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Treatment of domestic wastewater by anaerobic denitrification: Influence of the type of support media on the production of extracellular polymer substances

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Eighteen Erlenmeyer flask containing six different support media [pozzolan, polyvinyl chloride1 (PVC1), polyvinyl chloride2 (PVC2), foam, polyethylene terephthalate (PET) and polystyrene (PS)] were subject to identical volumetric organic loadings and hydraulic retention time in treating synthetic protein ± carbohydrate waste. The objective was to examine the influence of support media on performance of anaerobic denitrification and retention and their resulting impact on system performance and failure. According to the results relative to every control support media, it was noticed that the best support media were the ones in PVC1 and PVC2, with successive reduction rates of 68.33 and 61.93% for chemical oxygen demand (COD), and 55 and 49% for nitrate. On the other hand, in two submerged anaerobic biofilter reactor packed with the support media of PVC1 and PVC2, the reactor with PVC1 media exhibited 89.93% COD and 78.75% nitrate removal efficiency attributable to its higher production of EPSp and EPSc.

Key words: Wastewater, anaerobic biofilm, extracellular polymeric substances (EPS), extraction, support media.

INTRODUCTION

Biologic denitrification of nitrates and nitrites present in wastewater is important and necessary. It is a process of nitrate and nitrite reduction in which nitrite serves as the terminal exogenous hydrogen acceptor when the oxygen tension in wastewater is sufficiently low. The normal end product of this nitrate and nitrite respiration is elementary nitrogen or nitrous oxide gas, which, being inert can be allowed to escape into the atmosphere (Narjari et al.,

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1984).

The literature containing numerous data has been reported in literature concerning the influence of different denitrification conditions on the rate of the process (Mazierski, 1984). Environmental conditions that must be optimized for denitrification are temperature, pH, and type of carbon substrate. In the present work, the system of biologic treatment was based on the oxidation of organic and nitrogen matter of synthetic wastewater. Micronutrients were added for good performance of microorganisms. Dissolved oxygen, pH, and temperature were monitored for the nitrification process.

Denitrifying bacteria are ubiquitous in nature (Gamble et al., 1977; Zumft, 1992), and biological denitrification treatment consists of the provision of suitable carbon and energy sources which may be organic or inorganic compounds. Treatment can take place in the aquifer, or the water may be pumped into above ground reactors.

Successful biological treatment of wastewater depends on the generation and maintenance of the appropriate sludge. The sludge is a complex dynamic biological structure composed of microflora (bacterial consortium) and micro fauna (mainly protozoa and metazoa). The bacteria naturally produce the extracellular polymeric substances (EPS) which form with bivalent cations a network where microorganisms are embedded (Wingender et al., 1999). Biofilms are dynamic environments, in which the microorganisms are optimally organized to make use of all available nutrients. In the biofilm, the EPS molecules provide the framework into which microbial cells are inserted, which is essential for the development of the architecture matrix (Sutherland, 2001).

Varesche et al. (1997) evaluated the anaerobic biomass attachment onto polyurethane foam matrices taken from the HAIB reactor treating a glucose-based substrate. The authors observed that polyurethane matrices offered excellent conditions for anaerobic growth and retention, due to the low level of microbial organization required by such a support material.

This paper presents some aspects of the influence of substrate on the process of biofilm formation onto polyurethane foam matrices. The results from the quantification of the biomass and extracellular polymers were used to investigate the role of substrate on the biomass adhesion onto polyurethane foam particles.

Substrate utilization in anaerobic filters has often been modelled based on fixed film fundamental. High media surface area seems to be desirable in AF applications for higher growth of biofilm. However, it has been reported that media surface area appears to have only a minor effect on the performance of upflow AFs (Young and Yang, 1989). The EPS are issued from bacteria metabolism and are considered as key components determining the physicochemical and biological properties of flocs and biofilms (Wingender et al., 1999; Flemming and Wingender, 2001).

This study has been initiated to examine the influence of support media on biomass growth and retention; either as suspended growth trapped within the interstitial void spaces or as attached biofilm adhered to the media surfaces in tow laboratory-scale anaerobic filters treating synthetic protein and some carbohydrate waste. The influence of the EPS on elimination of the chemical oxygen demand (COD) and the nitrate in treatment of domestic wastewater by anaerobic denitrification was evaluated quantitatively.

MATERIALS AND METHODS

Wastewater sampling

The origin of the poured residuary water in the sea comes from domestic wastewater or mixed with industrial wastewaters (95 and 5%). Samples of wastewater were collected and stored at 4°C (Figure 1).

Analytical design

Standard methods (AFNOR, 1986) were used for COD, nitrate and pH analyses of the samples (Table 1). The fixed biomass was quantified by the determination of EPS.

Support media used for biofilm adhesion

To study the effect of the nature of the medium on bacterial adhesion, six types of support were tested: pouzzolane irregularly shaped, foam (polyurethane) cube-shaped, two different types of poly vinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS) in small rings, the characteristics are presented in Tables 2 and 3. The development of biofilm on these different media was followed in 250 ml Erlenmeyer flasks (with 3 replicates) containing the medium and the nutrient medium inoculated with denitrifying flora diluted 1/20. The anaerobic condition was ensured by keeping the media submerged, the flasks were kept tightly closed at room temperature. Denitrifying biomass was allowed to develop in the media for 5 days with the monitoring of the denitrification and the addition of KNO_3.

EPS extraction

A range of different chemical and physical approaches have been used to remove EPS from bacterial cell surfaces (Comte et al., 2006). Quantification of the major species of EPS (proteins and polysaccharides) was performed by colorimetric methods. Colorimetric method was based on the color developed by chemical reactions between the chemical functions of the molecule to be assayed and reagents. The color obtained is a function of the concentration of the species to be assayed. Measurement of the absorbance of the color was performed by UV visible spectroscopy.

