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Nitrate Reduction, Sulfate Reduction and Methanogenesis Interrelation in Fixed and Suspended Bed Batch Reactors

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Abstract: This study was focused on the interrelation among nitrate reduction, sulfate reduction and methanogenesis in fixed bed and suspended reactors. Dried stems of the cactus *Opuntia imbricata* were used as substratum for biofilm growth. Three anaerobic treatment systems: Biofilm and Macerated Anaerobic Sludge (BMAS), Biofilm formed by macerated anaerobic sludge (B) and Macerated Anaerobic Sludge (MAS) were studied. Experiments were carried out in batch reactors containing 250 mL of synthetic wastewater (0.5, 1.2 and 7.6 g/L of nitrate, sulfate and acetate, respectively). The nitrate reducing rate was of 6.0, 5.9 and 3.29 g NO_3^- $l^{-1}d^{-1}\times10^{-1}$ for BMAS, B and MAS systems, respectively. The sulfate reducing rate was two times higher for BMAS and B systems (11.5 and 11.3 g SO_4^{2-} l^{-1} $d^{-1}\times10^{-2}$, respectively) than MAS system (4.8 g SO_4^{2-} l^{-1} $d^{-1}\times10^{-2}$). Methanogenic rate was 1.5 times higher for BMAS and B systems (4.2 and 3.8 g CH_4 l^{-1} d^{-1} $\times10^{-1}$, respectively) than MAS system (2.15 g CH_4 l^{-1} d^{-1} $\times10^{-1}$). In MAS system, the processes were not simultaneous but in stages, contrary to observed in the removal of nitrate, sulfate and organic matter in the biofilm containing systems. Biofilm containing systems carried out nitrate reduction, sulfate reduction and methanogenesis processes more efficiently than MAS system.

Key words: Biofilm system, macerated anaerobic sludge, *Opuntia imbricata*, substratum

INTRODUCTION

Sulfate reduction, methane production and denitrification are important processes responsible for the terminal electron removal during decomposition of organic matter in anaerobic environments. Sulfate reduction, nitrate reduction and methanogenesis are governed by several factors, such as, the nature of carbon source, COD:SO_4^{2-} and COD:NO_3 ratio, pH, concentration of sulfate or nitrate (it is well known, that the sulfate and nitrate are indirect inhibitors of both process, methanogenesis and sulfate reduction), redox potential, temperature, microbial populations, kinetic and thermodynamic competitions (Akunna *et al*., 1998; Chen *et al*., 2008; Her and Huang, 1995; Percheron *et al*., 1999; Stams *et al*., 2003; Wiesmann, 1994).

Some researches in this area have focused on the use of immobilized cells and biofilms on different supports to improve the processes efficiency, increasing biomineralization and biodegradation percentage. Several synthetic and natural materials have been used as substratum for biomass attachment for anaerobic digestion. These materials are differentiated based on their surface roughness and porosity (Andersson *et al*., 2008; Camargo and Nour, 2001; Cao *et al*., 2002; Celis-García *et al*., 2004; Gomez *et al*., 2003; Hanaki *et al*., 1994; He *et al*., 2010; Kus and Wiesmann, 1995; Meraz *et al*., 1995; Mohanakrishnan *et al*., 2009, Mohanakrishnan *et al*., 2011; Muñoz *et al*., 1997; Qian *et al*., 2001; Silva *et al*., 2006; Sorlini *et al*., 1990; Xia *et al*., 2010). Mixed populations of microorganisms, which are fixed on substratums and form biofilms or biomass aggregates are advantageous in various aspects: high biomass concentration by accumulation of nutrients in biofilm matrix, microbial growth while mediating simultaneously a variety of biological reactions, capacity to handle full scale flow rates, smaller reactor volumes and consequently smaller area required due to high organic loading rates, high biomass age and minimization of excess sludge production (Andersson *et al*., 2008; Skiadas *et al*., 2003).

The aim of this study was to determine the behavior of the nitrate reduction, sulfate reduction and methanogenesis using dry stems of *Opuntia*

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imbricata as substratum. Fixed bed batch reactors where compared with suspended bed batch reactors.

MATERIALS AND METHODS

Macerated anaerobic sludge (MAS): Granular sludge obtained from an anaerobic digester treating brewery wastewater was used as inocolum. This sludge was macerated in a blender under nitrogen bubbling conditions. The reactors were inoculated with macerated anaerobic sludge (3 g/L VSS-Volatile Suspended Solids).

