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Perchlorate degradation using an indigenous microbial consortium predominantly *Burkholderia* sp.

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ABSTRACT

An acclimatized mixed microbial consortium, predominantly *Burkholderia* sp., was isolated from an activated sludge and investigated for its potential to degrade perchlorate in batch shake flasks. The 16S rDNA analysis of the predominant strain in the mixed culture showed the closest homology (98%) with *Burkholderia* sp. ATSB16. For the first time mixed culture with predominantly *Burkholderia* sp., has been reported to be involved in perchlorate degradation. The substrate perchlorate was completely utilized within 10 days even at a high concentration of 1000 mg L^{-1} utilizing succinate as the sole carbon-source. Compared to other carbon-sources tested in this study, succinate proved to be better for perchlorate degradation by the mixed consortium. The optimum conditions for perchlorate degradation by the enriched mixed culture were found to be 30 °C and pH 7.0. The effect of co-pollutants on perchlorate removal by the mixed culture was also investigated at a mixed perchlorate concentration of 500 mg L^{-1}. Results showed that the degradation of perchlorate was affected to different extent due to the presence of an equal concentration (500 mg L^{-1} of each) of co-pollutants such as nitrate, nitrite, chlorate and phosphate.

1. Introduction

Perchlorate (ClO_4^-) contamination has been a significant concern in surface and ground waters for last two decades. Major sources of perchlorate contamination include solid propellants for rocket fuel, missiles, road flares and fireworks [1,2]. It has recently been added to the drinking water candidate contaminant list (CCL) by the United States Environmental Protection Agency (USEPA) [3] as it poses threat to indigenous wildlife as well as human health [4,5].

The perchlorate ion (ClO_4^-) is nonvolatile, highly soluble and very stable in the aqueous phase. The use of perchlorate-contaminated water interferes with iodine uptake by thyroid gland, which affects several vital body functions. Several studies indicate that low concentrations of perchlorate significantly inhibit iodide uptake in humans and animals; higher doses have been found to cause fatal bone marrow disorders [6]. In 1992, the U.S. Environmental Protection Agency reviewed the various health effects of perchlorate administered to patients with hyperthyroidism and found that doses of 6 mg kg^{-1} per day or more over a period of 2-months resulted in fatal bone marrow changes [7].

Perchlorate removal from contaminated water can be achieved both by physico-chemical and biological processes. Ion exchange is used as one of the effective methods for its removal, but it is an incomplete process because it is non-selective and separates only perchlorate from the contaminated sources [8]. Therefore, due to the high affinity of perchlorate for the resins, very high salt concentrations (7–12%) are needed to regenerate the whole column. Under such conditions, bioremediation offers an efficient technological solution as perchlorate can be eliminated in an environment-friendly manner [9].

It has been recognized over the years that microbial reduction of chlorine oxyanions under anaerobic conditions is possible [10]. Perchlorate is used as an electron acceptor by pure and mixed microbial cultures [11,12]. Therefore, biological reduction is a promising treatment approach as perchlorate can be reduced to chloride by dissimilatory (per)chlorate-reducing bacteria (PRB) [13]. Hackenthal et al. proposed the perchlorate reduction pathway as follows [12]:

$$ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2 \quad (1)$$

The reduction of perchlorate (ClO_4^-) to chlorate (ClO_3^-) and therefore to chloride (Cl^-) by bacteria has been confirmed by other researchers [10,14,15] and none of the intermediates has been reported to be accumulated in aqueous solution [16–18]. Chlorite (ClO_2^-) disproportionation to chloride and oxygen is a non-energy

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yielding step catalyzed by the enzyme chlorite dismutase [10,15]. All PRB are capable of dissimilatory reduction of chlorate, whereas many of these can also reduce nitrate.

The present study demonstrates effective degradation of perchlorate by an enriched mixed consortium isolated from a sewage treatment plant. Phylogenetic analysis of the predominant perchlorate degrading strain in the mixed consortium was also performed. In addition, the optimal environmental conditions and the effect of co-pollutants on perchlorate reduction by the mixed consortium were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents used in the study were of analytical grade, and inorganic salts used in preparing microbial growth media were of reagent grade. Sodium perchlorate ($NaClO_4{\cdot}H_2O$), procured from Merck, India, was used as the source of ClO_4^- in all the experiments. All the other chemicals used in this study were purchased from Merck, India.

