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Exploring the Strength of Pseudomonas –a & Pseudomonas B in Removal of ClO_4^- & ClO_3^-: an Outstanding Approach of Environmental Bioremediation

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Abstract Elimination of perchlorate (ClO_4^-) and chlorate (ClO_3^-) can be viewed as a very promising water treatment technology. The process is based on the ability of specific bacteria to use (per) chlorate as an electron acceptor in the absence of oxygen. The present research work was focused on the isolation and kinetic characterization of perchlorate reducing bacteria. The enrichment process started with a sludge sample taken from an anaerobic digester of a domestic wastewater treatment plant (Ankleshwar, India). Two perchlorate-reducing bacteria (A) and (B) were isolated using different selection methods, platting and liquid transfer respectively. The purity of the isolates was confirmed by genetic characterization of 16S rDNA. The BLAST search showed that the microorganims shared a 99% sequence similarities to the 16S rDNA of *Pseudomonas sp.* (A) and *Pseudomonas sp.* (B). Batch tests were performed under anaerobic conditions with acetate as the electron donor and perchlorate and/or chlorate as electron acceptor. During perchlorate reduction by *Pseudomonas sp.* (A) it was observed transient accumulation of chlorate. The isolates showed different behaviour concerning perchlorate and chlorate reduction. Chlorate was preferentially reduced when both electron acceptors were present, being perchlorate reduced after completely depletion of chlorate. The former performance was observed in both bacteria.

Keywords*: perchlorate and chlorate reduction, kinetic characterization, pseudomonas*

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1. Introduction

Perchlorate (ClO_4^-) and chlorate (ClO_3^-) have been produced on large scale by the chemical industry for use in a wide range of applications. The improper storage and/or disposal of these oxyanions have led to harmful concentrations in surface and groundwater supplies, as they are extremely soluble and not significantly broken down in the environment. These characteristics make them persistent and problematic environmental pollutants of drinking waters. Moreover, ClO_4^- and chlorate are also a health concern, as they can cause serious diseases such cancer. In the medium-term the removal of ClO_4^- from drinking water will become necessary in order to protect the environment and human health. The long-term solutions must involve a reduction in the release of ClO_4^- into the environment and wastewater treatment should be done more efficiently. The biological removal of these anions can be viewed as a very promising water treatment technology. The process is based on the ability of specific bacteria to utilize (per) chlorate as a physiological electron acceptor in the absence of oxygen and reduce it completely to innocuous chloride. The main advantages of this process are the selectivity, its fastness and the low operating costs. Although a number of investigators are currently working on bioreduction processes, studies are needed to identify and characterize more of the microorganisms that reduce ClO_4^- so as to optimize conditions for maximal destruction while minimizing by-product formation, wasteful sidereactions and nutrient consumption. Also more effort must be expended in elucidating the mechanism by which microorganisms reduce ClO_4^-, including isolation, purification and characterization of the active enzyme (s). It may be possible to exploit the mechanism whereby the bacteria are capable of perchlorate reduction, but only if we have a better understanding of that mechanism. The first studies published on the biological reduction of chlorine oxyanions indicated that microorganisms rapidly reduced chlorate that was applied as an herbicide for thistle control (Aslander, 1928). Initial investigation of the microbiology of chlorate reduction suggested that it was mediated by nitrate-respiring organisms in the environment and chlorate uptake and reduction was simply a competitive reaction for the nitrate reductase system of these bacteria (Coates and Achenbach, 2004; de Groot et al., 1969). The ability of bacteria to use ClO_4^- as a terminal electron acceptor was not reported until 1976 (Romanenko et al., 1976). Initially, it was supposed that all chlorate reducing bacteria (CRB) were able to reduce ClO_4^-, leading to the

early speculation of the abbreviation (per)chlorate. However, current studies have provided evidence that not all CRB are perchlorate reducing bacteria (PRB) and consequently there is a subset of CRB that cannot use ClO_4^- as an electron acceptor for respiration (Logan et al., 2001b). PRB are nearly ubiquitous and have now been isolate from a broad diversity of environments, including rivers, sediments, soils, farm animal waste lagoons and wastewater treatment plants (Bruce et al., 1999; Wolterink et al., 2002; Achenbach et al., 2001; Waller et al., 2004; Bardiya et al., 2006). The present research work was focus on the isolation, purification and characterization of possible new perchlorate reducing bacteria. Since there is hardly any ClO_4^- and ClO_3^- degradation kinetic data available, it was studied the kinetic characterization of the isolates obtained. Furthermore, the improved understanding of the biological ClO_4^- reduction will lead to better biological remediation processes.