Substratum: Dry canes of *Opuntia imbricata* were recollected and used for this purpose. *Opuntia imbricate* is a cactus found in arid lands of South USA and North of Mexico, which is highly rough and porous (dry state). Canes were cut in pieces (7 cm × 1 cm × 0.4 cm aprox.), subsequently were brushed, washed and autoclaved at 121ºC (252ºF) and 1.4 bar (20 psi). SEM Micrographs were taken with a Leica-Cambridge electron microscope model Stereoscan 440. The samples of substratum were taken before inoculation with MAS and after biofilm formation (at 40th day).

Synthetic wastewater (SW): 0.5, 1.2 and 7.6 g/L of nitrate, sulfate and acetate (as COD) respectively were diluted in deionized water. The pH was adjusted 7.0 with NaOH and HCl solutions (1 M).

Biofilm formation: Fifteen batch reactors with a volume of 500 mL were inoculated with 2 g/L VSS macerated sludge, 200 mL of inducer medium (mix of 10 ml of mineral salt medium ($(NH_4)_2SO_4$: 2 g, Na_2HPO_4: 3.61 g, KH_2PO_4: 1.75 g, $MgSO_4,7H_2O$: 0.2 g, $CaCl_2$: 50 mg, $FeSO_4,7H_2O$: 1 mg, $CuSO_4,5H_2O$: 50 mg, H_3BO_3: 10 mg, $MnSO_4,5H_2O$: 10 mg, $ZnSO_4,7H_2O$: 70 mg, $(NH_4)6Mo_7O_{24},4H_2O$: 10 mg per liter of distilled water) and 190 mL of SW) and 50 g of substratum. The initial pH in all reactors was 7.0, the incubation temperature was 37ºC. To allow the growth of a biofilm, the reactors were incubated for 40 days.

Experimental setup: After 40 days, in BMAS system the inducer media was removed, biofilm and macerated anaerobic sludge remained; in B system, macerated anaerobic sludge (non attached on *Opuntia imbricata*) and inducer media were withdrawn from reactor washing with distilled water (B); MAS system only kept macerated anaerobic sludge, removing the inducer media and withdrawing the pieces of substratum. After this, each reactor was fed with 250 mL of synthetic wastewater, incubated at 37ºC and monitored under these conditions. Each system was replicated five times. Five reactors with 50 g of substratum without sludge were monitored to discard adsorption of synthetic water components by the substratum.

Analytical methods: Total sulfides (hydric and gas phase), sulfate, nitrate, nitrite, ammonium, COD and VSS (macerated anaerobic sludge and detached biomass) were determined by standard methods (APHA *et al*., 1998). For biomass detachment from the substratum, all stems pieces were removed from the reactor and placed in glass flask with 200 mL of phosphate buffer solution. The flasks were agitated during 30 min. Finally, the VSS content of biofilm was determined.

Methane was measured by gas chromatography equipped with thermal conductivity detector using helium as carrier gas. pH was determined by a conventional glass electrode. Nitrate reducing, sulfate reducing and methanogenic activities were assessed taking into account the consumption rate of nitrate and sulfate and formation rate of methane per gram sludge (VSS) per day.

Statistical analyses: Statistical analyses of the results were performed using SAS (1996). Analysis of variance was carried out using a data set of five replicates. The resultant data were statistically evaluated applying ANOVA at a 5% level of significance. Duncan's critical range tests (DCRT) were used to determine the significant difference between dependent variables.

RESULTS AND DISCUSSION

No adsorption of synthetic wastewater components by the substratum was observed in controls. The substratum used in the experiments is highly rough and porous (Fig. 1a), and these characteristics support adherence of microorganisms and biofilm formation. Fig. 1b shows microorganisms attached to substratum, although treatment given to samples in this case before taking the scanning electron micrographs tended to damage the integrity of biofilm grown on the substratum.

Nitrate was completely consumed after 26 h in the BMAS and B systems, while it took 44 h in the MAS system. Seven hours lag phase was observed in the MAS system, while there was no lag phase in the other systems (Fig. 2). In spite of the nitrite formation and consumption in BMAS and B systems were similar. The nitrite concentration formed was higher in MAS than BMAS and B systems, in spite of this; it was completely consumed in all systems at 100 h. The behavior of nitrite in MAS system was consistent with results reported by Akunna *et al.* (1994). They carried out a research in batch reactors employing was used on this study (Fig. 3). Nitrate reduction results, indicate that it is metabolized by the route of anaerobic sludge at different ratio concentrations

