Use of biopolymers as solid substrates for denitrification

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Abstract

The conventional process to remove nitrate from water, the biological denitrification, uses the addition of dissolved organic carbon that has the potential risk to further deteriorate water quality. Thus, this work aimed to evaluate the specific denitrification activity of a mixed microbial culture and a pure culture of Pseudomonas stutzeri with solid substrates such as polycaprolactone (PCL), polylactic acid (PLA), and starch. The highest nitrate reduction activity was obtained with a microbial mixed culture using starch, $104 \mathrm{mgN_2-N/(gVSS.d)}$, and PCL, $97 \mathrm{mgN_2-N/(gVSS.d)}$, followed by PLA, $53 \mathrm{mgN_2-N/(gVSS.d)}$. A considerable advantage of using biopolymers in water denitrification is the reduced risk of contaminating the water with soluble biodegradable organic carbon. Key words | denitrification, polycaprolactone, polylactic acid, solid substrate, starch

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INTRODUCTION

Nitrate removal by conventional physico-chemical water treatment technologies is usually very expensive and/or very energy consuming (Rocca et al. 2007). Biological denitrification of nitrate to harmless N_2 gas and nitrous oxide, by heterotrophic bacteria, represents a suitable alternative for remediation of water contaminated with nitrate due to the high specificity of denitrifying bacteria, high denitrification efficiency, and low cost (Wang et al. 2009).

Typically, groundwater contaminated with nitrate and secondary treated effluents are both severely limited in organic carbon and the addition of an external substrate, such as acetic acid, sucrose, ethanol and methanol, has been the usual procedure to achieve nitrogen removal (Gomez et al. 2000). Nevertheless, the costs associated, and the risk of additional contamination of the environment, with in this procedure demand the use of alternative substrates. In this regard, several solid substrates have been studied such as cotton, chitin, polyhydroxyalkanoats, and biodegradable plastics (Rocca et al. 2007; Soejima et al. 2010).

Polycaprolactone (PCL) and polylactic acid (PLA) are two well known biodegradable aliphatic polyesters (Moura et al. 2011). PCL can be easily degraded by aerobic and anaerobic microorganisms that are widely distributed in various ecosystems (Tokiwa et al. 2009). PLA decomposes to lactate that might be used as an electron provider in biological denitrification; however, it has been reported that PLA biodegradation does not occur readily (Suyama et al. 1998; Teeraphatpornchai et al. 2003). The biodegradation of PCL and PLA is favoured by a low melting temperature, crystallinity, and molecular weight of the polymers (Tokiwa et al. 2009). Starch is a polysaccharide known to be highly biodegradable (Moura et al. 2011).

Until now, there have been very few studies using biopolymers as solid substrates for water denitrification. Therefore, the aim of this work was to evaluate the specific denitrification activity of a mixed microbial culture and a pure culture of Pseudomonas stutzeri with solid substrates such as PCL, PLA, and starch.

MATERIALS AND METHODS

Batch tests

A pure culture of Pseudomonas stutzeri (DSMZ, 7136) and a microbial mixed culture from a sand filter treating surface river water were used as inocula. The reduction of nitrate by both inocula with monomeric substrates ($0.14\ g/L$ ε-caprolactone, $0.27\ g/L$ lactic acid, and $0.27\ g/L$ acetate) and solid polymeric substrates ($0.17\ g/L$ PCL, $0.27\ g/L$ PLA, and $0.24\ g/L$ starch) was assessed in batch tests. The concentration of nitrate used was $0.10\ gNO_3{}^- - N/L$ and the amount of each substrate needed to reduce the nitrate to N_2 was calculated according to the respective stoichiometric equations. Moreover, two sets of control tests were done: with nitrate and without a substrate, as well as without nitrate but with the substrates. Experiments were done in duplicate.