2. Experimental

2.1. Chemicals

The chemicals used for preparation of media were procured from Sigma-Aldrich & Hi-Media, India. All media were prepared using ultrapure water (Milli Q system) and research grade chemicals in the amounts indicated in grams per liter. For the enrichment, the basal medium was amended with 1 mL/L of mineral solution SL-10. For growth kinetics of the isolate (A), 1 mL/L of medium KL was added to the basal medium and for the isolate (B), 1 mL/L of medium SLA was added to the basal medium. Sodium acetate was used as the sole electron donor in 1:2 molar ratio to sodium perchlorate and/or sodium chlorate, final electrons acceptor. Solid agar plates were prepared by adding 15 g/L agar on the medium as described below.

Table 1. Media and reagents used for enrichment and isolation (g/L)

Reagent	Basal Medium	SL-10	Medium KL	Medium SLA
K_2HPO_4	1.55			
$NaH_2PO_4{\cdot}H2O$	0.85			
NH_4Cl	0.25			
$MgSO_4{\cdot}7H_2O$	0.1			
HCl (37%)		10 ml		
Na_2SeO_3		0.0017		
$Na_2SeO_3{\cdot}5H_2O$			0.15	0.1
$FeCl_2{\cdot}4H_2O$		1.5		18
$FeSO_4{\cdot}7H_2O$			4	
$NaMoO_4{\cdot}2H_2O$		0.036	0.4	0.3
$NiCl_2{\cdot}6H_2O$		0.024	0.1	0.1
EDTA			3	
H_3BO_3			0.6	5
$ZnCl_2$				1
$MnCl_2{\cdot}4H_2O$		0.07		0.7
$CoCl_2{\cdot}6H_2O$		0.19		2.5
$CuCl_2{\cdot}2H_2O$		0.002		0.1

2.2. Source of Organism

A sludge sample was collected at the anaerobic digestor from wastewater treatment plant. The primary inoculum used to start the enrichment was obtained by the dilution of the sludge sample (1:10 in 0.6% NaCl).

2.3. Bacterial Isolation Procedures & Culturing Conditions

All the enrichment was performed under anaerobic conditions with basal media + SL-10. The media was made O_2-free by flushing continuous Argon and were prepared in 50 mL bottles capped with butyl rubbers stoppers, crimped with aluminium capsules and sterilized by autoclaving. Incubation was carried out 37°C under constant shaking (100 mot/min). A concentration of 5 mM ClO_4^- was selected to start the enrichment. During a period of two months, continuous transfers (10% by volume) were made in sterilize conditions in a laminar flow. Cultures became turbid in 7 to 14 days. For further enrichment, the ClO_4^- concentration was increased to 10 mM. Subsequently, two different selection methods were applied to reach pure cultures. In the first one, a sample was serially diluted to 10^{-3} and spread onto agar plates. The anaerobic growth in agar plates was performed with kit Microbiologie Anaerocult® mini. Select colonies were picked and then re-grown in fresh liquid Basal medium + SL-10. The second method applied consisted in the continuous transfers of the enriched liquid culture at exponential phase to fresh Basal medium + SL-10 during a period of 20 days.

