Nitrate removal properties of solid-phase denitrification processes using acid-blended poly(l-lactic acid) as the sole substrate

T Yamada, H Matsuoka, J Sun, S Yoshikawa, H Tsuji, and A Hiraishi

1Department of Environmental and Life Sciences, Toyohashi University of Technology, Toyohashi 441-8580, Japan
2Toyo Seikan Group Corporate R&D, 240-0062, Kanagawa, Japan
*Corresponding author. E-mail: tyamada@ens.tut.ac.jp

Abstract. The large amount of waste that is discharged along with the diffusion of poly(l-lactic acid) (PLLA) articles in use is persistent concern. Previously, we studied solid-phase denitrification (SPD) processes using PLLA to establish an effective re-use of PLLA waste. We found that PLLA with a weight-average molecular weight ($M_w$) of approximately 10,000 was suitable for SPD processes; however, the recycling of PLLA waste consumes a high energy. A new PLLA plastic including 5% poly(ethylene oxalate) (PEOxPLLA) as a blend material has attracted attention because recycling of PEOxPLLA consumes less electricity than that of PLLA. In this study, our main objectives were to evaluate whether PEOxPLLA can be used for SPD processes by changing its $M_w$ and to investigate the bioavailability for denitrification of hydrolysates released from PEOxPLLA. The predicted hydrolysates, including oxalic acid, ethylene glycol, and lactate, are abiotically released, leading to different biological nitrate removal rates. Consequently, the nitrate removal rate of PEOxPLLA ranged from 0.9–4.1 mg-NO$_3$--N·g-MLSS·h$^{-1}$ by changing the $M_w$ in the range of 8,500–238,000. In culture-dependent approaches, denitrifying bacteria using each substrate as an electron donor are found in activated sludge, suggesting that all hydrolysates functioned in the SPD processes using PEOxPLLA.

1. Introduction

Poly(l-lactic acid) (PLLA) has attracted much attention as a biodegradable plastic material that can be employed for general use, as an alternative to petrochemical plastics. The diffusion of PLLA plastics in use would bring about a discharge of large amount of PLLA waste, which calls for an effective way of using the waste as an earth-friendly resource circulation system. A promising method for this subject involves the use of PLLA instead of externally added soluble...
substrate (e.g., methanol) as reducing power for denitrification during nitrogen removal from wastewater.

Thus far, biological denitrification with biodegradable plastics, such as poly (3-hydroxybutyrate) (PHB) [1,2,3], poly [(R)-3-hydroxybutyrate-co-hydroxyvalerate] (PHBV) [4,5], and poly (ε-caprolactone) (PCL) [6,7], termed solid-phase denitrification (SPD) [8], has been intensively studied. However, much less effort has been directed toward the use of PLLA in SPD processes, because of its relatively low biodegradability. In fact, nitrate removal during SPD processes with commercially available PLLA has been shown to be much lower than that with PHBV or PCL [8]. For practical use of PLLA as the source of electron donor in the SPD processes, it is particularly important that PLLA be modified for enhancing hydrolysis and degradability. One of the effective and simple ways to achieve this is the adjustment of the weight-average molecular weight ($M_w$) to enhance physicochemical hydrolysis and degradability of PLLA. Our previous study has shown that good nitrate removal efficiency takes place with lactate abiotically released from the PLLA when its $M_w$ is adjusted to 10,000 [9]. However, a persistent problem is the concern that the adjustment of $M_w$ requires large energy consumption. A new PLLA plastic including 5% poly(ethylene oxalate) (PEOxPLLA) as a blend material has attracted much interest as an environmentally-friendly PLLA resin. Physicochemical hydrolysis and degradation of PEOxPLLA are faster than those of PLLA in the environment, because oxalate and ethylene glycol are first released, enhancing the hydrolysis PLLA region in PEOxPLLA materials. Considering the physicochemical characteristics, PEOxPLLA can be made with less energy, and serve as an electron donor for SPD processes. However, little information has so far been available on SPD processes with PEOxPLLA.