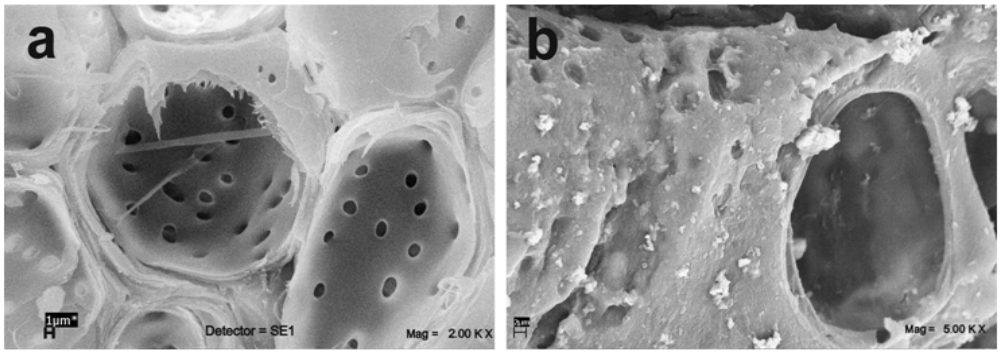

Fig. 1: Scanning electron micrographs, (a) *Opuntia imbricata* (magnification 2000), and (b) biofilm formed on *Opuntia imbricata* (magnification 5000)

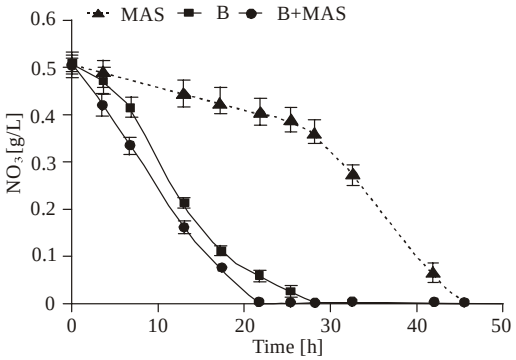

Fig. 2: Nitrate consumption in three systems; B+MAS: Biofilm with macerated anaerobic sludge, B: Biofilm without macerated anaerobic sludge, MAS: Macerated anaerobic sludge.

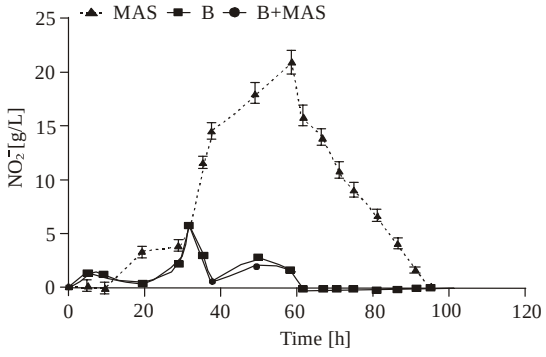

Fig. 3: Nitrite formation and consumption in three systems; B+MAS: Biofilm with macerated anaerobic sludge, B: biofilm without Macerated anaerobic sludge, MAS: macerated anaerobic sludge

COD/NO_3^- 15.7 (2450 mg/L of COD and 156 mg/L of NO_3), 7.6 (2450 mg/L of COD and 312 mg NO_3/L), and 3.9 (2450 mg/L of COD and 635 mg NO_3/L). For the case

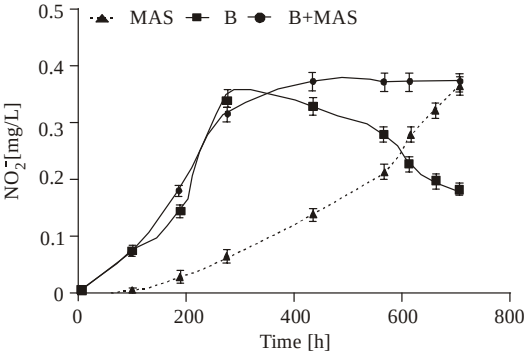

Fig. 4: Sulfide formation in three systems; B+MAS: Biofilm with macerated anaerobic sludge, B: Biofilm without macerated anaerobic sludge, MAS: Macerated anaerobic sludge

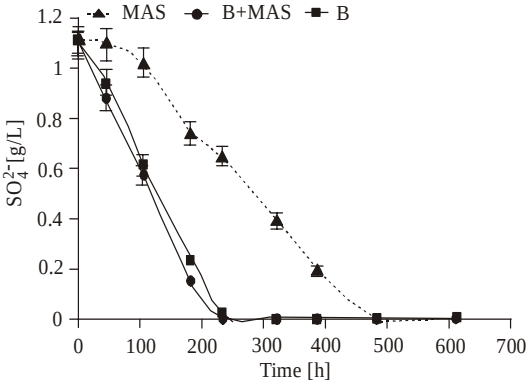

Fig. 5: Sulfate consumption in three systems; B+MAS: Biofilm with macerated anaerobic sludge, B: Biofilm without macerated anaerobic sludge, MAS: Bacerated anaerobic sludge

of 15.7 ratio they reported that nitrite was totally consumed after 120 h, this ratio concentration was similar