Quantification of protein

In the case of wastewater, the choice of the most appropriate method for the quantification of proteins is not easy. However, the Lowry method was more frequently used in various scientific studies. The Lowry et al. (1951) method is based on two chemical reactions. First, the biuret reaction which involves the processing of

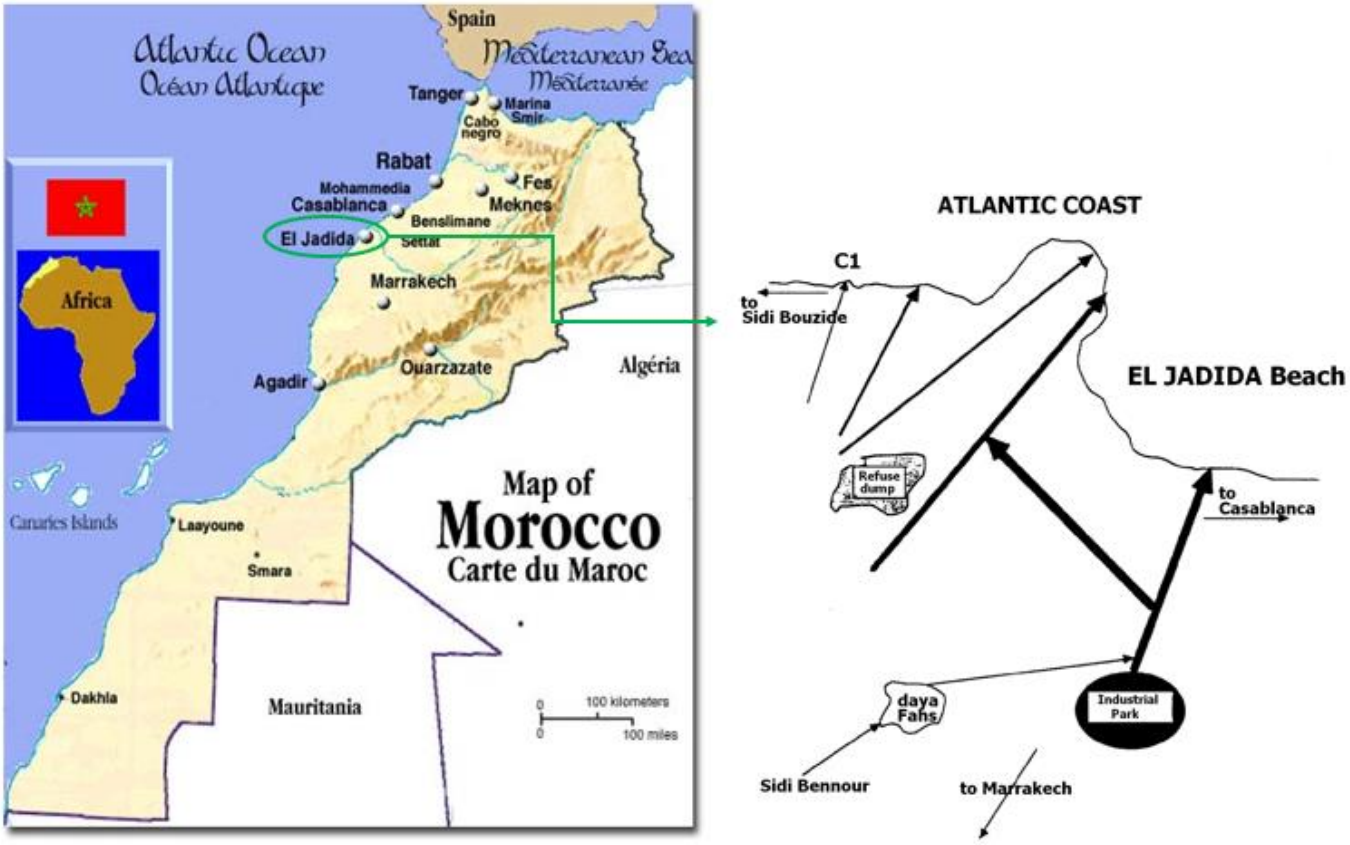

Figure 1. Location of sampling site in study zone: C1 (Lower town collector).

Table 1. Characterization average of pH, temperature, nitrate and COD in the effluent collected at the collector of sewage from the city of El Jadida.

Composition	Minimum	Maximum	Average	Standard deviation
pH	6.2	6.7	6.51	0.17
T°C	20	27	23.9	2.61
COD (mg/L)	545	624	575.2	28.12
Nitrate (NO^-_3) (mg/L)	1.90	4	2.9	0.68

Table 2. Characteristics of packing media in batch mode.

	Materials					
	Pouzzolane	**PVC1**	**PVC2**	**Foam**	**PET**	**PS**
Surface texture	Rigorous	Striated	Smooth	Rigorous	Smooth	Rigorous
Outside diameter (mm)	-	25	17	-	2	-
Height (mm)	12	12	25	18	30	20
Thickness (mm)	-	2	2	-	1	-
Specific surface (m^2/m^3)	115	99	187	292	957	324
Equivalent pore diameter (mm)	3.5	20	11	1	2	2

Table 3. Characteristics of packing media in continuous mode.

Materials		
	PVC1	**PVC2**
Surface texture	Striated	Smooth
Outside diameter (mm)	25	17
Height (mm)	45	25
Thickness (mm)	2	2
Specific surface (m^2/m^3)	99	187
Equivalent pore diameter (mm)	20	11

Figure 2. Experimental batch mode system.

peptide bonds with copper sulfate in alkaline solution. Second, the reduction of the complexes formed was carried out by the Folin-Ciocalteu. As a result, a blue solution was obtained. Measuring the absorbance of the solution formed was carried out by UV-visible spectroscopy at 750 nm.

Quantification of polysaccharides

The method used for the quantification of polysaccharides was that of Dubois al. (1956). In this method the polysaccharides were hydrolyzed through the heating by a strong acid (sulfuric acid). Then, saccharides reacted with the reagent specific to each method. The method of Dubois used phenol. This reagent produced the same intensity of color for all polysaccharides.