2.2. Enrichment, isolation and identification of the mixed consortium

An indigenous mixed microbial culture, potent to degrade perchlorate, was collected and enriched from a sewage treatment plant located in Guwahati, India. To enhance the capacity of the mixed culture to degrade perchlorate, 0.5 L sludge with a microbial count of $15 \times 10^4 \pm 0.02 \times 10^4$ cfu mL^{-1} from the treatment plant was added with 1 L medium containing 1 g L^{-1} perchlorate (with low initial concentration of chloride as given by Kim and Logan [19]) and incubated at 28 °C in an incubator. The medium used for growing the mixed culture contained (per liter of deionized water): 1555 mg K_2HPO_4, 850 mg NaH_2PO_4, 500 mg $NH_4H_2PO_4$, 100 mg $MgSO_4{\cdot}7H_2O$, 5 mg $FeSO_4{\cdot}7H_2O$, 0.2 mg $Na_2MoO_4{\cdot}2H_2O$, 10 mg EDTA and trace minerals: 2 mg $ZnSO_4{\cdot}7H_2O$, 1 mg $MnCl_2{\cdot}2H_2O$, 1 mg $CaCl_2{\cdot}2H_2O$, 0.2 mg $CuSO_4{\cdot}5H_2O$, 0.4 mg $CoCl_2{\cdot}6H_2O$, pH 7.0. Initial pH of the media was adjusted by adding required amounts of 0.1 M HCl and 0.1 M NaOH solutions. A magnetic agitator was used to stir the suspension for 30 min every day, and 100 mL of the spent medium was replaced with fresh medium. To maintain anaerobic condition in the culture flask, oxygen free nitrogen was purged at regular intervals. The culture was then acclimatized, over a period of one and half month, to degrade perchlorate starting from 200 mg L^{-1} up to a concentration of 1000 mg L^{-1}. Following the enrichment procedure, which took about one and a half months, 1% of the microbial culture was subcultured twice using a fresh medium for isolating the predominant strain responsible for perchlorate degradation. Preliminary examination of the mixed culture under light microscope revealed presence of rod shaped bacteria. The culture was also plated on a solid medium and incubated in an anaerobic jar containing the media with 1.5 g L^{-1} agar. The isolated pure bacterial strain, which was named AG, was sent to Genie (Bangalore, India) for 16S rDNA sequence analysis, and later the result was submitted to GenBank database to carry out similarity search for nucleotides by online BLAST tool (http://www.ncbi.nlm.nih.gov). The neighbor-joining phylogenetic tree was constructed and bootstrapped (1000 iterations) using Robust Phylogenetic Analysis for the Non-Specialist [20] to represent the relationship between the perchlorate degrading strain AG (HM104637) and related genera. The thermodynamic properties of the strain which was identified to be *Burkholderia* sp. culture were also determined by using the online tool BIOTOOL (http://www.unc.edu/~cail/biotool/oligo/index.html) [21].

2.3. Influence of different parameters affecting perchlorate removal

Most of the contaminated sites have more than one pollutant such as oxyanions of chlorine, and sulfur that can serve as electron acceptors during bioremediation. Therefore, several parameters affecting perchlorate removal were investigated through batch experiments, including the effect of different carbon sources, incubation temperature, pH and different electron acceptors. All experiments in this study were performed in triplicate sets of 150 mL Erlenmeyer flasks containing 100 mL of media containing perchlorate.

2.3.1. Effect of different organic acids as sole carbon source

The potential of the enriched mixed consortium to utilize different organic acids such as succinic acid, acetic acid, oxalic acid, formic acid and citric acid as the sole carbon source in degrading perchlorate was analyzed. Each of the carbon sources was added at an initial concentration of 500 mg L^{-1} with 500 mg L^{-1} of initial perchlorate. About 5% v/v of the enriched culture was added as the inoculums in these experiments. The influence of different concentrations of succinate as the sole carbon source on the perchlorate removal efficiency by the mixed consortium was also studied in the range 300–1000 mg L^{-1}. In all these experiments the pH of the media was set to 7.0 and temperature was maintained at 28 °C.

