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Heterotrophic denitrification by *Enterobacter hormaechei* collected from wastewater treatment plant in presence of methanol and sodium-succinate

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Abstract

Heterotrophic denitrification is one of the most selective and advanced method for nitrate removal, it is usually inhibited by insufficient carbon sources. From the wastewater treatment plant (WWTP) of El Menia (Constantine), Algeria, five bacterial strains were isolated but only the strains N5 and N6 were identified. The strains were preliminarily identified using biochemical tests and then a method based on the similarity of the PCR-16S rRNA. 16S rRNA gene sequence analysis indicated that both strains were most closely related to *Enterobacter hormaechei* with sequence similarities of 99%. Both strains showed an excellent capability to remove nitrate and to transform carbon substances namely methanol and sodium-succinate to carbon dioxide. This process was accompanied by a slight rise in pH with a simultaneous production of nitrogen gas as the end product at 35°C. Nitrate was removed after 48 h of incubation. Thus, the anaerobic denitrification ability of strains N5 and N6 has been proved. Sodium-succinate is the most favorable carbon source for denitrification by both strains (N5 and N6). Furthermore, strains N5 and N6 could effectively remove nitrate at low C/N ratios and under conditions where the temperature is elevated, which would benefit nitrate removal from the wastewater.

1. Introduction

The problem of water resources shortage is increasingly recurring, so that the reuse of wastewater after appropriate treatment has became a global consensus [1]. With population growth, the intensive use of nitrogen fertilizers and livestock waste in agriculture led to an accumulation of nitrate in water, which causes the degradation of their quality. Furthermore, a high concentration of nitrates in rivers will lead to an increase in the eutrophication of ecosystems [2]. Moreover, the consumption of this water will result in the occurrence of methemoglobinemia in humans, this has incited the World Health Organization (WHO) to classify nitrate as a dangerous pollutant threatening the public health and fixed its higher acceptable limit to 50mg.L^{-1} N-NO_3^- [3].

The removal of nitrates is based on various techniques and physicochemical processes: ion exchange, reverse osmosis and biological treatments, specifically, heterotrophic or autotrophic denitrification. However, biological denitrification has been proven to be one of the more advanced high-performance methods, the sole selective low cost method for complete nitrate elimination [4-5]. Heterotrophic denitrifying bacteria have a higher growth rate than autotrophic bacteria [6] and are the most abundant type of denitrifiers found in the natural environment [7].

This process requires the use of a final acceptor for the electrons transferred along a respiratory chain [8]. Biological removal of nitrate is widely used in the treatment of domestic and complex industrial wastewaters [9]. Most of the reported biological denitrification methods rely on heterotrophic bacteria that require an organic carbon source, like methanol which is one of external sources that has been used for this purpose [10-11]. It is also the most common source of organic carbon added to support biological denitrification. Denitrification was believed to be a strictly anaerobic process [12]. The biological denitrification is a process carried out by numerous types of bacteria. A great number of heterotrophic bacteria taxonomically and physiologically different are able to denitrify [13]. The main genera associated with the denitrification are: *Actinomyces*,

Aeromonas, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Bacteroides*, *Enterobacter*, *Escherichia*, *Pseudomonas*, *Rhizobium*, *Salmonella* and *Staphylococcus*. Many studies on the diversity of denitrifying bacteria are based on the amplification of functional genes of denitrification. These genes may be genes of the periplasmic or membrane-bound nitrate reductases (*napA* and *narG*, respectively) [14-15], or nitrite reductase *nirS* and *nirK* [16]. These studies revealed a significant diversity of denitrification genes in the environment, which are often divergent from denitrifying bacteria in culture [15, 17].

The main objective of this study is to isolate and to characterize some bacterial strains capable of completely removing nitrate and COD simultaneously and efficiently from a synthetic liquid medium using methanol and succinate as carbon sources, in a batch culture. Furthermore, determination of the optimal C/N ratio to achieve a high denitrification yield is also considered.