2.4. 16S Ribosomal Dna Extraction and Sequencing

2.4.1. Extraction and Confirmation

DNA extraction of the isolates was performed using FastDNA® SPIN Kit (for soil), according to the manufacturer instructions (Bio101Ó systems, Q-biogen, USA). Some changes were performed in order to adjust the kit to our sample. To the Lysing matrix E Tube was added 500 µl of centrifuged cell pellet, 650 µl sodium phosphate buffer and 80 µl MT buffer. The tube was processed in FastPrep® Instrument at speed 4.5. The supernatant was transfer to 2 new tubes (600 µl to each one) and 500 µl of Binding Matrix was also added to each tube. The supernatant (600 µl) was discarded and the remaining amount was resuspended in the Binding Matrix. The extraction was confirmed by gel electrophoresis. The agarose solution was prepared in TAE (trisacetate EDTA) with a final concentration of 1%. The solution was heated during 50 seconds in the microwave and was poured in a gel tray to allow cool at room temperature. To stain the gel, ethidium bromide was incorporated before gel polymerization. After polymerization, DNA samples and the mass ladder (1 kb) were loaded into the sample well. A loading dye (bromophenol blue) was used together with samples and the mass ladder. The gel was run at 100 V for a period of 40 min. The gel was then visualized directly upon UV light.

2.4.2. PCR Amplification and Purification

The 16S ribosomal DNAs were amplified by conventional PCR. The amplification program included initial denaturation at 94°C for 5 minutes followed by three steps repeated 30 times. Step 1: 94°C for 30 seconds; step 2: 48°C for 30 seconds; and step 3: 72°C for 2 minutes. The final elongation was at 72°C for 5 minutes. The primers 27f and 1492r, and Taq polymerase

(Invritogen) were used in this amplification. The PCR products were purified by gel electrophoresis and then cleaned with QIAquick® PCR purification kit (250). The gel electrophoresis was prepared in the same conditions as mentioned before for DNA extraction confirmation, with the exception of the run time and voltage, 1h and 80V, respectively. The purified products were then sequenced by Bangalore Genei, India.

2.4.3. Phylogenetic Analysis

For establishing the identity of the isolates by 16S rDNA nucleotide-nucleotide sequence homology, the BLAST (Basic Local Alignment Search Tool) network service, via the nucleotide collection (nr/nt) database at the National Center for Biotechnological Information (NCBI) was used.

2.4.4. Batch Growth Kinetics

After confirmation of purity the isolate *Pseudomonas* (A) show to grow better on basal medium + medium KL and *Pseudomonas* (A) on basal medium + medium SLA. These were the media used to grow the isolates in batch tests. The batch tests were performed in a reactor filled with 0.5 L of the appropriate medium with electron acceptor and donor. The medium was made O_2-free by flushing continuous Argon (Ar) during more than 12h. The growth kinetics was conduct at controlled temperature (37°C) and the redox potential was measured with a redox electrode *in-situ*. The pH measurements were made *ex*-situ and samples (5 mL) were taken in sterilized conditions, at regular time intervals for further analysis. To keep a positive pressure inside the reactor, Ar was supplied every time that it was taken a sample.

2.5. Analytical Techniques

Culture growth was monitored by optical density at 600 nm (OD600 nm) with a spectrophotometer and converted to dry weight (DW) using a calibration curve. The DW determination was made using the method described elsewhere (Olsson and Nielsen, 1997). The anions concentration was analyzed by HPLC. The concentration of perchlorate was determined by ion chromatography equipped with an Ion Pac AS16 column and a AG16 guard column (4 mm, Dionex), a self-regenerating suppressor (SRS Ultra II), and an autosampler. The eluting perchlorate was detected by a conductivity detector (Dionex) and the suppressor controller was set at 100 mA for the analysis. The samples were analyzed with a 50 mM NaOH mobile phase at a flow rate of 1 ml min-1. The injection loop volume was 30 µl. The chlorate, chlorite, chloride and acetate were determined with the same ion chromatography system described before. An Ion Pac AS9 column and a AG9 guard column were used. The eluent used was 9 mM Na_2CO_3 at a flow rate of 1ml min-1. The injection loop volume was 30µl and the suppressor controller was set at 50 mA for the analysis.