Reactor and batch mode system

Batch mode

Eighteen differential Erlenmeyer flask of 250 ml was used to evaluate the process of biofilm formation. Each erlenmeyer was filled with a media type (Figure 2). There, erlenmeyer was subjected to 260 ml of substrate with COD of 624 mg L^{-1} and 0.26 g KNO_3. The experiments were carried out in a batch mode. EPS was extracted from the media containing attached biomass using the cool aqueous extraction techniques (Sutherland and Wilkinson, 1971; Jia et al., 1991). The protein content of EPS (EPS_P) in the supernatant was measured according to the Lowry et al. method (1951) and the carbohydrate content (EPS_C) by the phenol/sulfuric-acid method. The sum of EPS_P and EPS_C represents the total EPS of the sludge.

Continuous mode

Tow 64.5 L columns packed with PVC1 and PVC2 ring was used as the anaerobic filters. Each reactor was 0.25 m in diameter and 1.05 m height, providing an empty bed of 64.5 L (Figure 3). The substrate was pumped into the bottom of the reactors through a variable speed pump “PERCOM N-M” Peristaltic and flowed upward

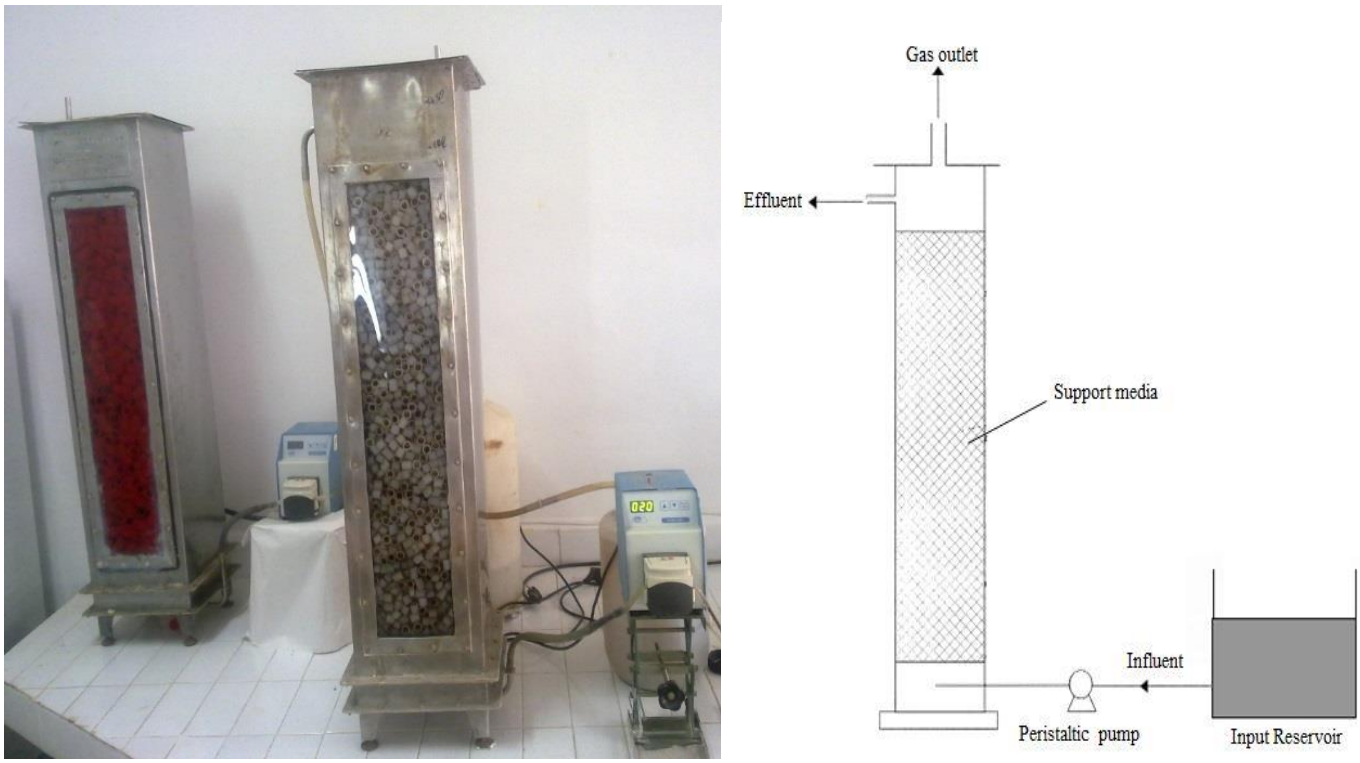

Figure 3. Experimental anaerobic bioreactor system.

through the porous medium. Sampling taps provided along the depth of the reactor allowed the extraction of samples for analysis at various stages of treatment. Both reactors were set at ambient temperature.

Denitrification studies and characterization of the isolates

This work has been the subject of a second study realized by our laboratory team. Denitrification experiments were performed in the medium, modified from Vossoughi et al. (1982), with the following composition (per liter of distilled water): $FeSO_4 \cdot 7H_2O$ 0.002 g, $CuSO_4 \cdot 5H_2O$ 0.02 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $MnSO_4 \cdot H_2O$ 0.12 g, $MgSO_4$ 0.8 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, K_2HPO_4 3.2 g, yeast extract 0.3 g, KNO_3 4.7 g, sucrose 3 g, at pH 7.5. The isolates were grown in nutrient broth for 24 h and centrifuged at 10,000 rpm for 7 min. The cell pellet was washed twice with NaCl 0.9% and re-suspended in NaCl 0.9% with absorbance of 1.0 at 620 nm for isolates and 25 ml of this was inoculated in 250 ml Erlenmeyer flasks containing 250 ml of the medium. The flasks were incubated at 30°C under static conditions up to 6 days by sampling at an interval of every 24 h for estimating growth, nitrate, nitrite and organic matter. The identification of denitrifying strains was carried out on two isolates ADR1 and ADR2 sampled from an anaerobic bioreactor system. The isolates were subjected to 16S rRNA gene sequence analysis. A species level match is based on a similarity greater than or equal to 99% (Drancourt et al., 2000)

Isolate ADR1 was observed to be gram-positive bacilli, and BLAST results of partial 16S rRNA gene showed 99% identity with *Bacillus cereus*; isolate ADR2 gram-positive bacilli showed 99% similarity with *Bacillus tequilensis* with partial 16S rRNA gene sequence (Table 4). Phylogenetic positions of isolates are shown in Figure 4, where the isolates ADR1 and ADR2 clustered with *B. cereus* and *B. tequilensis*, respectively.