2.3.2. Effect of temperature and pH

To investigate the effect of pH on perchlorate degradation by the mixed consortium, media containing 800 mg L^{-1} of perchlorate and 1 g L^{-1} of succinate were adjusted to different pH ranging from 5.0 to 9.0 and at 28 °C. The pH was adjusted by adding required quantity of 1 M HCl and 1 M NaOH. Similarly, effect of temperature on the reduction of perchlorate was investigated at five different values ranging from 20 °C to 40 °C at pH 7 of the media. The results were fitted to the following first order rate equation and perchlorate degradation rate constants (k_d) estimated for different pH and temperature conditions in the study:

$$\frac{dC}{dt} = -k_d C \tag{2}$$

where C_0 is the initial concentration of perchlorate, C is the concentration at time t. The perchlorate degradation rate constant k_d was obtained using the linearized form of the rate equation as shown below:

$$\ln(C_0 - C) = -k_d t \tag{3}$$

2.3.3. Effect of different co-anions

The capability of the consortium to utilize perchlorate (ClO_4^-) in the presence of other competing anions (which act as alternative electron acceptors) like, nitrate (NO_3^-), nitrite (NO_2^-), chlorate (ClO_3^-) and phosphate (PO_4^{3-}) was also analyzed by supplying the co-anions both individually and in mixture at equal concentration of 500 mg L^{-1} each to media containing 500 mg L^{-1} perchlorate. The experiment was performed at optimum pH and temperature, and succinic acid was added as the sole carbon source at 1000 mg L^{-1} in the media.

2.4. Analytical methods

All the anions, viz. perchlorate, nitrate, chlorate, sulfate, phosphate, and the organic acids such as acetate, formate, citrate, oxalate and succinate were measured using a Metrohm 792 Basic Ion Chromatograph (Metrohm AG, Herisau, Switzerland) equipped with a Dual 3 column (250 mm × 4 mm), a RP guard column, and a conductivity detector. Samples taken during the experiments were

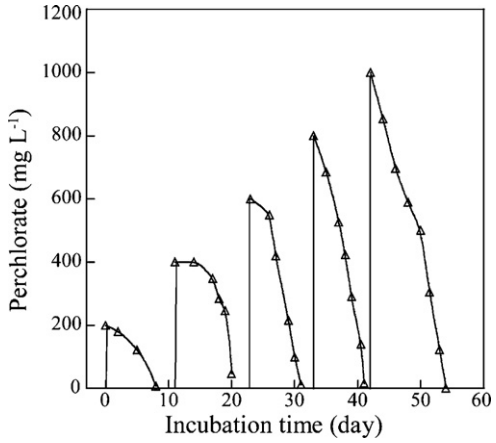

Fig. 1. Results of acclimation of the mixed microbial consortium to degrade perchlorate. Initial conditions: acetate, 1 g L^{-1}; temperature = 28 °C and pH = 7.0.

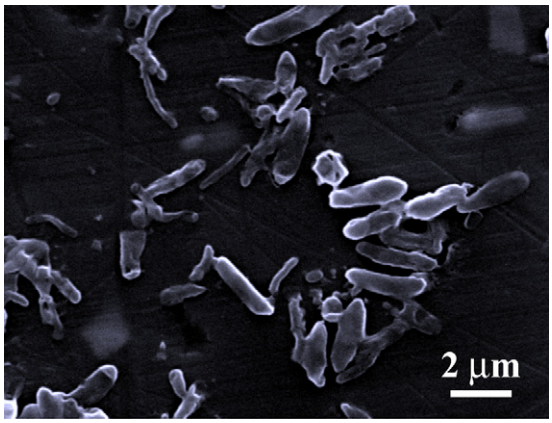

Fig. 2. Scanning electron micrograph of the anaerobically grown acclimated mixed consortium.

centrifuged at 8000 rpm for 10 min and were filtered through a C-18 reverse-phase cartridge and then through 0.45 μm filter for analysis. NaOH (5 mM) served as the eluent and sulfuric acid (2.0 mM) as the regenerant in the chromatogram analysis. Scanning electron microscope (SEM) images of the mixed microbial culture (glued to an aluminum stub and gold sputtered) were obtained by means of an electron microscope (Make: LEO, Model: 1430 VP, U.S.A.).

3. Results and discussion

3.1. Enrichment, isolation and identification of the mixed consortium

Enrichment of the mixed consortium was carried out by adding gradually increasing the amount of perchlorate in synthetic wastewater from 200 to 1000 mg L^{-1}. The detailed enrichment phase presented in Fig. 1 shows that the perchlorate removal rate was improved at each and every stage of acclimatization. Morphological characterization of the mixed consortium performed using SEM revealed that the cells were of various sizes and shapes, from small rods to large rods (Fig. 2). Most of the rods were between 0.2 μm × 1.7 μm and 0.6 μm × 1.8 μm in size (Fig. 2). The major rod shaped bacteria were also found to grow as clusters.