3. Calculations

3.1. Specific Growth Rate

The specific growth rate was determined based on the cell dry weight (DW) as

$$funtion\ of\ \ time: \mu = \frac{\triangle \ln(DW)}{\triangle t}$$

3.2. Specific uptake rate

The following formula was used to determine the specific uptake rate for acetate,

$$perchlorate\ and\ chlorate: -q_s = \frac{\triangle S}{\triangle t}$$

3.3. Electron Acceptor Yield over Acetate

The following formula was used to determine the chloride formation yield:

$$Y_{e-acceptor/CH_3COO^-} = \frac{\triangle e-_{acceptor}}{\triangle_{CH_3COO^-}}$$

3.4. Chloride Yield over Electron Acceptor

The following formula was used to determine the chloride formation yield:

$$Y_{e-acceptor/Cl^-} = \frac{\triangle e-_{acceptor}}{\triangle_{Cl^-}}$$

3.5. Biomass Yield

The following formula was used to determine the biomass yield for acetate,

$$perchlorate\ and\ chlorate: Y_{x/s} = \frac{\triangle X}{\triangle S}$$

4. Results

4.1. Genetic Characterization of the Isolates

The purity of the two isolates was confirmed by genetic characterization of 16S rDNA. The BLAST search showed that the microorganism (A) shared a 99% sequence similarities to the 16S rDNA of *Pseudomonas*. Concerning the genetic characterization by 16S rDNA of the microorganism (B), the sequencing showed that the microorganism shared a 99% sequence similarities to the 16S rDNA of *Pseudomonas*.

4.2. Growth Kinetics

4.2.1. *Pseudomonas* (A) (10 mM ClO_4^-)

Pseudomonas (A) was grown on basal medium + KL solution amended with 20 mM of CH_3COO- and 10 mM of ClO4$^-$ (Figure 1). Over perchlorate reduction by *Pseudomonas* (A), it was observed accumulation and subsequent degradation of the intermediate chlorate. Around 3.4% on a molar basis of perchlorate concentration was accumulated as chlorate. The chloride (Cl^-) formation was also detected during perchlorate reduction, indicating a completely conversion of perchlorate into innocuous chloride (data not shown). The

specific acetate uptake rate was two times higher compared with perchlorate uptake rate (data not shown). This information showed that the molar ratio acetate to perchlorate was 2:1 as it was expected. Concerning pH, it was observed over the growth a slightly increased from 6.99 to 7.33, which was not significant (data not shown). The maximum cell dry weight obtained was 1.53 g/L corresponding to an OD600 nm of 1.08. During the kinetic, some samples were examined by optical microscopy and no changes were observed in the shape of the microorganisms, neither other bacteria were present, which confirmed the purity of the isolate.

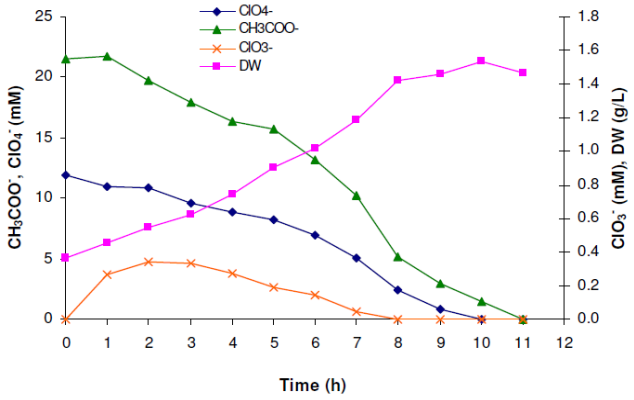

Figure 1. Acetate and perchlorate uptake and transient accumulation of chlorate as function of time during the reduction of 10mM of $ClO4^-$ by *Pseudomonas* (A). Note the different concentration scale for $ClO3^-$. Dry Weight (DW) as a function of time is also represented.

4.2.2. *Pseudomonas* (A) (10mM ClO_3^-)

The study of chlorate reduction by *Pseudomonas* (A) is represented in Figure 2. In this kinetic the basal medium + KL was amended with 10 mM of ClO_3^- and 20mM of $CH3COO^-$ *Pseudomonas* (A) showed the ability to reduce chlorate in the same concentration and conditions as used for perchlorate reduction. A completely reduction of chlorate into chloride was observed. The maximum value achieved for cell dry weight was 0.49 g/L, demonstrating a lower biomass yield compared with perchlorate reduction. The pH ranged from 6.96 to 7.29.