Table 4. Determination of denitrifying isolates by partial 16S rRNA gene sequence similarity.

Parameter	Isolate ADR1	Isolate ADR2
Similar species	*Bacillus cereus*	*Bacillus tequilensis*
Accession No.	KF484678	JX315319
% Similarity	99	99

RESULTS AND DISCUSSION

In batch mode

pH variation for each type of support media

The pH measured at the sampling point was 6.7. This value was consistent with the limit value of liquid discharges from the project dictated by Moroccan standards, which was between 6.5 to 8.5. The value of the temperature measured at the sampling point was consistent with the limit value of liquid discharges of the project dictated by Moroccan standards which sets a threshold temperature of 30°C for direct discharges and 35°C for indirect discharges.

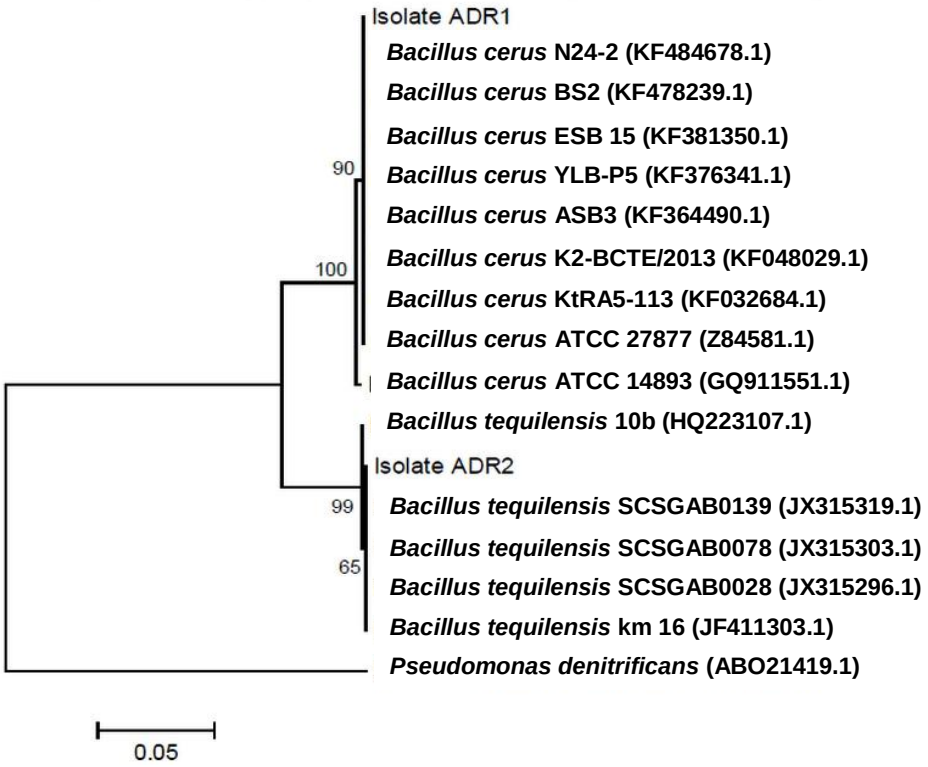

Figure 4. Phylogenetic tree constructed by neighbor-joining method showing position of the isolates with other related cultures. Bootstrap analysis of 10000 resampling by maximum-likelihood method was used to reconstruct tree. Parenthesis contains the accession number of the cultures. *Pseudomonas denitrificans* (AB021419) was used as an outgroup. Source: Moukhlissi et al. (2014).

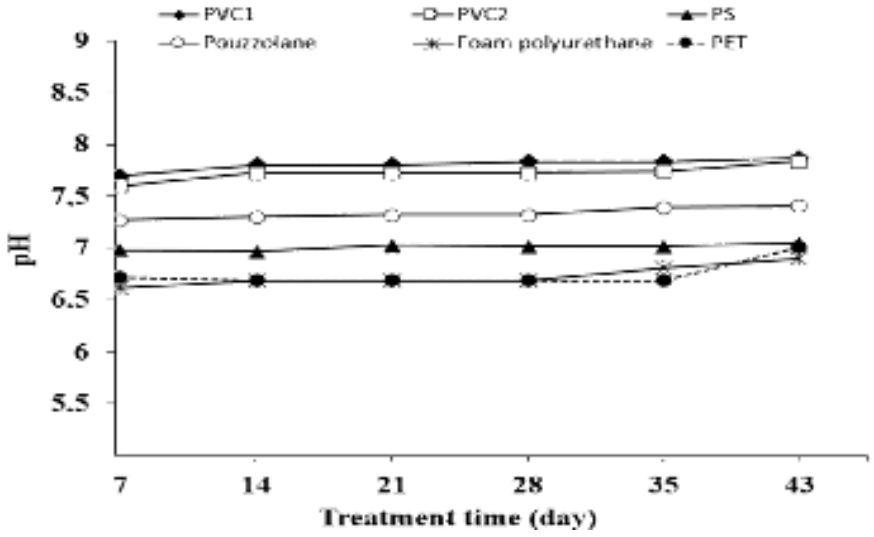

Figure 5. Evolution of pH in batch mode (initial pH is 6.1).