The batch tests were done in glass vials of 118 mL containing 88 mL of medium. Concentrated solutions of the monomers were previously prepared and then 1 mL of each solution was added to the vials to reach the desired initial concentration. The polymers were ground to powder and the mass of each polymer, corresponding to the concentrations described above, was weighed and inserted into the vials. Subsequently, the medium was added and the pH was adjusted to 8.5. A visible layer of suspended polymer was observed inside the vials. A concentrated suspension of the inocula, previously washed three times with a sterile buffer solution to remove soluble carbon in the medium, was added to obtain a final concentration of $0.5\ g/L$ of volatile suspended solids (VSS). At last, the volume inside the vials was adjusted to $88\ mL$. The vials were sealed and flushed with N_2 gas for 15 min to obtain anoxic conditions. Then, they were put into a shaker (150 rpm) at $30\ ^\circ C$ for 30 min to reach the gas/liquid equilibrium. After that, the pressure was equalised to the atmospheric one. The tests were started by injecting 1 mL of the concentrated $NaNO_3$ solution. The vials were incubated under the above referred conditions for 20 d for monomers and 35 d for polymers. The overpressure due to gas production was measured over time.

Sampling, analytical methods, and calculations

The pressure in the headspace was measured daily for the first week and then every three days during the experimental time using a differential pressure transducer (Sper Scientific 840065). The gas composition in CO_2, N_2O, and N_2 was determined at the end of the batch tests by gas chromatography (GC Chrompack CP 9001). The N_2 concentration was calculated with the headspace volume, the gas composition, and the measured overpressure, applying the ideal gas law. Liquid samples were collected at the beginning and at the end of the batch tests. They were filtered using a 0.45μ m filter before the determination of nitrate, nitrite, dissolved organic carbon (DOC) and monomers concentration. The concentration of the monomers was measured by high performance liquid chromatography (HPLC, Knauer) using a UV-Vis detector. The remaining parameters were determined according to Standard Methods (APHA, AWWA & WPCF 1998).

The specific denitrification activity was determined as the ratio of the highest amount of N_2 gas produced and the time needed to reach this amount multiplied by the amount of microbial culture. The amount of microbial culture was expressed as VSS.

Fourier transform infrared (FTIR) spectra of the polymers were recorded with a spectrometer (Perkin-Elmer 1720). Measurements were performed in a transmittance mode using a spectral range varying from 4,000 to $500\ cm^{-1}$ (Moura et al. 20II). An amount of 20 mg of each polymer dried at $60^{\circ}C$ and finely ground to powder were encapsulated in 200 mg of KBr to prepare translucent sample disks used for FTIR analysis. FTIR spectra were measured with undegraded and degraded polymers; the later was collected by filtration of the contents inside the vials at the end of the tests.

Statistical analyses

A t-test was used where specific means were compared. Acceptance or rejection of the null hypothesis was based on a α-level of 0.05 .

RESULTS AND DISCUSSION

Denitrification with monomers

PCL and PLA are two polyesters whose repeating units are based on ε-caprolactone and lactic acid, respectively. To better describe the factor that might limit the biodegradability of both polymers, the reduction of nitrate with the monomers ε-caprolactone and lactic acid was assessed in batch tests with P. stutzeri and a microbial mixed culture as inocula.

A parallel test was done with acetate as a positive control. The N_2 gas represented about 90% of the total gas produced in the test, with the remaining 10% composed of CO_2 (8.7%) and N_2O (1.3%). As it can be observed in Figure 1 (panels (a) and (b)), the N_2 gas production increased for all monomers in the first 3 d , remaining constant afterwards. The highest N_2 gas production by *P*. stutzeri and the microbial mixed culture with ε-caprolactone (7.9 ± 0.15mg and