The predominant perchlorate reducing strain was isolated from the enriched mixed consortium and designated as strain AG. Partial 16S rDNA sequencing result showed that strain AG had 1436 base pairs (bp). The sequence was submitted to the GenBank with HM104637 as accession number of the strain AG. Gene analysis by online BLAST tool indicated that the isolate contains sequences that are specific to the members of the β subdivision of the family Proteobacteria. The phylogenetic tree shown in Fig. 3 was prepared using neighbor joining method based on near-full-length 16S rDNA gene sequences recovered from the isolated strain and

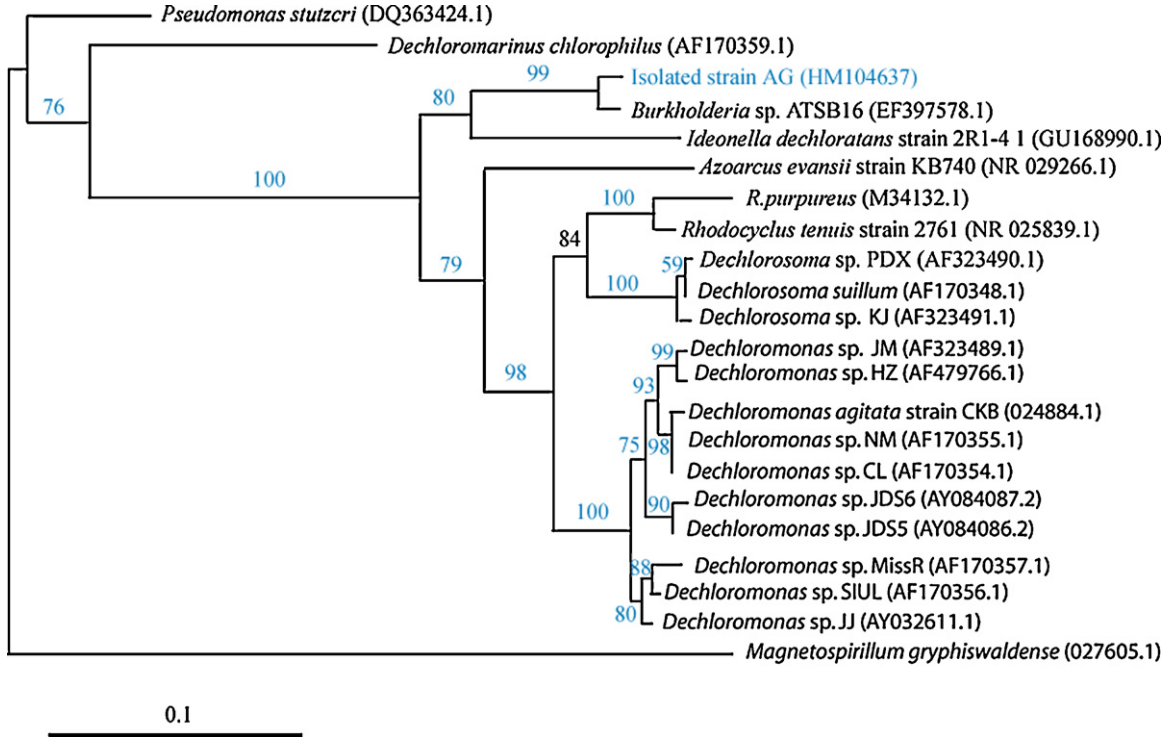

Fig. 3. Phylogenetic relationships between strain AG (HM104637) and other known perchlorate degrading strains based upon the analysis of aligned regions of 16S rDNA gene sequences. Bootstrap values are noted on the branch and the scale bar (= 0.1) represents nucleotide substitution per 100 nucleotide.