As shown in Figure 5, pH in influent was stabilized between 7.01 and 7.87, throughout the experiment. The optimum pH for denitrification is between 7.0 and 8.7 (Parkin et al., 1985; Šimeka et al., 2002). The pH in different Erlenmeyers with PVC1 then with the PVC2 increased from 7.6 to 7.87. For PS support media, the pH

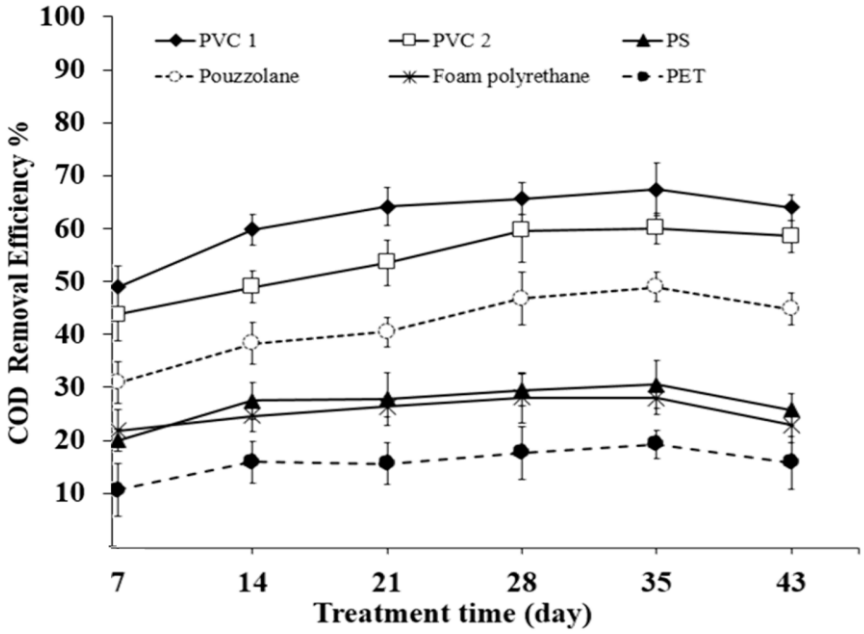

Figure 6. Steady-state COD removal efficiency.

was for the order of 7 and it remained constant. On the other hand, in different Erlenmeyers with Pozzolala, foam polyurethane and PET decreased on the whole from 6.98 to 6.69 to 28 days, and then it increased in 7.4 to 35 days and later it became stable. The degradation mechanism for the denitrification process could be deduced from the pH variation. The alkalinity and pH increased in heterotrophic and H_2-based autotrophic denitrification because nitrite reduction consumed protons (H^+). Proton consumption is illustrated in Equations (1 to 4) (Rittmann and McCarty, 2001).

Heterotrophic denitrification

$$NO_3^- + 0.263CH_3CH_2OH + 0.0445H^+ = 0.954NO_2^- + 0.0445C_5H_7O_2N + 0.655H_2O + 0.303\mathbf{CO}_2 \quad (1)$$

$$NO_2^- + 0.425CH_3CH_2OH + \mathbf{H}^+ = 0.455\ N_2 + 0.0912C_5H_7O_2N + 1.457H_2O + 0.393\mathbf{CO}_2 \quad (2)$$

Autotrophic denitrification

$$NO_3^- + 1.13H_2 + 0.01H^+ + 0.05\mathbf{CO}_2 = 0.99NO_2^- + 0.01C_5H_7O_2N + 1.1H_2O \quad (3)$$

$$NO_2^- + 0.122\mathbf{CO}_2 + \mathbf{H}^+ + 1.78H_2 = 0.488\ N_2 + 0.0244C_5H_7O_2N + 2.19H_2O \quad (4)$$

In both systems, nitrite reduction is the predominant source of alkalinity, consuming 1 H^+ equivalent per N equivalent of NO_2^- [highlighted by boldface in Equations (2) and (4)]. Another factor that affects pH is the net production of CO_2 in heterotrophic systems (highlighted by boldface in Equations 1 and 2 and net consumption of CO_2 in autotrophic systems (highlighted by boldface in Equations 3 and 4. CO_2 is a weak acid, and its addition partially suppresses the pH rise from proton consumption, as well as increases the concentration of total inorganic carbon species.

Evolution of COD removal

Effects of support media and times on purification efficiency was evaluated. As show in Figure 6, the COD removal efficiencies indicated that the support media PVC2 and PVC1 have significant purification efficiency at the loadings of 5 and 6 mg COD/L/day, with overall COD removal efficiency in excess of 60%. On the other hand, the support PET presents the lowest observed performance, with overall COD removal efficiency of 20%. This suggests that the anaerobic filters (AFs) can offer relatively high organic loading capacities compared with full-scale anaerobic contact plants which normally handle organic loadings of 1 to 3 g COD/L/day (Lawrence and McCarty, 1969). When the loading increased to 10 mg COD/L/day and subsequently to 15 and 25 mg COD/L/day, removal at the stages began to show relative superiority of support media PVC1 and PVC2 over foam polyurethane, pozzolana, PS and PET. The COD removal efficiencies was about 65% in both PVC1 and PVC2 and was 33, 36, 47 and 19% in PS, pozzolana,

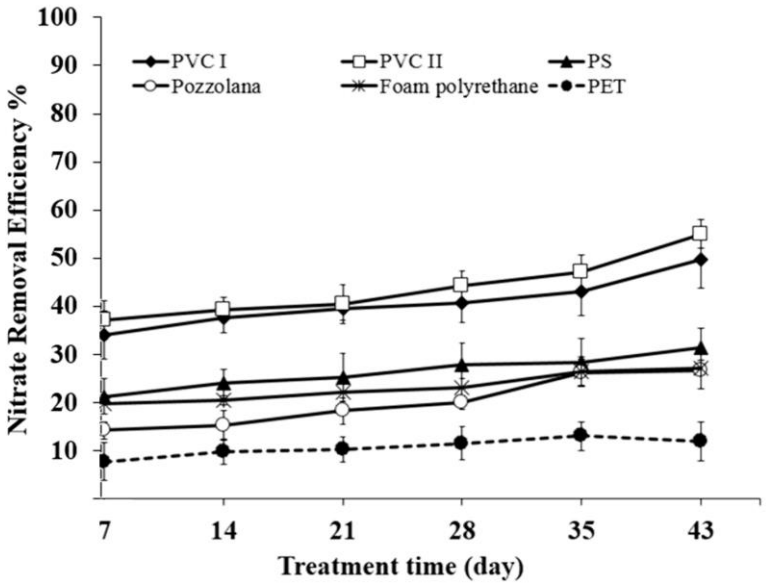

Figure 7. Steady-state Nitrate removal efficiency.