The main objectives of this study were to evaluate whether PEOxPLLA can serve as a solid substrate for denitrification by changing its $M_w$, and to investigate whether the oxalate, ethylene glycol, and lactate released from PEOxPLLA could be utilized as electron donors by denitrifying bacteria. Also, viable denitrifying bacteria that can utilize each hydrolysate were measured and compared with the total cell count and the total viable count in activated sludge.

2. Materials and methods

Preparation and characterization of PEOxPLLA For the production of poly(ethylene oxalate) (PEOx), 354 g (3.0 mol) of dimethyl oxalate, 223.5 g (3.6 mol) of ethylene glycol, and 0.30 g of tetrabutyl titanate were introduced into a 1 L separable flask equipped with a mantle heater, a stirrer, a nitrogen inlet, and a condenser. The flask was heated under nitrogen stream until the inside temperature increased from 110 to 170°C, while methanol was distilled off. After the reaction proceeded for 9 h, 210 mL of methanol was distilled off at the end of the reaction. Thereafter, stirring was performed for 1 h at an inside temperature of 150°C and at a reduced pressure of 0.1 to 0.5 mmHg. After the reaction proceeded for 7 h at an inside temperature of 170 to 190°C, the PEOx products were taken out from the flask. Oval PEOxPLLA pellets, measuring 5 mm in length and 4 mm in width and consisting of poly(lactic acid)/poly(ethylene oxalate) (95/5% [v/v]), were melted and blended at 200°C using a twin-screw extruder (ULT Nano 05-20AG, Technovel Corporation, Osaka, Japan). The $M_w$ of the PEOxPLLA pellets was approximately 238,000 ($M_w$/number-average molecular weight ($M_n$)=2.0). The pellets were autoclaved at 121 or 130°C under high pressure of 1.05 or 1.9 kgf cm$^{-2}$ to decrease their $M_w$ (Table 1).
The $M_w$ of the PEOxPLLA pellets was determined by HLC-8120 gel permeation chromatography (GPC) (Tosoh, Tokyo, Japan) with a TSK gel Super HM-H column (Tosoh). For comparison, oval PLLA pellets (5 mm long and 4 mm wide) which had an initial $M_w$ of approximately 238,000 ($M_w$/number-average molecular weight ($M_n$)=1.74), were also autoclaved at 121°C under high pressure of 1.05 kgf cm$^{-2}$. The $M_w$ of the autoclaved PLLA pellets was determined by GPC (Tosoh, Tokyo, Japan) as described [10].

**Table 1** Autoclave conditions for production of PEOxPLLA and PLLA having different $M_w$ applied for SPD processes

| Biodegradable plastics | Temperature ($^\circ$C) | Pressure (kgf cm$^{-2}$) | Time (min) | $M_w$  |
|------------------------|-------------------------|--------------------------|------------|--------|
| PLLA                   | 120                     | 1.05                     | 0          | 193,300|
|                        | 120                     | 1.05                     | 120        | 45,100 |
|                        | 240                     | 1.05                     | 240        | 14,100 |
|                        | 270                     | 1.05                     | 270        | 11,500 |
|                        | 300                     | 1.05                     | 300        | 9,900  |
|                        | 360                     | 1.05                     | 360        | 7,900  |
|                        | 480                     | 1.05                     | 480        | 5,300  |
|                        | 600                     | 1.05                     | 600        | 3,800  |
| PEOxPLLA               | 121                     | 1.05                     | 0          | 238,000|
|                        |                         |                          | 5          | 99,600 |
|                        |                         |                          | 15         | 43,000 |
|                        |                         |                          | 30         | 28,400 |
|                        |                         |                          | 120        | 15,500 |
|                        |                         |                          | 60         | 8,500  |
|                        |                         |                          | 90         | 5,000  |

The $M_w$ of the PEOxPLLA pellets was determined by HLC-8120 gel permeation chromatography (GPC) (Tosoh, Tokyo, Japan) with a TSK gel Super HM-H column (Tosoh). For comparison, oval PLLA pellets (5 mm long and 4 mm wide) which had an initial $M_w$ of approximately 238,000 ($M_w$/number-average molecular weight ($M_n$)=1.74), were also autoclaved at 121°C under high pressure of 1.05 kgf cm$^{-2}$. The $M_w$ of the autoclaved PLLA pellets was determined by GPC (Tosoh, Tokyo, Japan) as described [10].