2. Material and Methods

2.1. Microorganisms isolation and culture media

The wastewater samples were collected from the anaerobic sludge of the sewage treatment plant of El Menia, Constantine. The samples were refrigerated at 4°C and keep for future use. Isolation of microorganisms was achieved after a serial dilution of the sample in normal saline within the range of 10^{-2}-10^{-5} and plating on mineral agar medium containing (g/l): K_2HPO_4, 1.00; KH_2PO_4, 1.00; KNO_3, 1.00; NaCl, 1.00; $MgSO_4$, 0.20; $CaCl_2$, 0.02 and agar, 15.00 in 1000 mL of deionized water ; pH was adjusted to 7.2 [18]. The inoculated media were incubated with constant shaking (120 rpm) at 35°C, until visible colonies were formed (48 hours). Different forms of colonies were distinguished on the plate's surface. In order to compare the isolated microorganisms and to select the best denitrifying one, a liquid batch test was carried out in 500 mL reactors in anaerobic conditions. The initial NO_3–N concentration was 300 mg.L^{-1} and the carbon sources (methanol and sodium succinate) were added separately to the mineral medium at a final concentration of 1 mg.L^{-1}.The denitrification under the batch tests was checked with different isolated microorganisms.

2.2. Nitrate removal

Experiments were carried out in 500 ml batch reactors to test nitrate reduction. Each clean sterile reactor was filled with 250 ml of the mineral medium supplemented with the appropriate carbon source and 10 ml of the biomass suspension. The initial NO_3–N concentration was 300 mg.L^{-1} and carbon sources (methanol and sodium succinate) were added separately to the mineral medium at a final concentration of 1 mg.L^{-1}. The reactor was operated at anaerobic conditions with the denitrifier with constant shaking (120 rpm). Prior to inoculation, helium was bubbled to remove air and 5% acetylene was introduced to inhibit any oxide nitrate reductase activity [19]. The C/N ratio was kept constant at 3/2 and temperature was set at 35°C throughout the whole work accompanied with pH monitoring. Nitrate removal and effect of carbon source were monitored separately. On the other hand, for the study of the effect of C/N ratio, each batch reactor was inoculated with the N5 strain, the medium contained different C/N ratios (0.6, 1.0 and 3.0) with methanol and sodium succinate as a source of carbon and energy separately), pH was also monitored.

2.3. Identification of the denitrifying isolates

Bacteria identification was initially performed with biochemical tests, as suggested by the Bergey's Manual of Determinative Bacteriology [20] using an API system E kit. Molecular identification after amplification and sequencing of the 16S rRNA gene was performed. The bacterial 16S rRNA genes of the isolated strains (N5 and N6) were amplified by PCR using the universal primer pair: S-D-Bact-0008-S (5'AGAGTTTGATCCTGGCTCAG-3') and S-D-Bact1495-(5'CTACGGCTACCTTGTTACGA-3'). 16SrRNA sequences were compared to the sequences available in the Genbank database using the BLASTn program at NCBI (National Centre for Biotechnology Information).

2.4. Analytical methods

Bacterial growth monitoring was performed spectrophotometrically at 600nm [21]. The samples were centrifuged for 20 min at 7000 rpm (Sigma 1-15,).Thus; the cell-free supernatants were used for nitrate and Chemical Oxygen Demand (COD) analysis, using Standard Methods [22].

Nitrate concentration was monitored spectrophotometrically at λ=415 nm (Safas Monaco), by a colorimetric method using sodium salicylate [23], while the chemical oxygen demand (COD) was measured by introducing into a tube 3 ml of the concentrated acid solution H_2SO_4 (d=1.83), containing 6.6 g/L of $AgSO_4$), 0.1 g of pure $HgSO_4$ powder, 2 ml of sample, 1 ml of the potassium dichromate solution (2.4518g.L^{-1}). The tubes were hermetically closed and homogenized then placed in the COD reactor, the final analysis was carried

out spectrophotometrically at λ=340 nm [23]. The pH was regularly monitored throughout the trials using a pH-meter (HANNA instruments).

