sadiq et al., 2009). The growth rate at 100 mg/L is comparatively lower when compared to 75 and 50 mg/L concentrations, within the log phase. The 'μ' is negatively correlated with a concentration of the perchlorate. As the perchlorate concentration increases the cell growth rate decreases continuously and is given by the relation with ±2.5% deviation as,

$$\mu = -1.1\times10^{-3}x + 0.1557 \tag{3}$$

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 the important factors that decide the perchlorate degradation mechanism. Even though the microbial growth retardation was observed by increasing the concentration of perchlorate as depicted in Figure 1, the bacterial growth rate (μ) shows negative correlation with the perchlorate concentration (x) (Figure 4). This is due to the inhibitory effect of perchlorate on the growth mechanism of bacteria which is predominant at higher concentrations of perchlorate irrespective of the initial amount of inoculum injected was constant.

We speculate that, the lack of enzyme production (perchlorate reductase and chlorite dismutase) with respect to increase in perchlorate concentration in the culture medium may be another reason which causes a negative impact on the microbial degradation. The mechanism of action involves 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 (O'Conor and Coates, 2002). The biochemical characterisation results of *Proteus* sp. LMNCRE reveals that, the test organism is having nitrate reducing property. This is due to the presence of $(NH_4)_2SO_4$ in the growth medium, which may lead to the release of nitrate in the culture medium and which could act as a protonophore. The previous studies reported that, if nitrate is present acting as an inhibitor in the perchlorate removal mechanism (Chaudhuri et al., 2002). The inhibitory effect of nitrate in perchlorate reduction is due to the toxic effect of the accumulating nitrite in the medium rather than the competition with the nitrate acting as an electron donor.

4 Conclusions

The application of a batch bioreactor system for the removal of an anthropogenic environmental pollutant called perchlorate is demonstrated in this study. The hypothesis in this study proves that perchlorate at the tested concentrations (0–100 mg/L) inhibits growth of the test microorganism under the defined experimental conditions. Addition of degradable substrate like acetate is required to enhance perchlorate reduction in the system, where the process mechanism is dependent upon the amount of initial inoculum and varying ClO_4^- concentrations used in the bioreactor. Kinetic analysis revealed that, zero order substrate-utilising the kinetic model was found to be reasonably fit the entire degradation data with a high degree of accuracy. The strain has a higher growth-inhibitory effect at ClO_4^- concentration more than 75 mg/L. The overall results revealed that, a good potential of *Proteus* sp. LMNCRE can be employed for perchlorate degradation in a bioreactor system with an average degradation rate of 1.95 mg/L/h. The environmental behaviour is more complex in nature which depends upon the environmental factors which affect the growth of bacteria, medium components and the presence of an active microbial population inside the reactor. Therefore, the concentration-dependent negative effect on strain may overcome by a slow adaptation under the stable environmental conditions. In future, using strain LMNCRE in an immobilised cell form could be employed in a bioreactor system for the perchlorate removal from contaminated water.

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