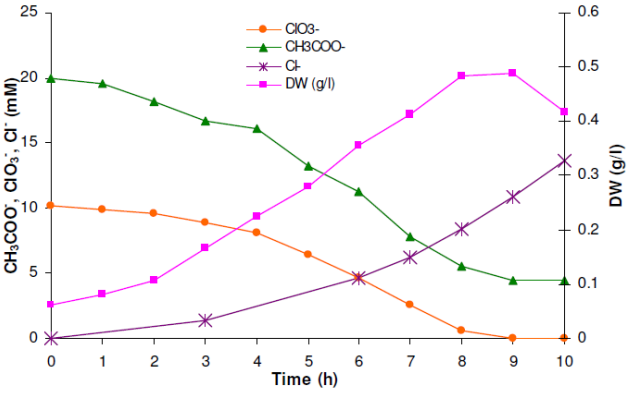

Figure 2. Acetate and chlorate uptake as function of time during the reduction of 10mM of $ClO3^-$ by *Pseudomonas* (A). Dry weight (DW) and chloride formation as a function of time are also represented.

Pseudomonas (A) (5 mM ClO_4^- + 5 mM ClO_3^-) In order to study the growth kinetic of *Pseudomonas* (A) with perchlorate and chlorate, it was performed a kinetic with 5 mM ClO_4^- and 5 mM ClO_3^- simultaneously in the media. Again, basal medium + KL was used as growth media and CH_3COO- was used in a concentration of 20 mM (Figure 3).

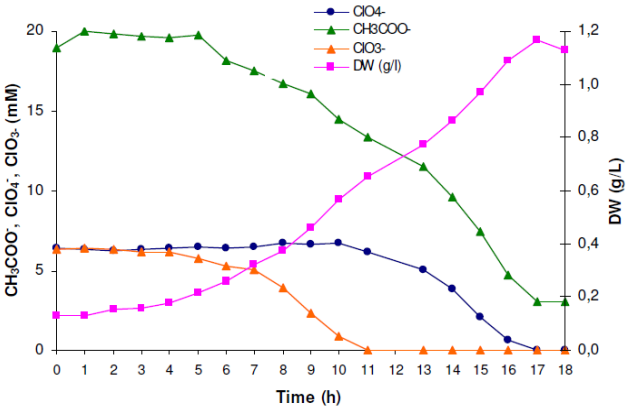

Figure 3. Acetate, perchlorate and chlorate uptake as function of time during the reduction of 5mM of $ClO4^-$ + 5mM of $ClO3^-$ by *Pseudomonas* (B). Dry weight (DW) as a function of time is also represented.

Concerning acetate, it can be observed two different uptake rates, near related with chlorate and perchlorate reduction respectively and similar to each substrate individually. The chloride produced was identical to the sum of perchlorate and chlorate amounts, showing once more a completely conversion of both electron donors in chloride. The pH showed again a small variation starting with 7.02 and ended with 7.34. The maximum cell dry weight produced was 1.11 g/L. In this kinetic it should be stressed the observed preference for chlorate when both chlorate and perchlorate were present in the media. From Figure 4 it can be observed in more detail the reduction of the electrons acceptor, perchlorate and chlorate. Perchlorate was not reduced unless chlorate was almost reduced. This observation probably indicates chlorate inhibition over perchlorate reduction when both were present at the same concentration of 5 mM. The same batch tests were performed with *Pseudomonas* (B). It was performed a kinetic study with 10 mM of ClO_4^-, one with 10 mM of ClO_3^- and a last one with 5 mM ClO_4^- + 5 mM ClO_3^-. In this case, the medium used in all tests was basal medium + SLA as described before.

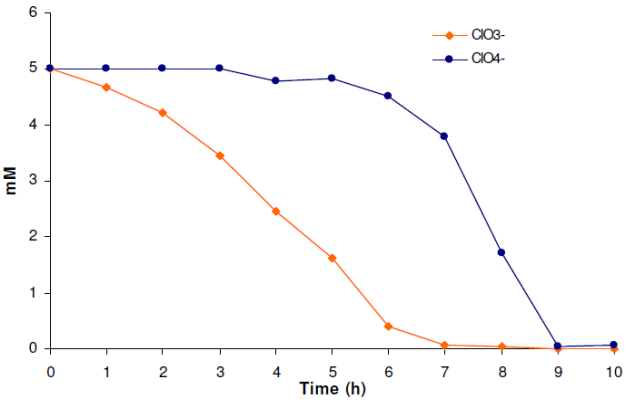

Figure 4. Perchlorate and chlorate uptake as function of time during the reduction of 5mM of $ClO4^-$ + 5mM of $ClO3^-$ by *Pseudomonas* (A).