3. Results and discussion

3.1. Isolation and screening of the denitrifying bacteria

A total of five bacterial isolates were collected from the WWTP of El Menia (Constantine) based on their ability to grow on MMS. In order to estimate the ability of the five bacterial isolates to completely remove nitrate from the medium, their growth in presence of methanol and sodium-succinate separately was first monitored in parallel to nitrate removal and pH change. The results on bacterial growth monitoring are shown in figure 1 (a, b). The pattern of the bacterial growth of the five isolates corresponds to a typical growth curve in a batch culture; however, they displayed different specific growth rates (μ). The highest were recorded for N5 and N6 isolates to be equivalent to 0.07 h^{-1} and 0.06 h^{-1}, in presence of methanol, and 0.09 h^{-1} and 0.08 h^{-1}, in presence of sodium-succinate, respectively. Higher growth rates were obtained when succinate was used as a carbon source; this is due to the fact that succinate is a key intermediate metabolite of the tricarboxylic acid pathway (TCA), suggesting that it was readily utilized for the growth [24-25].

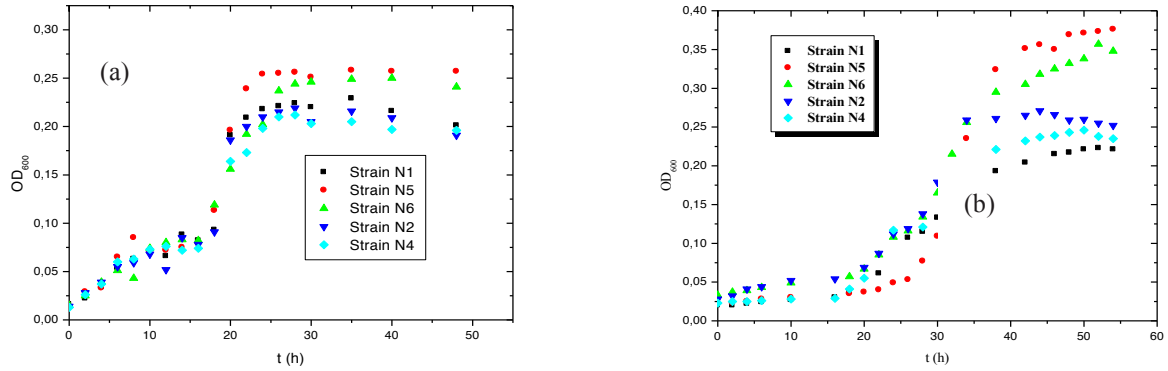

Figure 1: Growth kinetics of the isolated strains with different sources of carbon. (a) Methanol. (b) Sodium-succinate

3.2. Nitrate removal performance

The nitrate removal rate was studied in a batch reactor of 500 ml for 72 hours (Figure 2) using a spectrophotometer. The results obtained using methanol and sodium-succinate separately showed a complete nitrate removal after 56 hours and 48 hours, respectively for the strain N5; whereas with the strain N6, a nitrate concentration of 25.6 $mg.L^{-1}$, corresponding to a biodegradation rate of 91.67%, was observed in presence of methanol (Figure 2a). On the other hand, and in presence of sodium-succinate, the concentration of the residual nitrate was 22 $mg.L^{-1}$ with the strain N6, which correspond to a removal rate of about 92.67% (Figure 2b).