**Measurement of hydrolysis release rate and nitrate removal rate** The hydrolysate release rate was measured by monitoring the hydrolysates (oxalate, ethylene glycol, and lactate) from PEOxPLLA pellets having different $M_w$. This measurement was performed using vials containing 50 ml RM2 medium [11] supplemented with 20 mM KNO$_3$ and PEOxPLLA pellets (1% [v/v]). The concentration of oxalate and lactate was determined by HPLC with an organic acid column (Waters, Milford, MA, USA), as described [4]. Ethylene glycol was measured with a GC353B gas chromatograph (GL science, Tokyo, Japan) equipped with a CP-porabond Q fused silica column (Crawford Scientific, Scotland, UK) and a flame ionization detector. To measure the nitrate removal rate with PEOxPLLA having different $M_w$, an activated sludge sample was collected from the main aerobic treatment tank of a sewage treatment plant in Toyohashi, Japan. The sludge sample was washed twice with 50 mM phosphate buffer (pH 7.0) and subjected to the nitrate removal assay. The washed sludge suspension at a final concentration of 2,000 mg L$^{-1}$ was introduced into rubber-plugged test tubes (75 mL capacity) containing 50 mL RM2 medium supplemented with 20 mM KNO$_3$ and PEOxPLLA pellets (1% [v/v]). Nitrate removal tests were also conducted using the same medium with 20 mM oxalate, 30 mM ethylene glycol, and 20 mM...
lactate to determine the bioavailability of each hydrolysate. The nitrate removal rate was measured by monitoring the change in the concentration of nitrate by ion chromatography as described previously [4,9].

**Numeration of total cell count, total viable count, and culturable denitrifying bacteria**

The total cell count in the activated sludge samples was measured by the SYBR Green-based total cell counting method [5]. Using the 1/10 TSA agar-medium (tryptone, 1.7 g·L⁻¹; soytone, 0.3 g·L⁻¹; glucose, 0.25 g·L⁻¹; NaCl, 0.5 g·L⁻¹; K₂HPO₄, 0.25 g·L⁻¹; agarose, 15 g·L⁻¹, pH7.0), diluted sludge samples were plated and incubated under aerobic conditions at 25°C for 1 week to measure the total viable count in the activated sludge. Culturable denitrifying bacteria in the sludge were measured by a pour-plating method with agar medium containing mineral base RM2 [11], 20 mM KNO₃, and 1.8 % (w/v) agar as the basal medium, to which 20 mM oxalate, 30 mM ethylene glycol, or 20 mM lactate as the sole carbon/energy source was added. On the media, diluted sludge samples were plated and cultivated under anaerobic condition at 25°C for 3 weeks. In all cases, plates recovering colonies in a range of 20–200 were taken for counting of colony-forming units (CFU).

3. Results and discussion

PEOxPLLA pellets with different $M_w$ were produced by autoclaving at different temperatures and times and compared with PLLA pellets treated under similar conditions. PEOxPLLA pellets with low $M_w$ could be made with less energy, because the autoclaving time for varying the $M_w$ of the PEOxPLLA pellets was much shorter than that for PLLA (Table 1). This can be explained by inferring that water easily approaches the pore where oxalate and ethylene glycol as the acid-blended materials in PEOxPLLA are released during the first step of autoclaving, thereby stimulating the hydrolysis of the PLLA region in PEOxPLLA.

![Graph A](image1.png) ![Graph B](image2.png)

**Fig. 1** Changes in abiotic hydrolysate-release rates for PEOxPLLA having different $M_w$ (A) and biological nitrate removal rates using them as the substrate (B). Symbols for hydrolysates (A): oxalate (closed triangles), ethylene glycol (open squares), and lactate (closed circles).
By using PEOxPLLA with different $M_w$, we measured the abiotic hydrolysate release rate by detecting lactate, oxalate, and ethylene glycol. The lactate release from the PEOxPLLA increased with lower $M_w$ (Fig. 1A) whereas oxalate and ethylene glycol release rates showed different tendencies in terms of $M_w$. The oxalate release was observed in a range of approximately 15,500 to 100,000 of $M_w$, but no hydrolysis release occurred in $M_w$ less than approximately 8,500 or more than 100,000 (Fig. 1A). On the other hand, ethylene glycol was released from all $M_w$-type PEOxPLLA tested (Fig. 1A).