4.2.3. *Pseudomonas* (B) (10mM ClO_4^-)

In this kinetic the basal medium + SLA was amended with 10 mM of ClO_4^- and 20 mM of CH_3COO^-. The

results showed that concerning perchlorate reduction, *Pseudomonas* (B) had similar behaviour compared with *Pseudomonas* (A). The chloride (Cl^-) formation was detected during perchlorate reduction, indicating a completely conversion of perchlorate into innocuous chloride (data not shown). The biomass production was 0.89 g/L in cell dry weight and conversely to *Pseudomonas* (A), no chlorate accumulation was observed for the same detection limit of 0.06 mM ClO_3^-.

4.2.4. *Pseudomonas* (B) (10 mM ClO_3^-)

The study of chlorate reduction by *Pseudomonas* (B) is shown in Figure 5. For this kinetic the basal medium + SLA was amended with 10 mM of ClO_3^- and 20 mM of CH_3COO^-. *Pseudomonas* (B) can also reduce chlorate as a single electron donor. Chlorate was completely reduced to chloride, which proves the total reduction of chlorate into chloride. Biomass produced was less than the observed in perchlorate reduction and the maximum value of cell dry weight was 0.75 g/L. The pH ranged from 6.96 to 7.29.

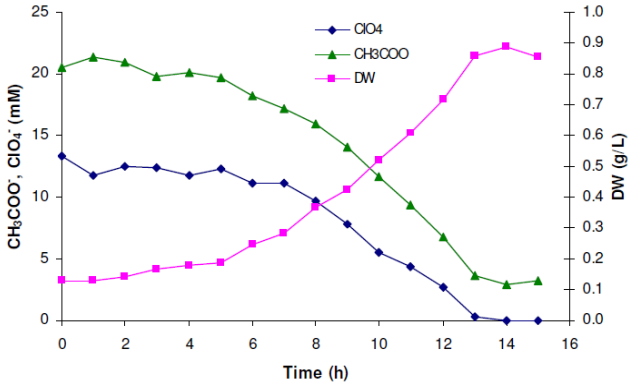

Figure 5. Acetate and perchlorate uptake as function of time during the reduction of 10mM of $ClO4^-$ by *Pseudomonas* (B). Dry weight (DW) as function of time is also represented

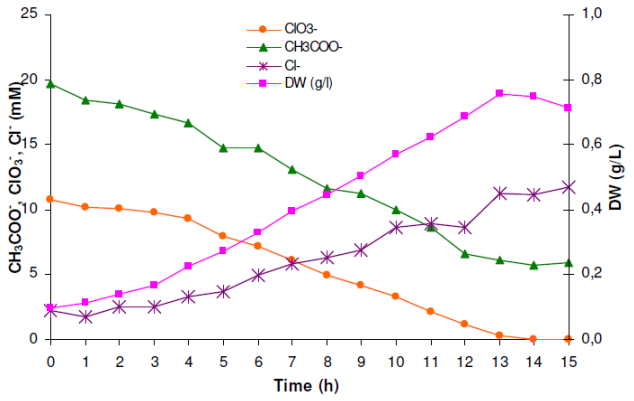

Figure 6. Acetate and chlorate uptake as function of time during the reduction of 10mM of$ClO3^-$ by *Pseudomonas* (B). Dry weight (DW) and chloride formation as function of time is also represented

4.2.5. *Pseudomonas* (B) (5 mM ClO_4^- + 5 mM ClO_3^-)

It was also performed a kinetic study with 5 mM ClO_4^- and 5 mM ClO_3^- simultaneously in the media, in order to study the growth kinetic of *Pseudomonas* (B). The basal medium + SLA was used as growth media and CH_3COO- was used in a concentration of 20 mM. Again in this kinetic study, it should be stressed the observed preference for chlorate when chlorate and perchlorate are present in the same media. It can also be observed two different acetate uptake rates, near related with chlorate and perchlorate reduction respectively, as it was observed for *Pseudomonas* (A). Once more, a completely conversion of both electron donors into chloride was observed (data not shown). The maximum cell dry weight produced was 1.17 g/L.