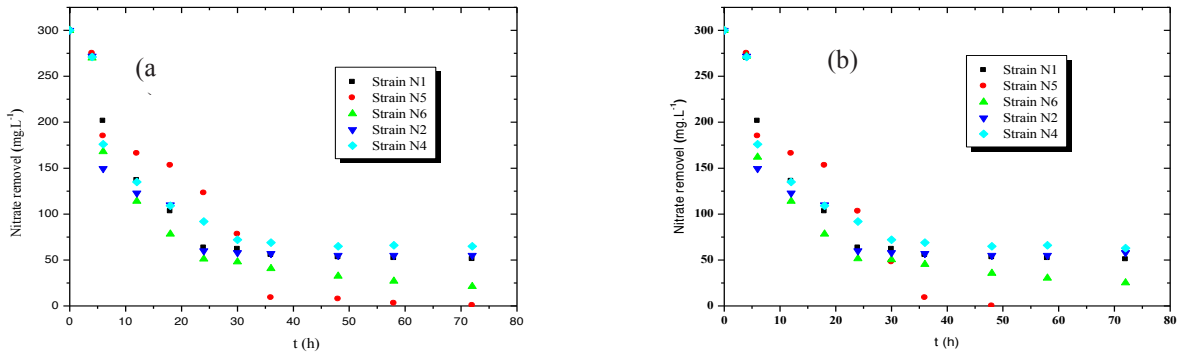

Figure 2: Anaerobic removal of nitrate by the isolated bacterial strains in presence of two carbon sources separately. (a) Methanol and (b) Sodium-succinate

The obtained removal rate is higher than many reported in the literature, for example, Pungrasmi et al reached an average nitrate removal efficiency of 85.17% using methanol at a COD:N ratio of 5:1 in a batch system [26]. The yield of the process was better when succinate was used as carbon source with both isolates (N5 and N6). Therefore, it could be suggested that, of the two tested carbon sources, sodium-succinate is more appropriate to be used for the treatment of wastewater contaminated with nitrate.

For strain N 5, it could be noticed that the bacterial growth ends after almost total nitrate is consumed. therefore, it is suggested that denitrification here is an alternative to conventional oxygen respiration and nitrates are then used as final electron acceptors which are transferred along the respiratory chain [8, 18]. However, the other strains, grew and reached a stationnary phase which coincides with a stabilization in nitrate levels, this could be attributed to the carbon source depletion. The age of cell culture is another factor that influences nitrate removal rate, indeed, the performance of the process is greater in early growth phase (from 0 to 30 hours) (figure 2), consequently, fresh culture has to be used in microorganisms-based biodepollution processes. Furthermore, prior adapatation to carbon sources like methanol and ethanol, could effectively enhance nitrate removal [27].

pH is one of the most important parameters that affect the denitrification process. pH monitoring revealed that it varies from 6.5 to 8.5 (Figure 3a and b) throughout the denitrification period. The slight increase in pH during the 20 first hours of incubation resulted from the depletion of NO_3^- [28]. Based on the obtained results, the isolates N5 and N6 were selected to be identified and further tested for their ability to reduce nitrates since they showed the highest denitrification activity and a total nitrate removal, in least than 48 hours.

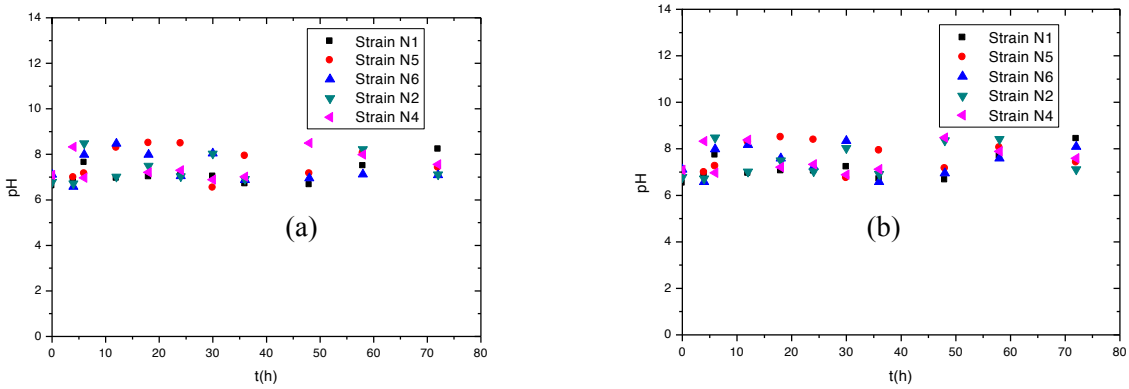

Figure 3: Monitoring of pH with carbon sources separately. (a) Methanol. (b) Sodium succinate.