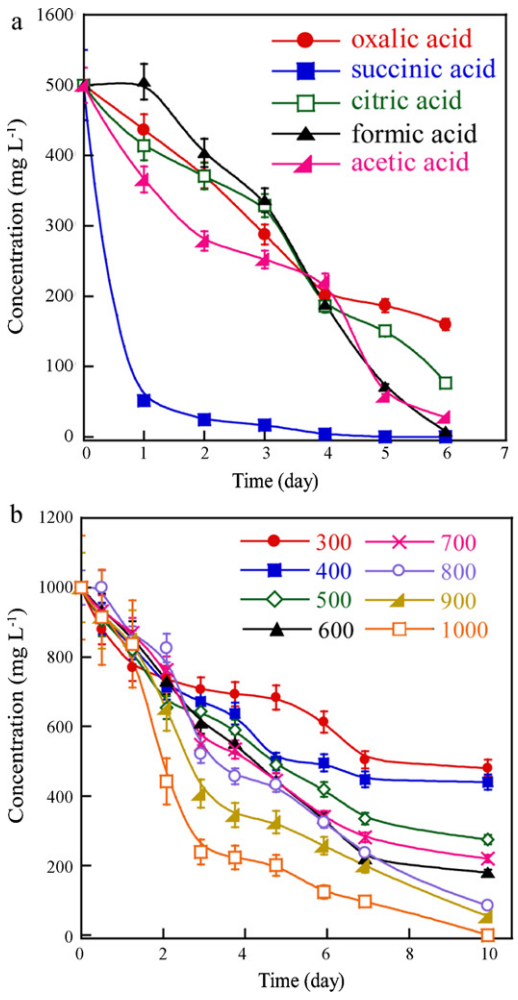

Fig. 4. (a) Effect of different organic acids on perchlorate degradation by the mixed consortium. Initial conditions: perchlorate, 500 mg L^{-1}; temperature = 28 °C and pH = 7.0. (b) Perchlorate degradation by the mixed consortium for different concentrations (300–1000 mg L^{-1}) of succinic acid in the media. Initial conditions: perchlorate, 1000 mg L^{-1}; temperature = 28 °C and pH = 7.0.

other sequences obtained from the GenBank database (GenBank accession number has been indicated with the generic name in the tree). The high bootstrap support of the tree shown in Fig. 3 derived from the 16S rDNA analysis demonstrated that strain AG is a typical member of the genus *Burkholderia* sp. and it has closest relation (98%) to *Burkholderia* sp. ATSB16. This is the first report of the *Burkholderia* sp. involved in perchlorate degradation. The thermodynamic properties as obtained by the online tool (BIOTOOL) indicated that strain AG has 54% GC content with 2399.1 kcal mol^{-1} Gibbs free energy (ΔG), 33,093.3 kcal mol^{-1} enthalpy (ΔH) and 31,576.1 cal mol^{-1} K^{-1} entropy (ΔS).

3.2. Effect of different organic acids

Both pure and mixed cultures of bacteria are shown to respire perchlorate as terminal electron acceptor with oxidation of several carbon sources, and acetate has been used extensively as a single substrate for heterotrophic perchlorate reduction [18]. Therefore, the ability of the mixed consortium to utilize different organic acids including acetic acid during perchlorate degradation was investigated in the present study. Fig. 4a shows the degradation profile of perchlorate by the mixed consortium in the presence of five different organic acids: succinic acid, acetic acid, citric acid, formic acid and oxalic acid. The probable reaction mechanisms of perchlorate degradation by the mixed consortium utilizing the five different organic acids are given below:

for succinic acid,

$$2C_4H_6O_4 + 4ClO_4^- \rightarrow 4Cl^- + O_2 + 8CO_2 + 6H_2O \quad (4)$$

for acetic acid,

$$C_2H_4O_2 + 2ClO_4^- \rightarrow Cl^- + 2O_2 + 2CO_2 + 2H_2O \quad (5)$$

for citric acid,

$$2C_6H_8O_7 + 5ClO_4^- \rightarrow 5Cl^- + O_2 + 12CO_2 + 8H_2O \quad (6)$$

for formic acid,

$$2CH_2O_2 + 2ClO_4^- \rightarrow 2Cl^- + 3O_2 + 2CO_2 + 2H_2O \quad (7)$$

for oxalic acid,

$$4C_2H_2O_4 + ClO_4^- \rightarrow Cl^- + O_2 + 8CO_2 + 2H_2O \quad (8)$$

Among the organic acids tested, the mixed consortium showed the ability to degrade perchlorate completely by utilizing succinic acid as the sole source of carbon. With oxalic acid, the degradation was only 68.1% after 6 days of incubation. The lowered efficiency of the mixed consortium to degrade perchlorate using oxalic acid could be due to insufficiency of the carbon source added to the media. From the balanced equation it can be calculated that the molar ratio of oxalic acid and perchloarate is 4:1 which is much higher than the ratio 1.1:1 at which the two compounds were taken in the experiments. Influence of succinic acid concentration on degradation of perchlorate was further examined by addition of different amounts of succinic acid ranging from 300 to 1000 mg L^{-1}. Increase in succinic acid initial concentration favoured removal efficiency of perchlorate and with 1000 mg L^{-1} of initial succinate concentration the mixed consortium was able to completely degrade the total amount of perchlorate within ten days (Fig. 4b). The degradation rate constant (k_d) increased from 0.15 to 0.29 day^{-1} with the increase in initial succinic acid concentration from 300 to 1000 mg L^{-1}. These results showed that at lower succinic acid concentration i.e. below 500 mg L^{-1}, the mixed consortium could not achieve significant perchlorate removal. Better perchlorate degradation efficiency at higher concentrations of succinic acid could be attributed to enhanced biomass growth of the culture at sufficiently high concentration of the carbon source provided in the experiments, which, however, needs further investigations to confirm. However, compared with perchlorate degradation rate at low initial concentrations of 500 mg L^{-1} of both succinic acid and perchlorate in the media, the culture exhibited lower degradation rate at 1000 mg L^{-1} each probably due to the initial biomass added as inoculum in the experiments that may be insufficient to degrade a higher concentration of perchlorate. Therefore, it could be surmised that biomass concentration (both initial and during the experiments) plays an important role in the perchlorate degradation, particularly when the concentration is high.

3.3. Effect of pH and temperature on perchlorate degradation

Fig. 5a shows perchlorate degradation profile by the mixed consortium over the pH range of 5.0–9.0 with initial 800 mg L^{-1} of perchlorate, which indicates that the culture could substantially degrade ClO_4^- in the pH range 5.0–7.0; the degradation efficiency, however, considerably reduced at pH 8.0 and 9.0 with values ~65% and ~55%, respectively. The maximum degradation (~98%) of ClO_4^- was observed at pH 7.0 within 6 days with a k_d value of 0.28 day^{-1}. In a similar study by Wu et al. [23], an optimum pH value of 8 was reported for maximum removal of perchlorate by a mixed consortium from an indigenous source with 50 mg L^{-1} of initial

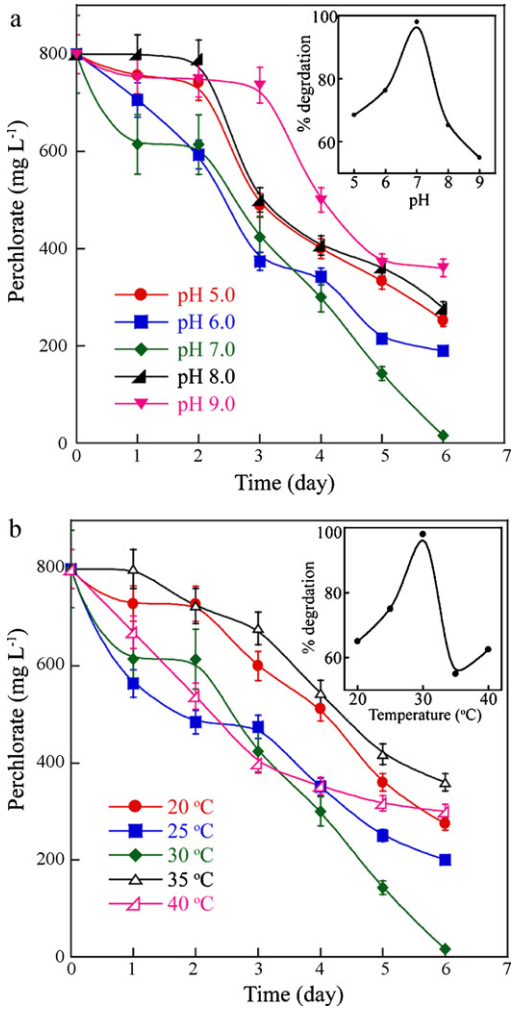

Fig. 5. (a) Effect of different pH on perchlorate degradation by the mixed consortium. Initial conditions: perchlorate, 800 mg L^{-1}, succinate, 1 g L^{-1}; temperature = 28 °C. (b) Effect of different temperature on perchlorate degradation by the mixed consortium. Initial conditions: perchlorate, 500 mg L^{-1}; succinic acid, 1 g L^{-1} and pH = 7.0.

perchlorate concentration and 1.2 g L^{-1} of acetate at temperature 30 °C.