Using PEOxPLLA with different $M_w$ noted above and activated sludge, the nitrate removal rate was measured to confirm the bioavailability of PEOxPLLA as electron donor for SPD processes. The highest nitrate removal rate (4.1 mg-NO$_3$·N·g-MLSS·h$^{-1}$) was shown with the PEOxPLLA with a $M_w$ of approximately 15,500 (Fig. 1B). This nitrate removal rate was similar to that in PLLA-added SPD processes achieved in our previous study [9], suggesting that PEOxPLLA may be utilized as an electron donor for denitrification. Interestingly, SPD using the PEOxPLLA of high $M_w$ (>15,500) still exhibited nitrate removal rates (0.9 to 3.6 mg-NO$_3$·N·g-MLSS·h$^{-1}$), contrasting with our previous finding that no nitrate removal took place with such $M_w$ of PLLA [9]. The nitrate removal rate of the PEOxPLLA with high $M_w$ might result from the use of hydrolysates other than lactate as electron donors (Fig. 1B). These findings suggested that the nitrate removal could be engineeredly controlled with the PEOxPLLA at a range wider than that with PLLA [9].

The nitrate removal tests with PEOxPLLA showed that all hydrolysates released could be utilized as electron donors for SPD, although the difference in nitrate removal rates was noted among the substrates used (Fig. 2). To explain this difference, denitrifying bacteria that can utilize oxalate, ethylene glycol, and lactate as the hydrolysates from PEOxPLLA were measured in the seed activated sludge. In the sludge, the total cell count and total viable count were $9.9 \times 10^8$ cell·mL$^{-1}$, $4.9 \times 10^7$ CFU·mL$^{-1}$. The plate counts of denitrifying bacteria with oxalate, ethylene glycol, and lactate as the sole substrate were $4.2 \times 10^4$, $4.7 \times 10^5$, and $2.4 \times 10^6$ CFU·mL$^{-1}$, respectively, accounting for 0.09, 0.9, and 4.9% of the total viable count. These results suggest that the difference of nitrate removal rates was affected by the absolute number of denitrifying bacteria in the seed sludge rather than by the difference in bioavailability of the substrates.

**Acknowledgements**

This study was supported in part by a Grant-in-Aid for Scientific research (No.21510084) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. This study was also supported by the Toukai Foundation for Technology and Kurita Water and Environmental Foundation (24083).
References
[1] Bernat K and Wojnowska-Baryla I 2008 Environ. Technol. 29 (1) 81–89
[2] Boley A, Muller W -R and Haider G 2000 Aquacul. Eng. 22 (1) 75–85
[3] Gibbs B M, Shephard L R, Third K A and Cord-Ruwisch R 2004 Wat. Sci. Technol. 50 (10) 181–188
[4] Karn S T, Horiba Y, Yamamoto M and Hiraishi A 2002 Appl. Environ. Microbiol. 68 (7) 3206–3214
[5] Karn S T, Horiba Y, Takahashi N and Hiraishi A 2007 Microbes Environ. 22 (1) 20–31
[6] Boley A, Mergaert J, Muller C, Lebrenz H, Cnockaert M C, Müller W -R, and Swings J 2003 Acta Hydrochim. Hydrobiol. 31 (3) 195–203
[7] Horiba Y, Karn S T and Hiraishi A 2005 Microbes Environ. 20 (1) 25–33
[8] Hiraishi A and Karn S T 2003 Appl. Microbiol. Biotechnol. 61(2) 103–109
[9] Takahashi M, Yamada T, Tanno M, Tsuji H and Hiraishi A 2011 Microbes Environ. 26(3) 212–219
[10] Tsuji H and Tenzuka Y 2005 Macromol. Biosci. 5 135–148
[11] Hiraishi A and Kitamura H 1984 Bull. Jpn. Sci. Soc. Fish. 50 1929–1937