3.2. Taxonomic identification of the N5 and N6 isolates

The morphological and biochemical profile of the selected bacterial isolates is presented in table 1.

Table 1: Morphological and biochemical profile of the two bacterial isolates (N5 and N6) using API 20 kits

Sr.No.	STRAIN CODE(Test)	N5 isolate	N6 isolate
1.	Cell shapes (Morphology)	Rod	Rod
2.	Gram	-	-
3.	Citrate (CIT)	+	+
4.	Indole (IND)	-	-
5.	Methyl Red (MR)	+	+
6.	Voges-Proskauer (VP)	-	-
7.	H_2S Production	-	-
8.	Catalase	+	+
9.	ADH (Arginie Dehydrogenase)	+	+
10.	Urease (URE)	-	-
11.	Amylase (AMY) (Starch Hydrolysis)	+	-
12.	Gelatinase (GEL)	+	+
13.	LDC	+	+
14.	ONPG	+	+
15.	TDA	-	-
16.	ODC	+	+
17.	INO	+	+/-
18.	RHA	+	+
19.	Sugar Utilization Test	Sugar Utilization Test	Sugar Utilization Test
a)	Glucose	+	+
b)	Mannitol	+	+
c)	Sucrose SAC	+	+

The cells of the isolate N5 are gram-negative motile rods, catalase positive that ferment glucose, surose, mannitol, sorbitol and arabinose. Indole and urease are not produced. Moreover, the 16S rRNA gene sequences of N5 and N6 were aligned with the sequences deposited in the GenBank database using a BLAST program. The phylogenetic tree (figure 4) was generated by Tree Dyn program (v198.3) proposed by Methods and Algorithms for Bioinformatics [29]. Results revealed that 16S rRNA gene sequences of both isolates were 99% identical to *Enterobacter hormaechei*. The given accession numbers were KM604662 and KM604663, for isolates N5 and N6, respectively. Bacterial denitrifying strains belonging to the genus *Enterobacter* are poorly investigated, a strain identified as *Enterobacter cloacae* was isolated from activated sludge, it removed almost total nitrate aerobically with an initial NO_3^--N concentration of 110 mg/L within 30 h [30].

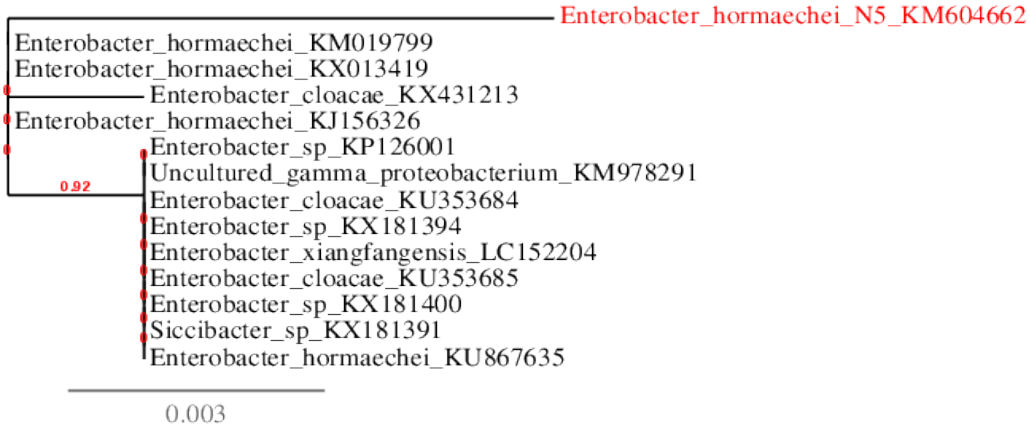

Figure 4: Phylogenetic tree for *Enterobacter hormaechei* N5 (accession number KM604662) obtained from 16S rDNA sequence analysis. The tree was generated by the neighbour-joining method using tree Dyn program [29].