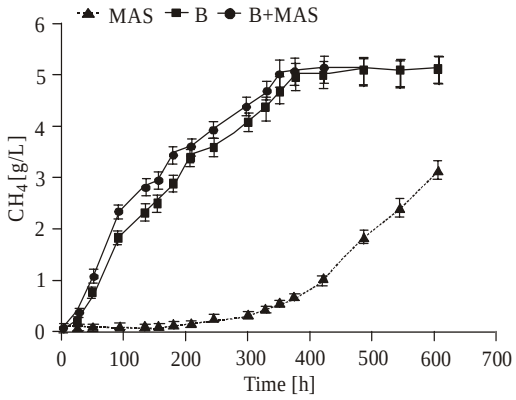

Fig. 6: Methane formation in three systems; B+MAS: biofilm with macerated anaerobic sludge, B: biofilm without macerated anaerobic sludge, MAS: macerated anaerobic sludge

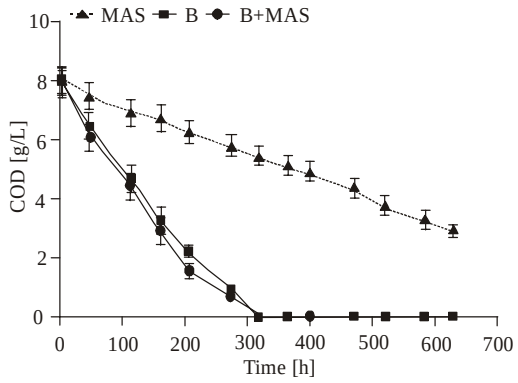

Fig. 7: COD consumption in three systems; B+MAS: biofilm with macerated anaerobic sludge, B: biofilm without macerated anaerobic sludge, MAS: macerated anaerobic sludge

to 16.4 (8200 mg/L of COD and 500 mg/L of NO_3) that dissimilatory reduction from nitrate to ammonium (DNRA) due to the fact of which was detected NH_4^+. Expression of denitrification pathway cannot be discard due to amount of ammonium form was not as expected (theoretically), although N_2 was not determined. BMAS and MAS systems were governed by DNRA since 85 and 128 mg/L of NH_4^+ respectively were detected. On the other hand, in B system, a smaller quantity of NH_4^+ (63 mg/L) was observed, but it diminished to 7.5 mg/L (behavior not detected in other systems). In this case, it must be consider as an assimilative nitrate reduction due to at the same time also diminished S^{2-} concentration from 350 to 200 mg/L (Fig. 4).

Sulfate was completely consumed approximately at 250 h in BMAS and B systems, and at 520 h in MAS system (Fig. 5). It is well known that sulfate reduction and methanogenesis are inhibited by nitrate (He *et al*., 2010; Mohanakrishnan *et al*., 2011; Percheron *et al*., 1999; Xia *et al*., 2010), because thermodynamically nitrate reduction is more favorable than the other two processes and the intermediate compound are toxic for these.

Other reasons for which nitrate inhibits sulfide and methane production are the increase in the redox potential of the media and the toxic effects of intermediate of denitrification (Mohanakrishnan *et al*., 2009; Scholten and Stams, 1995). However, the sulfide production was less inhibited in the biofilm containing systems compared to the MAS system (Fig. 4). This could be explained due to that was detected a low concentration of accumulated nitrite of 6 mg/L in the biofilm containing systems compared to 21 mg/L in the MAS systems, also ammonium was detected earlier in the biofilm containing systems, in which the intermediates of nitrate reduction were consumed faster.

It has been demonstrated the methanogenesis inhibition by sulfide using anaerobic granular sludge (Zhou and Fang, 1998), even though also has been demonstrated that biofilms systems showed a high tolerance to sulfide present in media (Hanaki *et al*., 1994; Muñoz *et al*., 1997). In the BMAS and B systems, the methane formation occurred more rapidly, where lag phase was not observed, whereas in MAS system this phase was approximately of 260 h (Fig. 6). Scholten and Stams (1995) observed a lag phase of 240 h in the methane formation with electron acceptors (nitrate or sulfate) and without electron acceptors in the media using freshwater sediment.