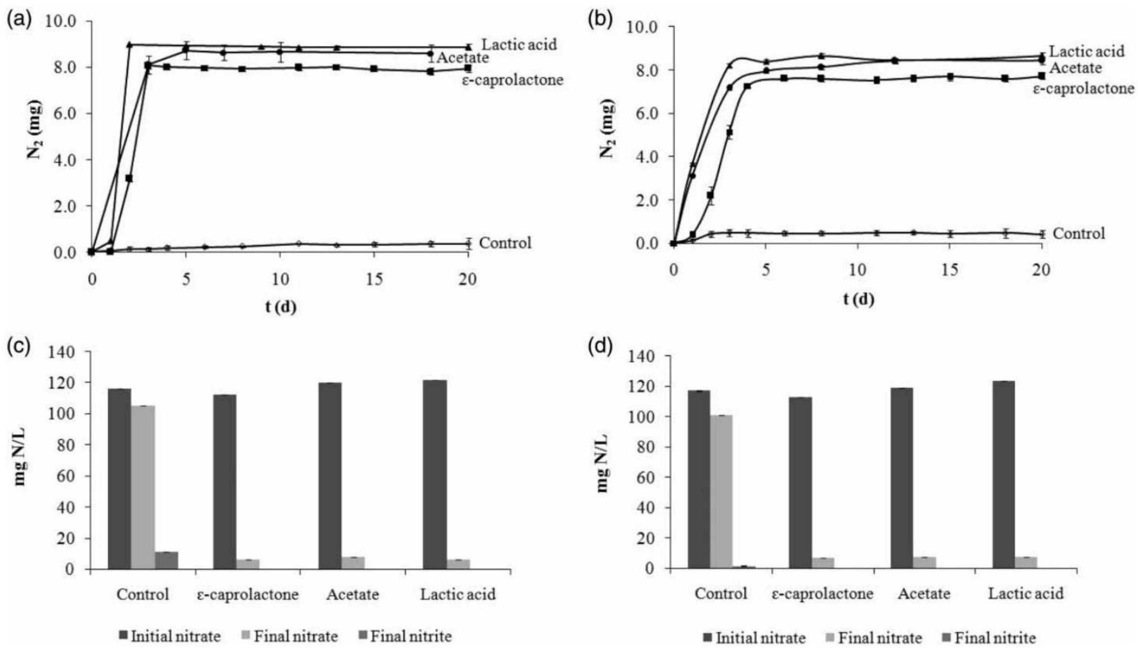

Figure 1 | N_2 gas production and nitrogen conversion by P. stutzeri (panels (a) and (c)) and a microbial mixed culture (panels (b) and (d)) with monomers. The control test was done without monomers.

7.7 ± 0.07mg, respectively), lactic acid (8.8 ± 0.06mg and 9.2 ± 0.3mg, respectively), and acetate (8.6 ± 0.08mg and 8.4 ± 0.1mg, respectively), was very close to the stoichiometric value of 8.8 mg . For all monomers, statistical analysis of the N_2 gas production revealed that there were no significant differences between the two inocula used in the batch tests ($\alpha = 0.05$).

Independently of the inocula and monomer tested, the concentrations of nitrate (Figure 1, panels (c) and (d)) and dissolved organic carbon (DOC) (data not shown) decreased in the batch tests without significant accumulation of nitrite. The final nitrate concentration was about $6.8\text{mg}NO_3{}^- - N/L$ and the final DOC concentration was on average close to 11mg/L. The remaining concentration of nitrate might be explained by carbon limitation because the final concentrations of ε-caprolactone, lactic acid, and acetate were lower than 0.01mg/L. The presence of 11 mg DOC/L might have originated from cell lysis or extracellular polymeric substances excreted by the biomass. N_2 gas production and nitrate were removed in the control test done with nitrate and without the monomers; however, these values were significantly lower than that obtained in the presence of the monomers. This result might be explained by the presence of a residual amount of organic carbon in the medium.