3.3. Effect of C/N ratio on heterotrophic nitrate removal

Bacterial growth evolution, the denitrification potential and pH were monitored for the two carbon sources in presence of different C/N ratios ranging from 0.6 to 3.0. C/N is a factor influencing on the amount of metabolites. The higher the carbon source concentration is, the faster the denitrification will be [31]. In addition, different C/N can result in different biochemical process and metabolites in denitrification process, it then influences on the nitrate removal rate. Cell growth of the strain *E. hormaechei* N5 showed higher levels with sodium-succinate at C/N=3.0 (Figure 5a), whereas with methanol at C/N=0.6, the growth rate was maximal (Figure 6a).

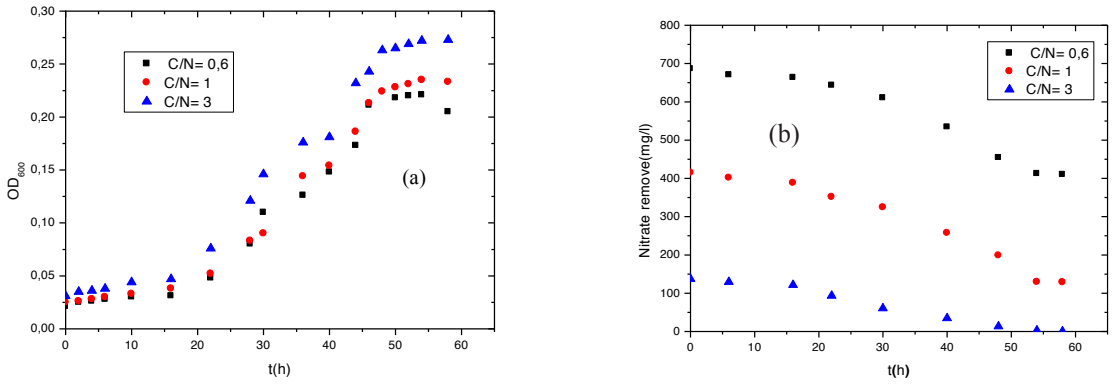

Figure 5: Effect of C/N ratio on the efficiency of nitrate removal by *Enterobacter hormaechei* N5 after incubation with sodium-succinate at 35 ° C. The data are averages of three experiments. (a) Cell growth; (b) C/N nitrate removal ratio

Furthermore, a complete nitrate reduction by the strain N5 was obtained after 58 hours of treatment with sodium-succinate giving a yield of 98.54% denitrification when C/N ratio was equal to 3, these results are in agreement with those found [32]. On the other hand, in presence of methanol, after 68 hours of treatment, a yield of 97.23% was shown when a C/N ratio equal to 1.0 was used. Therefore, the best C/N ratio of nitrate removal was equal to 3.0 when using sodium-succinate. Similarly, this ratio was also favourable according to Kim et al. [33]. In these conditions, the pH varied between 6.8 and 8.5, as seen in Figure 7a. The pH value has increased relatively in the denitrification process (Figure 7a).

The nitrate removal efficiency is closely related to the organic carbon source. Whatever was the nature of the carbon source, determination of the utilized carbon to the removed nitrate ratio is a preliminary prerequisite step to all optimization processes of biological treatment by denitrification [34]. The theoretical stoichiometric equations for the denitrification with methanol as carbon source were defined by Cheng and Lin [35]. This equations estimate that a C/N ratio of 0.71 is necessary for complete nitrate reduction to molecular nitrogen. Our results suggested that C/N ratios for denitrification with methanol and sodium-sucinate were of 1.0 and 3.0, respectively. From figure 5a, it can be supposed that the methanol stimulated the growth of the methanol-utilizing denitrifiers, which improved their capability to use it (figure 5b), however, it reduced their potential to use wastewater COD for denitrification (figure 7b), these results are in agreement with those of Shao et al. [36].