With respect to the effect of temperature on perchlorate degradation in the range 20–40 °C, maximum degradation of 98% was observed at 30 °C (Fig. 5b) with a k_d value of 0.27 per day. Results indicated that at 25 and 30 °C perchlorate degradation by the culture was sufficiently high; on the other hand, at lower (20 °C) or higher (40 °C) temperatures the degradation efficiencies were not significant enough. Several studies have also reported maximum perchlorate degradation at 30 °C for enriched mixed consortium [8,22]. On the contrary, Wu et al. [23] reported that a slight increase in the reaction temperature from 20 °C to 40 °C had a stimulatory effect on the rate of perchlorate reduction by an indigenous mixed culture using acetate as sole carbon source. From the results obtained in the present study, it can be well said that the mixed culture, which is predominantly *Burkholderia* sp., is a mesophilic bacterial consortium preferring 30 °C and neutral pH 7.0 for perchlorate biodegradation.

3.4. Effect of different co-anions

Anions like nitrate, nitrite, chlorate and phosphate co-exist with perchlorate in wastewaters from several industries, especially fireworks, fertilizer, electroplating, electro-polishing and army ammunitions, Among these anions, nitrate is a common co-contaminant that inhibits microbial reduction of perchlorate in many systems [12,18]. The effect of different co-anions on perchlorate reduction by the enriched mixed consortium is depicted in Fig. 6 which shows that the perchlorate degradation was affected in the presence of these co-anions. In case of the medium containing nitrate and perchlorate together at same concentration (500 mg L^{-1}), although perchlorate degradation was initiated quickly without any lag phase (Fig. 6a) the degradation efficiency was only 47% at the end of six days and did not improve further. The decrease in perchlorate concentration during the first 48 h was also supported by an increase in the cell density (OD_{600}) from 1.0 to 1.2. Several studies have reported the influence of nitrate on perchlorate degradation by denitrifying perchlorate reducers [24,12]. The inhibition of perchlorate degradation in the presence of nitrate is attributed mainly to the suppression of (per)chlorate reductase enzyme by nitrate [25]. However, existence of separate pathways for the two electron acceptors has also been proposed [26]. The preference of ClO_4^- to NO_3^- as electron acceptor is also likely to be associated with a different enzyme involved with lowered activation energy [27]. In mixture containing nitrite as the coanion, nitrite was almost completely degraded by the mixed consortium within the first day of the culture (Fig. 6b). The degradation rate of nitrite was also found to be higher than nitrate in the presence of perchlorate. Further, the observations that (a) no nitrite was found to be accumulated in the media in the presence of nitrate and (b) nitrite was completely reduced revealed nitrogen removal potential of the mixed consortium starting from nitrate to nitrite and then to gaseous nitrogen. The higher rate of the nitrite reduction than nitrate reduction is also supported by the lower Gibb's free energy value required for nitrite reduction than nitrate reduction. From the nitrate and nitrite concentrations used in the experiments, it can also be inferred that the perchlorate reducing mixed consortium can withstand sufficiently high amount nitrogen. Compared with the results obtained in the present study, Bardiya and Bae [9] observed nitrate to be toxic to an indigenous mixed culture even at a low concentration of only 100 mg L^{-1} in their study. In the presence of chlorate (ClO_3^-), perchlorate reduction by the mixed consortium was affected (Fig. 6c), and in which case chlorate was reduced much quicker than perchloarate. The consortium was able to degrade perchlorate only up to ~57% while chlorate was degraded up to ~81% (after 6 days of culture). It has been reported that (per)chlorate-reducing bacteria (PRB) use a single enzyme (per)chlorate reductase for the degradation of perchlorate (ClO_4^-) to chlorate (ClO_3^-) and chlorate to chlorite (ClO_2^-) [28]. Chlorite (ClO_2^-) is then converted into chloride (Cl^-) and molecular oxygen by the enzyme chlorite dismutase [29,30]. This indicates that the mixed consortium possesses different enzymes than only (per)chlorate reductase for the degradation of perchlorate and chlorate, which however needs further investigations to confirm. In the presence of phosphate, the culture utilized both the anions simultaneously at a significantly high rate (Fig. 6d) with sufficient biomass growth (OD_{600} = 1.5). However, the perchlorate degradation efficiency was low at only ~54% at the end of 6 days of culture compared with media containing only perchlorate. The reduced efficiency of perchlorate degradation in the presence of equal concentrations of the co-pollutants was analyzed based on the utilization profiles of succinic acid, which is manifested in Fig. 7. From the results shown in the figure, it can be observed that succinic acid was completely utilized by the mixed consortium after 6 days of culture. The depletion profiles of the carbon source in the medium in the presence of the co-anions could thus be attributed to reduced perchlorate degradation by the mixed consortium.