The specific denitrification activity of P. stutzeri with ε-caprolactone, lactic acid, and acetate was $107 mgN_2$ - $N/(g VSS \cdot d)$,$101 mgN_2 - N/(g VSS \cdot d)$, and $102 mgN_2 - N/$ (g VSS d), respectively. In the case of the microbial mixed culture with ε-caprolactone, lactic acid, and acetate, the specific denitrification activity was $101 mgN_2 - N/$ (g VSS $\cdot$ d), $109 mgN_2$-N/(g VSS $\cdot$ d), and $112 mgN_2$-N/ (g VSS-d), respectively. Thus, no significant differences in the specific denitrification activities were observed for the different monomers and inocula. Most studies on groundwater denitrification have been done with several soluble carbon sources: namely, ethanol, methanol and acetate (Vrtovšek & Roš 2006). Acetate, due to its high rate of denitrification, has been used as a benchmark to compare with other carbon sources. Groundwater denitrification with acetate in a biofilm reactor has been reported by Vrtovšek & Roš (2006). However, their values cannot be compared with that of the present study because they did not present the biomass concentration values necessary to determine the specific denitrification activity. In the wastewater treatment field, Strong et al. (2011) obtained a higher specific denitrification activity with acetate, $22 mgNO_3{}^- -$ $N/(g$ VSS $\cdot d)$, than with other substrates: formate, methanol, and ethanol. Previous studies on groundwater denitrification with lactic acid are not available. Examples include the work of Peyton et al. (2001) that obtained a lower specific denitrification activity with lactic acid, $300 mgNO_3{}^- - N/(gVSS.d)$, than with acetate, $650 mgNO_3{}^- N/(g$ VSS.d), with a pure culture of

Haloamonas campisalis in a halophilic environment. A similar trend was reported by Cuervo-López et al. (1999). They obtained a lower specific denitrification activity with lactic acid, $40 mgNO_3{}^- - N/(gVSS.d)$, than with acetate, $100 mgNO_3{}^-$-N/(g VSS.d), in an upflow anaerobic sludge blanket reactor. To the authors' best knowledge, the present study is the first to test ε-caprolactone as a substrate for groundwater denitrification. There is a lack of information regarding the use of lactic acid and ε-caprolactone in groundwater denitrification. Concerning the specific denitrification activities obtained in the wastewater field for both substrates, the values are in the range of $40 mgNO_3{}^- -$ $N/(gVSS.d)$ to $300 mgNO_3{}^-$-N/(g VSS.d) for lactic acid, and between $100 mgNO_3{}^-$-N $/(gVSS.d)$ and $650 mgNO_3{}^-$-N $/(gVSS.d)$ for acetate. The denitrification activities obtained in the present study are encompassed by literature values. The two possible explanations for the range of values found are the source of the inoculum and the limiting nutrient of the denitrification reaction (nitrate or the carbon source).

Denitrification with polymers

Two polyesters (PCL and PLA) and a polysaccharide (starch, positive control) were used as solid substrates to reduce nitrate in batch tests with P. stutzeri and a microbial mixed culture. The experimental results are depicted in Figure 2. The highest N_2 gas production (Figure 2, panel (b)) was obtained with starch (7.4 ± 0.02 mg), followed by PCL (6.4 ± 0.3mg), and at last PLA (3.1 ± 0.4mg) using the microbial mixed culture. Concerning P. stutzeri, the N_2 gas production followed the same trend as that of the microbial mixed culture (Figure 2, panel (a)), but the values obtained were significantly lower, namely 1.6 ± 0.08mg for starch, 1.2 ± 0.03mg for PCL, and 1.1 ± 0.02mg for PLA. For each polymer, statistical analysis of the N_2 gas production indicated that there were significant differences between the two inocula ($\alpha = 0.05$). The concentration of nitrate decreased in the batch tests and presented a similar behaviour to that of the N_2 gas production for both inocula (Figure 2, panels (c) and (d)). No nitrite accumulation was observed in the tests with the microbial mixed culture. Concerning P.