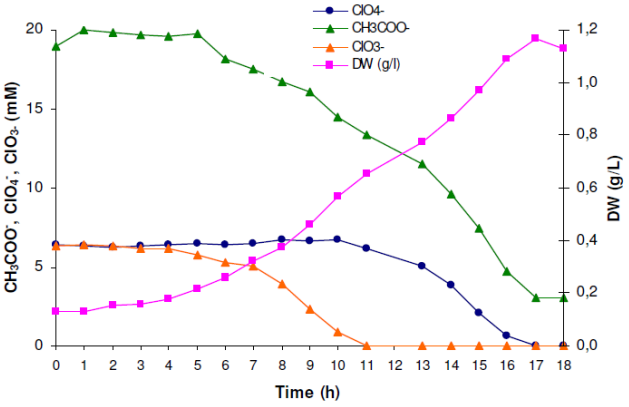

Figure 7. Acetate, perchlorate and chlorate uptake as function of time during the reduction of 5mM of $ClO4^-$ + 5mM of $ClO3^-$ by *Dechlorosoma* sp. PCC. Dry weight (DW) as a function of time is also represented

5. Discussion

Batch cultures of *Pseudomonas* (A) and *Pseudomonas* (B) were performed and kinetic parameters such as specific growth rate (μmax), specific acetate uptake rates (qCH_3COO-), specific perchlorate reduction rates ($qClO_4$-), specific chlorate reduction rates ($qClO_3$-) and biomass yield (g [DW]/ gCH_3COO-) were determined. Comparing *Pseudomonas* (A) with *Pseudomonas* (B) the specific growth rate determined for perchlorate reduction showed no significant difference. However, comparing both isolates during chlorate reduction, *Pseudomonas* (A) showed a specific growth rate higher than *Pseudomonas* (B). Comparing perchlorate and also chlorate reduction for each isolate individually, different values were found. *Pseudomonas* (A) showed higher specific growth rate for chlorate, while *Pseudomonas* (B) showed for perchlorate reduction. The difference found in specific growth rate for chlorate and perchlorate reduction within the same bacteria, possibly will indicate different mechanisms involved in each reduction. However, this result was not yet conclusive to predict if the same enzyme is responsible for both reduction or if different enzymes are present. Regarding the kinetics when both electron acceptors were present, it was observed in *Pseudomonas* (A) an increased of the specific growth rate compared with each electron acceptor separately and a mean value for *Pseudomonas* (B). The specific growth rates values find in this work were also compared with those reported by others. The specific growth rates determined in this study were within the values found in the literature, ranging from 0.07 to 0.26 h-1. Different uptake rates were found for both electron donor and acceptors. The acetate uptake rate for all kinetic studies was always twice than the value for uptake rate of perchlorate or chlorate. This fact means that the ratio acetate for electron acceptor was approximately 2:1 what is in accordance with the literature. Regarding *Pseudomonas* (A) the highest uptake rate was observed for

chlorate as the sole electron acceptor. Conversely, the uptake rates determined for *Pseudomonas* (B) were higher for perchlorate as a sole electron acceptor. This fact was in agreement with the values found for the specific growth rate, as mentioned before. It was also observed for *Pseudomonas* (A) that comparing perchlorate and chlorate uptake rates separately with the ones when both were present, the value for perchlorate reduction was very similar, while for chlorate reduction was half value. In this case it can be suggested that chlorate uptake rate could be influenced by chlorate concentration. On the other hand, perchlorate could have an inhibitor effect over chlorate reduction for the concentration used, although chlorate was preferentially reduced. For *Pseudomonas* (B), the uptake rates found when perchlorate and chlorate were present together at 5 mM each one, were both different compared with the kinetic study with the electrons acceptor present individually. It seems that uptake rate decreased with perchlorate concentration decrease and that the uptake rate increased with chlorate concentration decrease. In this case it can be suggested that both perchlorate and chlorate uptake rate could be influenced by concentration. In all batch test performed it was observed a completely conversion of the electron acceptors used in each kinetic into innocuous chloride, further confirming that chlorite dismutation occurred in the isolates of this study. The biomass yields for acetate (g [DW]/ gCH_3COO-) calculated in this work were generally higher than those reported by others in the literature (Table 2).