Approximately at 340 h, the acetate concentration (measured by COD) was totally consumed in the BMAS and B systems, whereas at 670 h in MAS system only reached 66.2% (Fig. 7). This result achieved in biofilm reactors was product of a simultaneous nitrate reduction, sulfate reduction and methanogenesis, which required a protons donor (acetate). Alvarado-Lassman *et al*. (2006) reported that in an inverse fluidized bed reactor with an anaerobic biofilm allowed the expression of denitrification and methanization activities simultaneously without physical or time separation.

Nitrate reducing rate and activity (NRR and NRA), sulfate reducing rate and activity (SRR and SRA) and methanogenic rate and activity (MR and MA) were remarkably superior in BMAS and B systems than in MAS system. The three activities in B system were increased drastically due to the low concentration of biomass (VSS), which came from biofilm, in spite of BMAS and B systems rates were similar (Table 1). While the NRR increased 1.5 times, the SRR and MR increased 2.3 and 1.6 times in BMAS and B systems in comparison with MAS system. It is important to emphasize that according to figures discussed above, in MAS system the processes occurred in stages. Nitrate reduction was practically the first stage, which finished at 44 h, in this

Table 1: Rates and activities obtained for the three systems

	Nitrate Reduction		Sulfate Reduction		Methanogenesis			
System	NRR (g NO_3^- $l^{-1}/d^{-1} \times 10^{-1}$)	NRA (g NO_3^- $l^{-1}/d^{-1}/g^{-1}$ VSS $\times 10^{-1}$)	SRR (g SO_4^{2-} $l^{-1}/d^{-1} \times 10^{-2}$)	SRA (g SO_4^{2-} l^{-1} d^{-1}/g^{-1} VSS $\times 10^{-2}$)	MR (g CH_4 l^{-1} $d^{-1} \times 10^{-1}$)	MA (g CH_4 l^{-1} d^{-1}/g^{-1} VSS $\times 10^{-1}$)	VSS (g/L)	Final pH
BMAS	6.0±0.06[a]	2.48±0.03[b]	11.52±0.48[a]	4.76±0.2[b]	4.18±0.22[a]	1.73±0.09[b]	2.42±0.30	8.22±0.30
B	5.9±0.36[a]	22.13±1.30[a]	11.28±2.82[b]	41.8±5.4[a]	3.76±0.28[b]	13.9±1.07[a]	0.27±0.04	8.10±0.49
MAS	3.9±0.27[b]	1.88±0.13[c]	4.8±2.58[c]	2.34±0.3[c]	2.15±0.15[c]	1.05±0.12[c]	2.05±0.21	8.13±0.19

*: Data expressed as means from five replicates; The values within a column followed by the same letter are not significantly different at a 0.05 level according to DCRT

lapse neither sulfate concentration decreased nor was methane production detected. The second stage was sulfate reduction, which started after 50 h and finished at 500 h, and the third stage was methanogenesis, which started at 260 h but was observed a "semi stationary" stage from 260 to 405 h, from 456 to 660 h increase was noticeable. Therefore, the biofilm systems reduce the inhibition caused by effect of toxic substances on the sulfate reducing and methanogenic bacteria. Similar to these results, Hanaki *et al*. (1994) reported that inhibitory effect of phenol, oleic acid, nickel, sulfide and propionic acid on the methanogenic bacteria was reduced by immobilizing the bacteria, this considering that the biofilms are a natural immobilization system on a surface.

CONCLUSION

Nitrate reduction, sulfate reduction and methanogenesis occurred in stages in MAS system, while in BMAS and B systems occurred simultaneously, demonstrating that biofilm systems are efficient for pollutant biodegradation, due to the higher tolerance to stress condition and extreme environment. The present work also demonstrates that *Opuntia imbricata* as substratum for biofilms development favored the anaerobic treatment of nitrate and sulfate rich wastewater. Further research is yet to be done for knowing the behavior of redox potential and other intermediate products such as nitrous and nitric oxides in biofilms.

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NOTATIONS

BMAS : Biofilm with macerated anaerobic sludge
B : Biofilm
MAS : Macerated anaerobic sludge
COD : Chemical oxygen demand
VSS : Volatile suspended solids
SEM : Scanning electron microscope
SW : Synthetic wastewater
DNRA : Dissimilatory nitrate reduction to ammonium
DD : Dissimilatory denitrification
NRR : Nitrate reducing rate
NRA : Nitrate reducing activity
SRR : Ulfate reducing rate
SRA : Sulfate reducing activity
MR : Methanogenic rate
MA : Methanogenic activity
CH_4 : Methane
NH_4^+ : Ammonium
NO_3^- : Nitrate
NO_2^- : Nitrite
SO_4^{2-} : Sulfate
S^{2-} : Sulfide

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