stutzeri, the highest value attained was $4.5 \pm 0.24 mgNO_2{}^{-}$-N/L. The N_2 gas production was very low in the control tests done without nitrate and with the solid substrates (Figure 2, panels (a) and (b), curves starch-C, PLA-C and PCL-C). Additionally, the concentration of dissolved organic carbon was low suggesting that PLA and PCL did not dissolve significantly. In the control test done with nitrate and without the solid substrates, the N_2 gas production was slightly higher than that of the controls without nitrate and was accompanied by a decrease in the concentration of

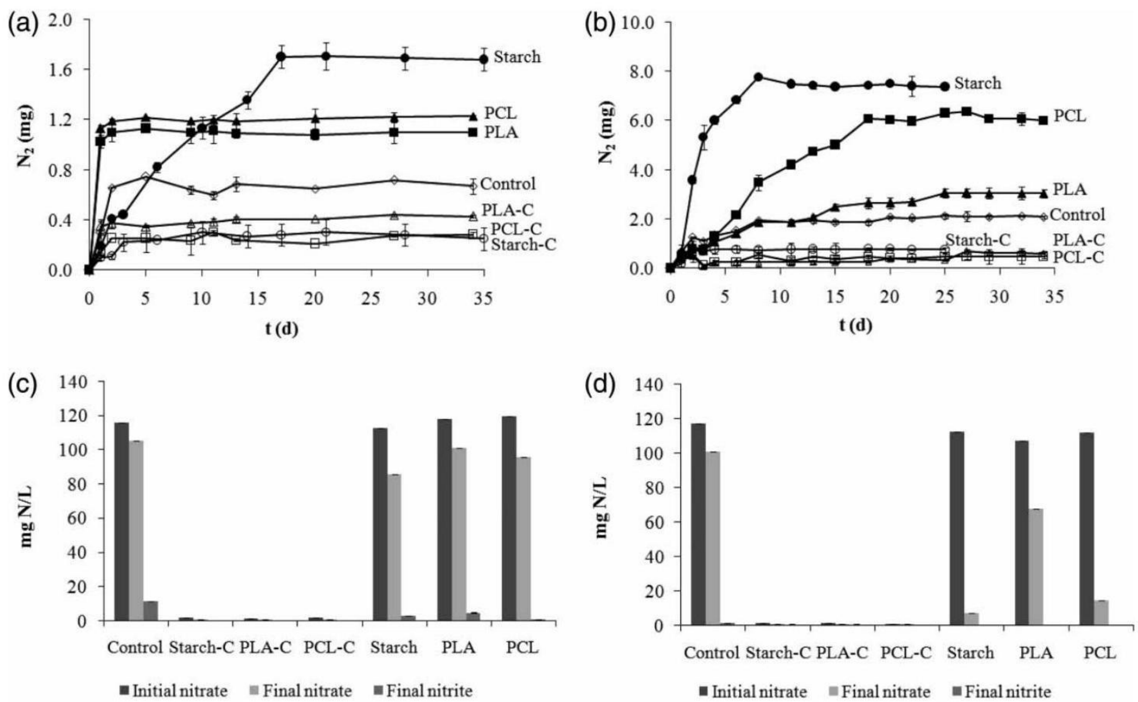

Figure 2 | N_2 gas production and nitrogen conversion by P. stutzeri (panels (a) and (c)) and a microbial mixed culture (panels (b) and (d)) with polymers. The control test was done without polymers. The tests Starch-C, PCL-C, and PLA-C were done without nitrate.

nitrate. A possible reason for these results is the presence of a residual amount of organic carbon in the medium, as already mentioned before.