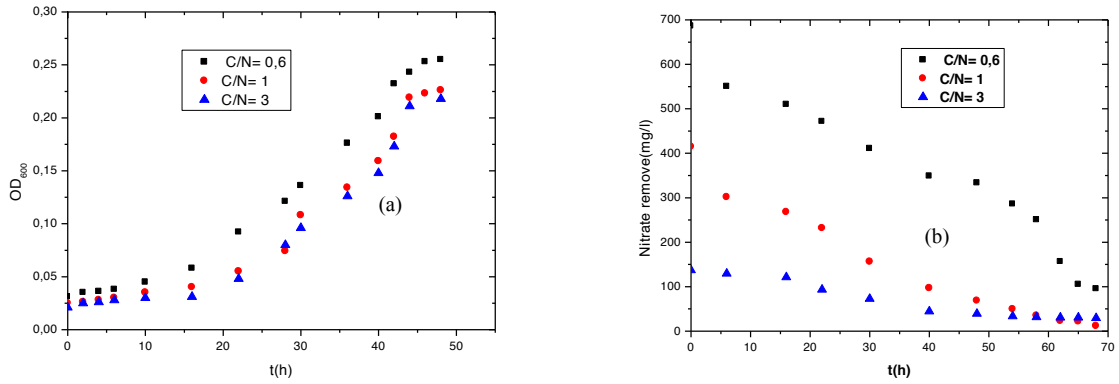

Figure 6: Effect of C/N ratio on the efficiency of nitrate removal by *Enterobacter hormaechei* N5, after incubation with methanol at 35 ° C. The data are averages of three experiments. (a) Cell growth; (b) C/N nitrate removal ratio

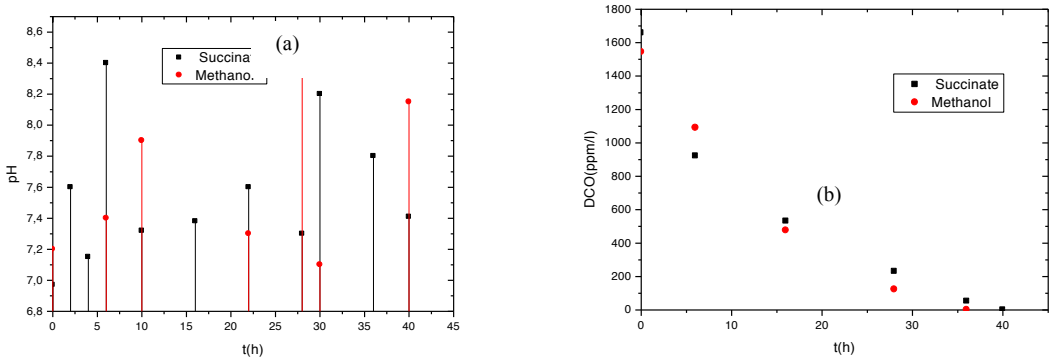

Figure 7: Effect of optimal C/N ratio (C/N=3.0 and 1.0) for sodium succinate and methanol, respectively on: (a) pH variation, (b) Dissolved oxygen (COD). The data are averages of three experiments.

Conclusion

According to our results, the two isolated strains belonging to the genus *Enterobacter* showed a significant ability to remove nitrates. *Enterobacter hormaechei* N5 in presence of sodium-succinate yields to a very high rate of denitrification (98.7%) at a C/N ration of 3.0 when compared to methanol. The results are promising suggesting that the isolated strains could be considered as potent candidates to form consortium for bio-denitrification of wastewater.

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