foam and PET, successively, at 10 mg COD/L/day. At higher loadings of 25 and 45 g COD and more, both PVC1and PVC2 showed similar COD removal of about 58.14 and 48.87% compared with the markedly reduced removal efficiencies of 5 and 57 in pozzolana.

The results indicated that this type of support media has a significant impact on the performance of purification efficiency. The higher removal efficiencies of PVC1 and PVC2 are likely attributed to higher growth of attached biofilm.

Evolution of nitrate removal

Biological denitrification is an efficient process for nitrogen removal from wastewater in which heterotrophic bacteria in the absence of oxygen (anaerobic conditions) convert nitrate-N and nitrite-N to nitrogen gas (Prosnansky et al., 2002; Van Rijn et al., 2006). As shown in Figure 7, the nitrate removal efficiencies indicated that all the erlenmeyers presented an important rate of elimination, but this elimination became stable between days 28 and 42. PVC1 and PVC2 presented a better performance in nitrate removing, with a maximum of 54.98%. On the other hand PET had low nitrate removal with 11.89% efficiency.

Evolution of the production of EPS

For years, carbohydrate was considered the main constituent of EPS in pure cultures (Sutherland, 2001; Sutherland and Kennedy, 1996). Recent studies of mixed cultures in wastewater treatment systems found that protein was also an important constituent in EPS, possibly due to the large quantities of exoenzymes entrapped in the EPS (Dignac et al., 1998). In this study, the protein content was greatest in the each type of media supports.

The production of EPSc and EPSp during all our experience showed similar evolutions (Figure 8). In the first phase, until the 21 days, a decrease of the ratio EPSp/EPSc was observed. This fact indicated that the EPS production can be related to adhesion of microorganisms onto the surface of each type of media support. Afterwards, it was verified that the ratio EPSp/EPSc decreased and kept constant until the end of last days of test. The findings were consistent with the results of the previous studies (Fenxia et al., 2011; Shim et al., 2001).

In continuous mode

pH variation for PVC1 and PVC2

As shown in Figure 9, pH in influent was stabilized between 8 and 8.1 in reactor with PVC2. The optimum pH for denitrification was between 7.0 and 8.7 (Parkin et al., 1985; Šimeka et al., 2002). In the reactor with PVC1, the pH increased from 8.3 to 8.7 towards 28 days.

Evolution of COD removal

COD conversion in all reactors was very high and stable during all the period of the experience. COD conversion from reactor with PVC1 was greater than 89.93 and

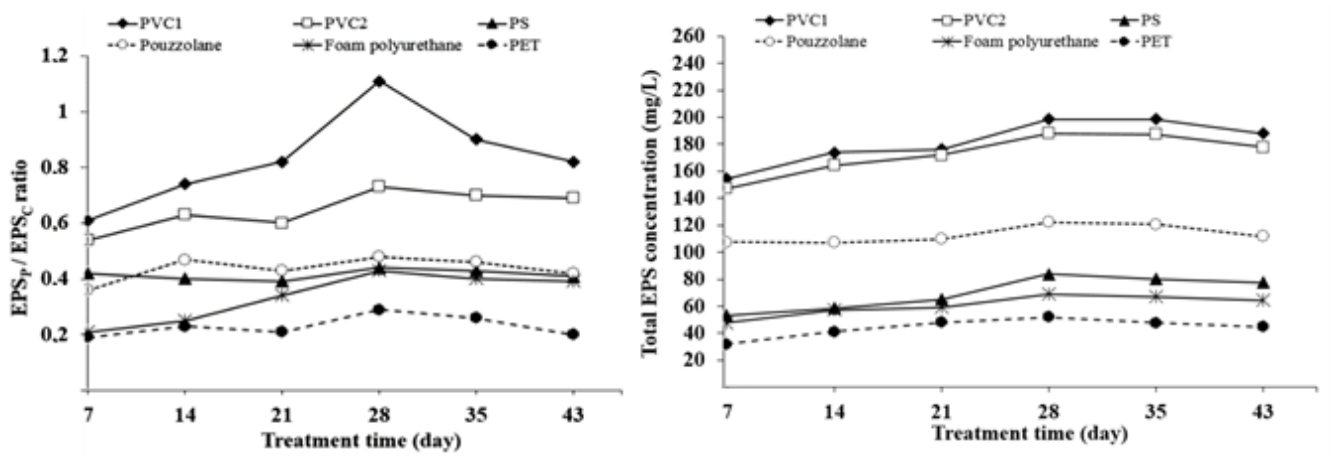

Figure 8. Ratio of EPSp/EPSc throughout the experiment.