Table 2. Biomass yields in the presence of different electron acceptors determined in this study and reported by others

Isolate	Electron Acceptor	Cell yield (g[DW]/g CH_3COO^-	Reference
Pseudomonas (A)	Chlorate Perchlorate	0.45±0.01 0.72±0.02	This study
Pseudomonas (B)	Chlorate Perchlorate	0.84±0.01 0.92±0.02	This study
Alcaligens	Oxygen Chlorate Perchlorate	0.45±0.05 0.48±0.07 0.50±0.08	Logan et al., (2001b)
Bacillus	Oxygen Chlorate Perchlorate	0.25±0.01 0.28±0.02 0.22±0.01	Rikken et al., (1996)
ET79	Oxygen Chlorate	0.15±0.02 0.12±0.02	Olsen S., (1997)

For *Pseudomonas* (B) the biomass yield for acetate was similar for perchlorate and chlorate reduction individually. The same was verified in two other perchlorate reducing bacteria found in the literature, in which no significant changes were found related with perchlorate and chlorate reduction. Concerning *Pseudomonas* (A) the biomass yield determined for chlorate was half value of perchlorate. Among the biomass yield found in the literature it was observed that *Pseudomonas* (A) showed highest values for perchlorate reduction and that *Pseudomonas* (B) showed highest values for chlorate reduction compared with the other isolates. Chlorate accumulation can be explained based on the existence of two enzymes responsible for the conversion of perchlorate into chlorite, in which the conversion of chlorate into chlorite was the rate-limiting step. If a unique enzyme was present, then chlorate accumulation could be explained based on the idea that chlorate affinity decreased when perchlorate is present at 10 mM.

6. Conclusions and Further Research

Two different bacterial species were isolated using two different selection methods. The genetic characterization of 16S rDNA showed that both isolates have already its sequence deposited, but no other description was made. The purity of the isolates was easily confirmed by genetic characterization with 16S rDNA sequence homology. Regarding more characterization of these two isolates, for future work it should be done a 16S rDNA sequence homology for phylogenetic tree construction. This will further allow relating these isolates with other perchlorate reducing bacteria. Concerning description of these bacteria it should be done the G + C content, cytochrome oxidase presence, catalase activity, Gram staining, pH, salinity and temperature range for optimal growth, fatty acid profile, electron donor/acceptor use and also microbial size should be performed. It should be stressed that both bacteria were able to couple complete reduction of the electron acceptors with growth. This was confirmed by the increase of biomass in all kinetics performed. The bacteria isolated showed different perchlorate reduction system. The main evidence was the transient accumulation of chlorate by *Pseudomonas* (A) during perchlorate reduction, which was not observed in *Pseudomonas* (B). However, the results were not conclusive to predict if one enzyme was responsible for both reduction (perchlorate and chlorate) or if different enzymes were present. Chlorate accumulation during perchlorate reduction was hardly studied. For further investigation, the enzymes involved in the perchlorate reduction pathway of these two bacteria, should be purified and studied concerning its biochemical characterization. Furthermore, for future enzymatic studies it will be necessary a large scale biomass production of these bacteria, based in kinetic parameters determined in this research work such as biomass yield. *Pseudomonas* (A) showed the highest biomass yield even compared with other found in the literature. Kinetic studies starting with different perchlorate and chlorate concentration should be done to observe the effect of the initial concentration over the reduction of each electron acceptor. Regarding the batch test done with chlorate and perchlorate present in the same medium, it was observed the preference for chlorate over perchlorate in both bacteria. This observation could also indicate that chlorate inhibit perchlorate at 5 mM, although this finding was not in agreement with chlorate accumulation observed during perchlorate reduction in the first test. For a better understanding of the effect of chlorate during perchlorate reduction it should be test chlorate spike during perchlorate reduction.

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