P. stutzeri was used in the present study because of specific metabolic properties reported in literature, namely the degradation of starch and aromatic compounds and the synthesis of polyhydroxyalkanoates (Lewis et al. 2000; Tokiwa et al. 2009). Unexpectedly, the extent of starch degradation by *P*. stutzeri inferred by N_2 gas production was significantly lower than that of the microbial mixed culture under the conditions used in the batch testes (nitrate as final electron acceptor, 30°C, and pH 8.5). To the authors' best knowledge there is no information that indicates that *P*. stutzeri is able to synthesize the enzymes needed to degrade PCL and PLA under denitrification conditions. According to the review work of Tokiwa et al. (2009), mixed cultures degrade starch more easily than PCL or PLA. From a limited number of studies, it is known that the number of PCL-degrading bacteria isolated in several ecosystems was higher than that of PLA-degrading bacteria (Suyama et al. 1998; Teeraphatpornchai et al. 2003). Teeraphatpornchai et al. (2003) isolated a mesophilic bacterial strain that degraded PLA in the presence of another carbon source, but did not grow with PLA as the sole carbon source. This result suggested that this strain degraded PLA by a cometabolic process.

On average, the specific denitrification activity of the microbial mixed culture was 5 times higher than that of P. stutzeri. The specific denitrification activities of P. stutzeri and the microbial mixed culture were $21 \mathrm{mgN}_2-\mathrm{N}/(\mathrm{gVSS.\ d})$ and $104 \mathrm{mgN}_2-\mathrm{N}/(\mathrm{gVSS.d})$, respectively, with starch, $18 \mathrm{mgN}_2-\mathrm{N}/(\mathrm{gVSS.d})$ and $97 \mathrm{mgN}_2-\mathrm{N}/(\mathrm{gVSS.d})$, respectively, with PCL, and $13 \mathrm{mgN}_2-\mathrm{N}/(\mathrm{gVSS.d})$ and $53 \mathrm{mgN}_2-\mathrm{N}/($g VSS.d), respectively, with PLA. Literature values for specific denitrification activities with PCL (Boley et al. 2000; Wang & Wang 2009) and PLA (Soejima et al. 2010) obtained in groundwater denitrification studies cannot be directly compared with those of the present study because the surface area of the polymer instead of the biomass concentration was used to calculate the specific denitrification activity. Literature values of specific denitrification activities can be found in the field of wastewater treatment. Specific denitrification activities of $1.0 \mathrm{~gNO}_3{}^{-}-\mathrm{N}/(\mathrm{gVSS.d})$ with starch (Lee & Welander 1996), $110-170 \mathrm{mgNO}_3{}^{-}-\mathrm{N}/($g VSS.d) with PCL (Horiba et al. 2005), and $6 \mathrm{mgNO}_3{}^{-}$-N/ (g VSS.d) with PLA (Fan & Wang 2009) have been previously reported. Despite the different environmental conditions, the results presented in the literature and the ones obtained in the present work showed that the highest
specific denitrification activity was obtained with starch and PCL, followed by PLA.

To compare the changes in the polymers' chemical structure that occurred during the batch tests, FTIR spectra of the polymers before and after the tests were recorded. For PLA, only slight differences were found with both inocula. Transmittance data showed that all peaks in the FTIR spectrum of PLA obtained after the batch test decreased slightly in size compared to the initial spectrum (Figure 3, panels (a) and (b)). A reduction in the CH -assymetric ($2{,}920 \mathrm{~cm}^{-1}$) and CH -symmetric ($2{,}850 \mathrm{~cm}^{-1}$) stretches indicated a decrease in PLA's molecular weight and the reduction of peaks related to carbonyl ($1{,}800 \mathrm{~cm}^{-1}$ and $1{,}700 \mathrm{~cm}^{-1}$) and ether bonds ($1{,}100 \mathrm{~cm}^{-1}$) indicated chain scission. Together, the slight modification of the polymer's chemical structure and the low N_2 production in the batch tests suggested that PLA was minimally biodegraded under the conditions used in the present study. The differences between initial and final FTIR spectra of PCL were pronounced in the test with the microbial mixed culture and very small in the test with P. stutzeri. All peaks in the FTIR spectrum of PCL obtained after the batch test with the microbial mixed culture decreased considerably in size compared to the initial spectrum (Figure 3, panel (d)), namely, peaks related to CH ($3{,}000 \mathrm{~cm}^{-1}$ to $2{,}800 \mathrm{~cm}^{-1}$), carbonyl ($1{,}800 \mathrm{~cm}^{-1}$ and $1{,}700 \mathrm{~cm}^{-1}$), and ether bonds ($1{,}100 \mathrm{~cm}^{-1}$). The FTIR spectrum obtained after the batch test also presented a band at $3{,}500 \mathrm{~cm}^{-1}$ which is associated with $\mathrm{O}-\mathrm{H}$ bonds. A possible explanation for this result is the formation of alcohol endgroups resulting from the degradation of PCL (Moura et al. 2011). Regarding the starch, differences can be observed between initial and final FTIR spectra with the P. stutzeri (Figure 3, panel (e)): decreasing of OH peaks ($3{,}300 \mathrm{~cm}^{-1}$ to $3{,}900 \mathrm{~cm}^{-1}$) as well as CH peaks ($2{,}927 \mathrm{~cm}^{-1}$). It was not possible to recover starch after the batch test with the microbial mixed culture to perform the FTIR analysis suggesting that starch was completely biodegraded. In summary, the biggest changes in the chemical structure of PCL and starch were observed with the microbial mixed culture and PCL was more biodegradable than PLA.