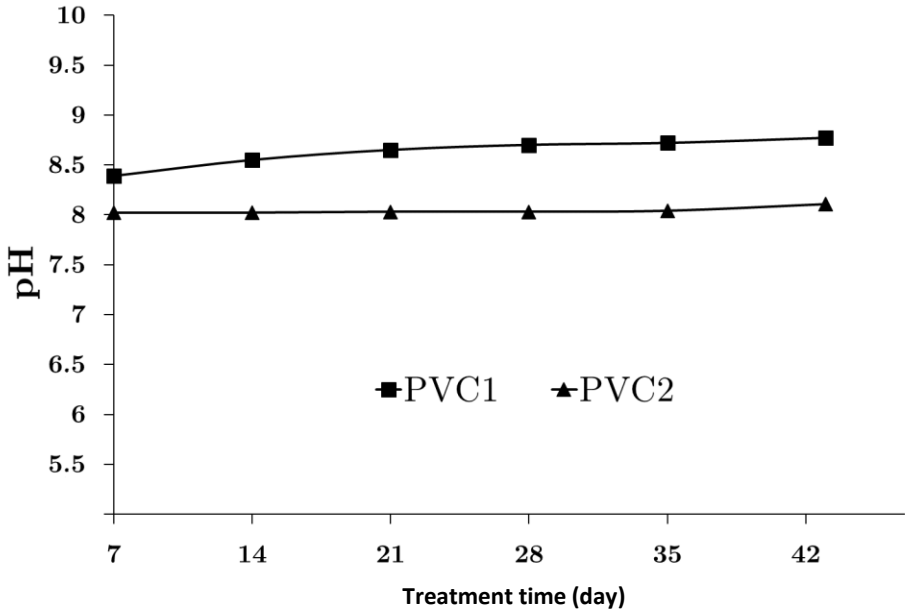

Figure 9. Evolution of pH in batch mode.

78.82% for reactor with PVC2 (Figure 10). The results indicated that the type of support media has a significant impact on the performance of purification efficiency. The higher removal efficiencies of PVC1 and PVC2 is likely attributed to higher growth of attached biofilm.

Evolution of nitrate removal

In this experience both reactors presented important performances with domination of the reactor with support media PVC1. As shown in Figure 7, The elimination of nitrate affected a maximum from 78.75% to 42 days for the reactor with PVC1. On the other hand, it was 66.81% for the reactor with PVC2 (Figure 11).

Evolution of the production of EPS in both reactors

The important factor determining the charge of the cell surface is the ratio of carbohydrates to protein in the EPS (Urbain et al., 1993). The production of EPSc and EPSp throughout the experiment showed similar evolutions (Figure 12). This production affected its optimum around day 35, with a ratio of 2.13 for the reaction with PVC2 and 2.22 for the reactor with PVC1. This resulted in an increase in the ratio of protein to carbohydrates, implying an important cell surface charge.

Denitrification pattern of the isolates

Based on 16S rRNA gene sequencing, isolates were

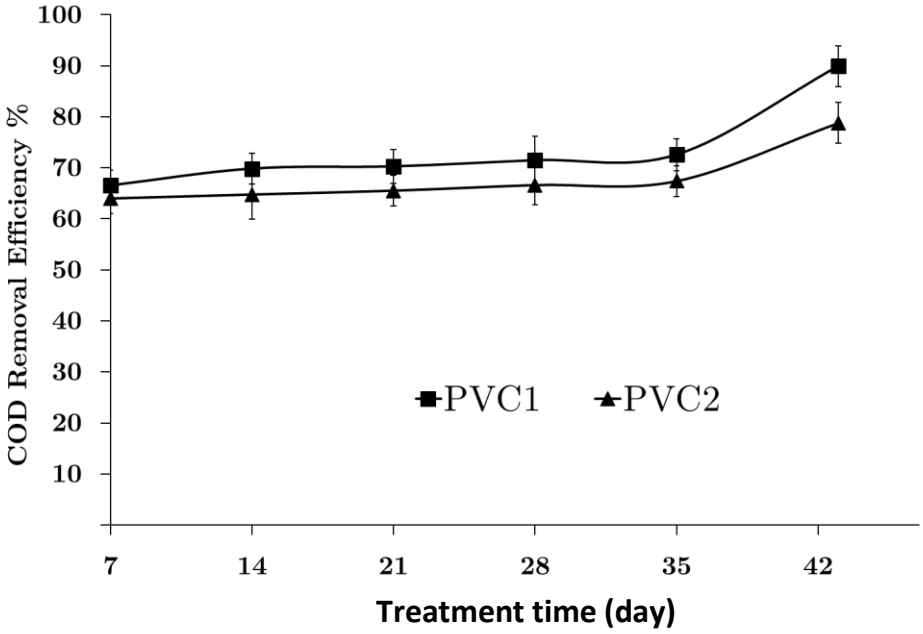

Figure 10. Evolution of COD removal efficiency.

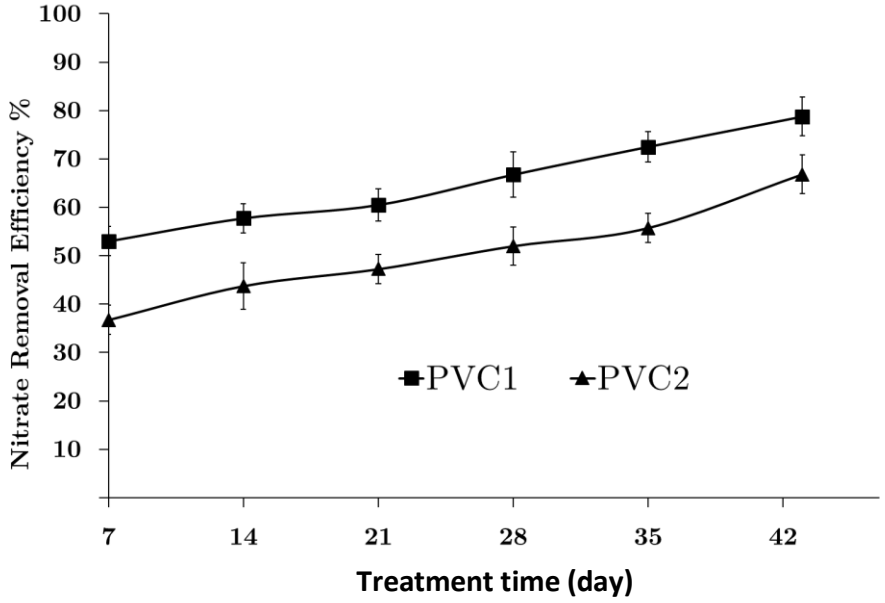

Figure 11. Evolution of nitrate removal efficiency.