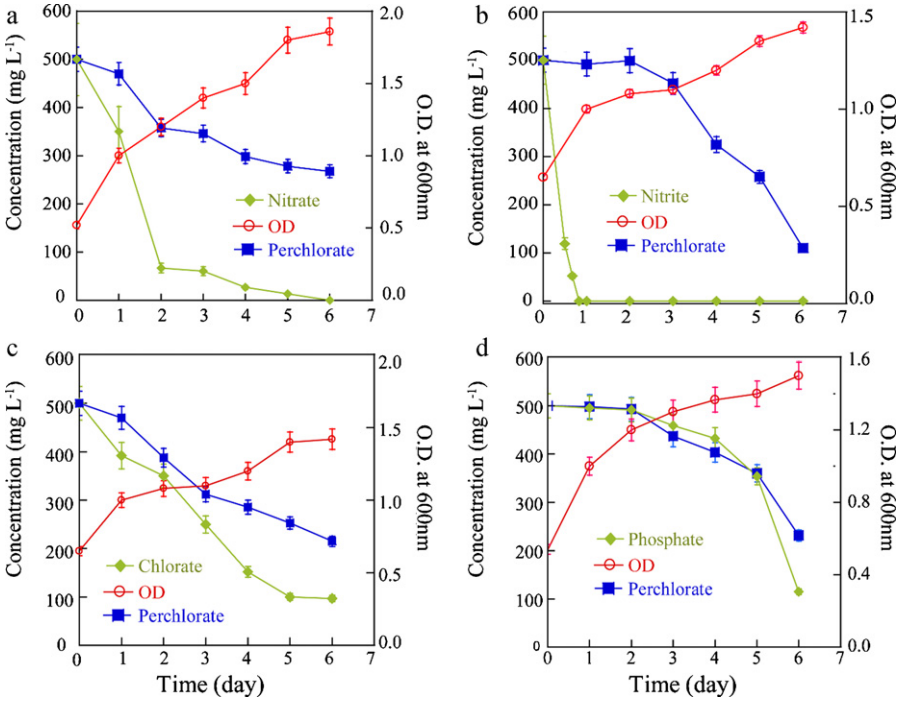

Fig. 6. Simultaneous degradation of perchlorate with (a) nitrate, (b) nitrite, (c) chlorate and (d) phosphate by the mixed consortium in a binary mixture. Initial conditions: perchlorate, 500 mg L^{-1}; succinic acid, 1 g L^{-1}; temperature = 30 °C and pH = 7.0.

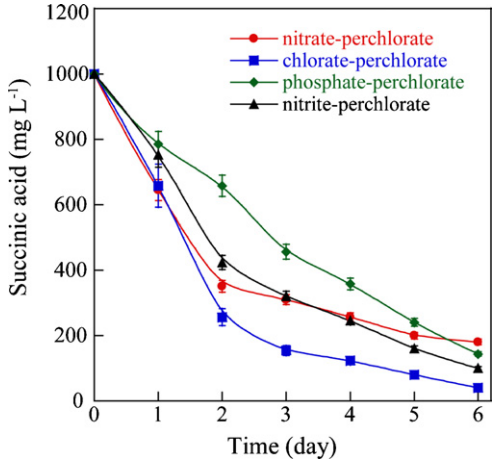

Fig. 7. Succinic acid utilization profile during simultaneous degradation of perchlorate with nitrate, chlorate, phosphate and nitrite by the mixed consortium in a binary mixture. Initial conditions: each anion concentration, 500 mg L^{-1}; succinic acid, 1 g L^{-1}; temperature = 30 °C and pH = 7.0.

4. Conclusion

Indigenous mixed microbial culture, isolated from an activated sludge, was highly effective in perchlorate removal from synthetic wastewater. The phylogenetic analysis of predominant strain in the mixed consortia revealed a close relation (98%) with *Burkholderia* sp. ATSB16. *Burkholderia* sp. found in the mixed consortium is reported for the first time to be involved in perchlorate degradation. The effect of different organic acids as the sole carbon source was investigated on perchlorate degradation by the mixed consortium. Among the various carbon sources tested in the study, succinic acid showed rapid and complete biodegradation of perchlorate. The optimum conditions of the mixed culture in perchlorate degradation were observed to be 30 °C and pH 7.0. In the presence of different co-anions, such as nitrate, nitrite, chlorate and phosphate, perchlorate degradation by the culture was found to be affected significantly. From the results obtained in the study, the potential of the mixed microbial culture can be implicated further in perchlorate removal from contaminated water systems.

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