Comparison between monomers and polymers

Comparing the N_2 gas production by a microbial mixed culture with monomers and polymers as substrates (Figures 1 and 2), it can be seen that the N_2 gas production with the monomers, ε-caprolactone and lactic acid, increased faster and was higher than that of the respective polymers, PCL and PLA. These results suggested that the limiting step of

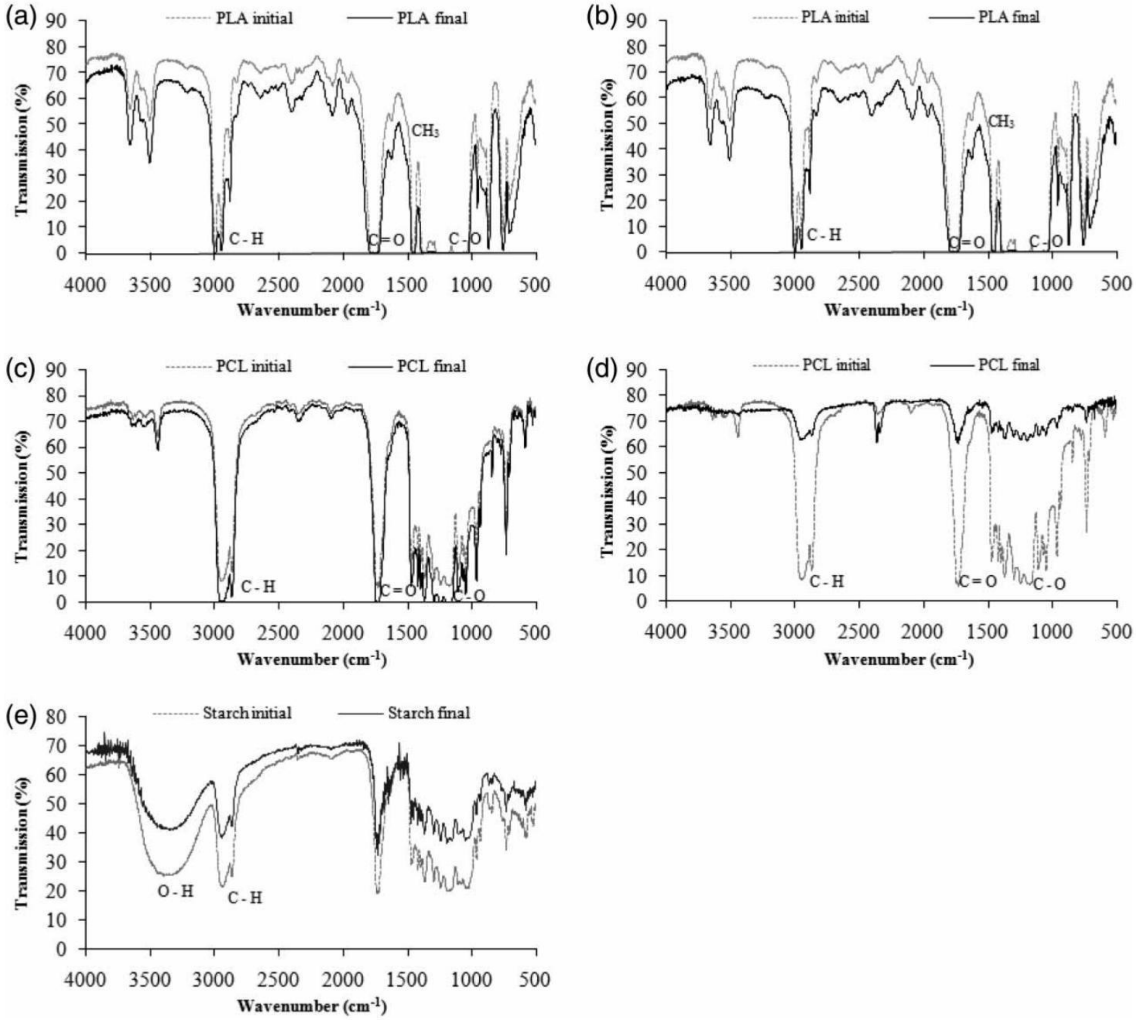

Figure 3 | FTIR spectra of PLA, PCL, and starch, at the beginning and at the end of the tests, using Pseudomonas stutzeri (panels (a), (c), and (e)) and a mixed culture (panels (b) and (d)) as inocula.

the biodegradation of the polymers was the hydrolysis reaction that cleaved the polymer into its monomers and that a portion of the polymer might not have been accessible to microbial attack. According to literature, the depolymerization of PCL is done by lipases and esterases (Tokiwa et al. 2009) and that of PLA by several serine proteases and esterases (Lim et al. 2005; Tokiwa et al. 2009). The detection of the activity of these enzymes was not in the scope of the present work. The rate and extent of biodegradation of polymers depends on its degree of crystallinity that is the proportion between the amorphous and crystalline regions. In fact, the enzymes mostly attack the amorphous regions of a polymer where molecules are weakly clustered leaving the crystalline regions untouched (Tsuji & Miyauchi 200I). Information was not available about the morphology of the PCL and PLA used in