affiliated with *Firmicutes*. The ability to denitrify has been identified in taxonomically diverse bacteria, including members of the *Aquificae*, *Deinococcus-Thermus*, *Firmicutes*, *Actinobacteria*, *Bacteroides* and *Proteobacteria* phyla (Zumft, 1997). Isolate ADR1 was related to *B. cereus*. *B. cereus* is a heterotrophic bacterium able to degrade organic matter under nitrate reducing conditions. Dou et al. (2010) reported that *B. cereus* could transform benzene to phenol and benzoate, and then used phenol and benzoate as carbon and energy source. Zhao et al. (2009) used the denitrifying *B. cereus* to remove nitrogen and organic matter from reclaimed wastewater used as landscape water. *B. cereus* is most likely involved in biogeochemical nutrient

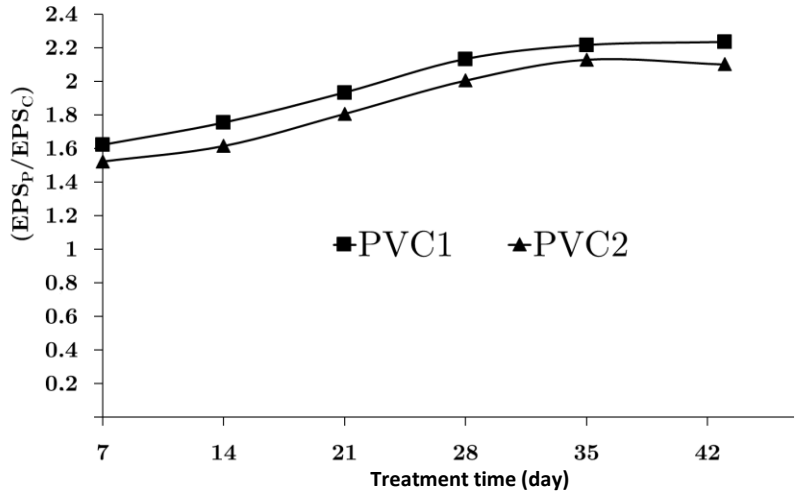

Figure 12. Variations of carbohydrate to protein ratio of EPS.

cycling, as it produces a wide range of extracellular enzymes and can grow on decaying organic matter (Borsodi et al., 2005). *B. tequilensis* could reduce nitrate to nitrogen, thus this species is a true denitrifier (Gatson et al., 2006). As reported by Das et al. (2014), *B. tequilensis* was chemoorganotrophic and could use hydrocarbons as sole carbon source.

Despite the fact that diverse denitrifiers have similar denitrification apparatus, each organism have its own activity. In this study, we compared the denitrification of two bacilli, ADR1 and ADR2, isolated from a denitrifying reactor and identified as *B. cereus* and *B. tequilensis*. The nitrate reduction rate was higher in *B. cereus*. However, two isolates have nitrite accumulation. Carlson and Ingraham (1983) revealed different patterns of denitrification between *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*. Betlach and Tiedje (1981) showed that transit nitrite accumulation in *Alcaligenes sp.* and *Pseudomonas fluorescens* was due to the differences in the reduction rates of nitrate and nitrite. The growth estimated by dry cell weight is more important in *B. tequilensis* than *B. cereus*. Otherwise, the increase of cell number leads to enhanced quantity of biomass and the sludge in the system of wastewater. Thus, *B. cereus* is more efficient because this strain reduces more amount of nitrate than *B. tequilensis* and produces less sludge.

The aforementioned results showed that isolates ADR1 and ADR2 isolated from denitrifying reactor were identified as *B. cereus* and *B. tequilensis*. The experimental results showed that *B. cereus* could reduce 29.46 mM of nitrate and degrade 4240 mg/L of organic matter within 6 days. However, *B. tequilensis* is less efficient and could reduce 13.82 mM of nitrate and 4500 mg/L of organic matter. In addition, *B. cereus* produced less biomass, avoiding clogging of wastewater treatment system. These results concerning *B. cereus* showed that the isolated bacterium could potentially remediate wastewater with high level of nitrate and organic matter. (Moukhlissi et al., 2014).

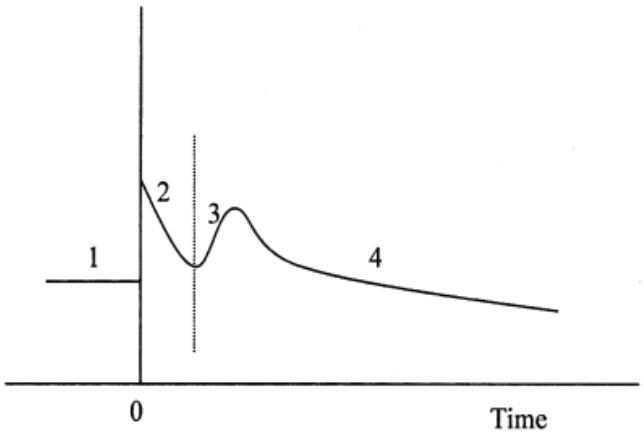

Figure 13. EPS biodegradability mechanisms: (1) pulse source of substrate from the added EPS; (2) easily biodegradable EPS was consumed; (3) produced soluble EPS plus minimally biodegradable EPS left; (4) newly produced EPS was further consumed and activity gradually stopped.

Conclusion

This study indicated that biofilm EPS was biodegradable by its own producers as well as by other microorganisms. Based on the aforementioned experimental evidence, we infer that the following events occurred during the EPS biodegradability study (Figure 13). The removal efficiency of COD and nitrate and production of EPS was closely

related with the type of support media. The PVC1 and PVC2 were favorable for the biofilm formation and therefore a better efficiency of wastewater treatment was obtained.

Conflict of interests

The authors have not declared any conflict of interests.

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