the present study and it can only be speculated that this parameter might have influenced the results observed. The N_2 gas production by P. stutzeri with the polymers, PCL and PLA, was considerably lower than that of the respective monomers, ε-caprolactone and lactic acid. These results suggested that the enzymes involved in cleaving PCL and PLA were poorly expressed by P. stutzeri or even not synthesized.

The main advantage of using solid electron donors for denitrification was that high nitrate reduction rates were achieved especially with starch and PCL without accumulation of noxious intermediates (e.g. nitrite, N_2O) and release of soluble organic carbon to the water. Future applications of solid electron donors may be denitrification of groundwater and tertiary treatment of wastewaters.

CONCLUSIONS

The highest nitrate reduction in batch tests was obtained with starch ($104 \mathrm{mgN_2-N/(gVSS.d)}$) and PCL ($97 \mathrm{mgN_2-N/(gVSS.d)}$) followed by PLA ($53 \mathrm{mgN_2-N/(gVSS.d)}$) with a microbial mixed culture. P. stutzeri was not able to degrade polymeric substrates probably due to the lack of enzymes that cleave the polymers. The risk of contaminating the water with soluble biodegradable organic carbon is reduced due to the slow solubilisation of the biopolymers.

ACKNOWLEDGEMENTS

The present study was financed by the FCT project 'A new generation of biofilm supports with controlled carbon release properties' (PTDC/BIO/77936/2006).

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