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Hydroxylation and hydrolysis: Two main metabolic ways of spiramycin I in anaerobic digestion

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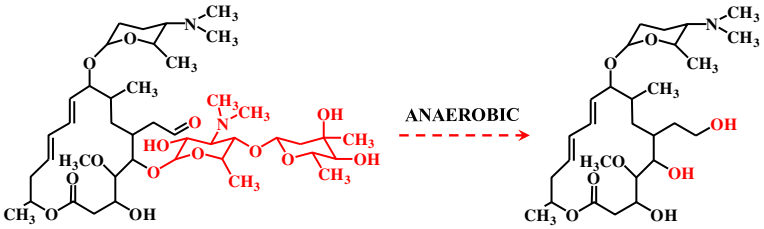
HIGHLIGHTS

- Antibiotic like macrolide could be degraded in anaerobic digestion.
- Over 95% spiramycin was degraded in 32 day's anaerobic digestion.
- Hydroxylation and hydrolysis were two metabolic ways of SPM I in anaerobic process.
- Structural analysis confirmed that P-3 was a new compound.

GRAPHICAL ABSTRACT



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ABSTRACT

The anaerobic degradation behaviors of five macrolides including spiramycin I, II, III, midecamycin and josamycin by sludge were investigated. Within 32 days, 95% of spiramycin I, II or III was degraded, while the remove rate of midecamycin or josamycin was 75%. SPM I degradation was much higher in nutrition supplementation than that just in sludge. The degradation products and processes of spiramycin I were further characterized. Three molecules, designated P-1, P-2 and P-3 according to their order of occurrence, were obtained and purified. Structural determination was then performed by nuclear magnetic resonance and MS/MS spectra, and data indicated that hydroxylation and hydrolysis were main reactions during the anaerobic digestion of spiramycin I. P-1 is the intermediate of hydroxylation, and P-2 is the intermediate of hydrolysis. P-3 is the final product of the both reaction. This study revealed a hydroxylation and hydrolysis mechanism of macrolide in anaerobic digestion.

1. Introduction

Antibiotics play a very important role in the treatments of human and animal diseases and are widely used as animal growth promoter in feed. Thousands of tons of antibiotics have been consumed in China each year; whereas, the resultant mass excretion of undegraded antibiotics poses emerging environment issues. Indeed, the residual antibiotics have been detected in all tested soils and rivers in China (Xu et al., 2007; Zheng et al., 2012; Zou et al., 2011). Of the administered antibiotics, only 10–70% will be absorbed by the body, while the rest will be excreted, mostly through poultry manure and human excrement (Sarmah et al., 2006). Therefore municipal sewage and manure is one of the major exposure routes that transport antibiotics or their metabolites into the environment (Mohring et al., 2009; Motoyama et al., 2011). Another major exposure route of antibiotics is the plants and hospitals where a large amount of antibiotics-polluted liquid and solid is released to the environment.

Macrolides are a group of antibiotics widely used in human and veterinary medicine. Meanwhile, they are also largely excreted into sewage with unchanged forms at extraction rates greater than 60% (Hirsch et al., 1999). It has been reported that the concentration of macrolides in raw sewage from Switzerland varies between 0.01 and 0.6 μg L^{-1} (Gobel et al., 2005a,b), and the wastewater treatment plants (WWTPs) influent in the USA

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contains macrolides at a concentration of 1.5 μg L^{-1} (Karthikeyan and Meyer, 2006). In individual cattle, chicken, and swine manures, the peak concentrations of tylosin reach 119, 35, and 62 mg kg^{-1} (dry weight basis), respectively (Scott Teeter and Meyerhoff, 2003). Thus, substantial amounts of macrolides could go to rivers and soils through effluent discharge and manuring. Several investigations (Xu et al., 2007; Zheng et al., 2012; Zou et al., 2011) have demonstrated that residues of some macrolides have been found in soils and adjacent environmental compartments such as surface and groundwater. Gullberg et al. (2011) suggested that the low antibiotic concentrations found in the environments were favorable for the enrichment and maintenance of resistance in bacterial population. Recent years, researches (Calza et al., 2009; Lange et al., 2006; Vione et al., 2009) have been focusing on the nature environmental degradation ways on macrolides such as photolysis, ozonation and hydrolysis. However, the effects of possible bioactivity of certain antibiotic degradations in the environment are still unknown.

Anaerobic digestion is an important way to reduce antibiotic pollution by the interaction between anaerobic microorganisms and antibiotics. Increasing numbers of recent researches have focused on the behaviors and kinetics of antibiotic degradation in anaerobic treatments of manure (Alvarez et al., 2010; Arikan, 2008; Beneragama et al., 2013; Dreher et al., 2012), pharmaceutical wastewater (Shimada et al., 2008; Sponza and Demirden, 2010) and municipal sewage (Gartiser et al., 2007; Le-Minh et al., 2010). The antibiotic concentrations have more or less inhibitive effects on methane production or methane percentage. Beneragama demonstrated that cefazolin showed no inhibition for methane production and oxytetracycline showed 70% inhibition (Beneragama et al., 2013). Dreher also found that chlortetracycline did not inhibit biogas production, however the methane percentage was approximately 15% decreased (Dreher et al., 2012). Shimada demonstrated that tylosin can inhibit propionate- or butyrate-oxidizing syntrophic and fermenting bacteria, and consequently lead to unfavorable effects on methanogenesis (Shimada et al., 2008). The biochemical mechanism of antibiotics degradation involved in anaerobic fermentation has not been elucidated in detail, mainly because of failures in isolation and purification of antibiotic metabolites to a detectable concentration from complicated anaerobic digestion systems. Up to data only one metabolite, formed by hydroxylation at the pyrimidine ring 4-OH-sulfadiazine, has been identified and partly quantified by mass spectrometry (Mohring et al., 2009). The unknown metabolites discharged into the environment directly after anaerobic treatment may also bring potential negative effects.

Spiramycin is a 16-member macrolide mainly used to treat infections of oropharynx, respiratory system and genito-urinary. It is a broad-spectrum antibiotic against most of Gram-positive and Gram-negative cocci, but not against *Enterobacteriaceae* (Rubinstein and Keller, 1998). The basic structure of spiramycin is a lactone ring bearing 16 carbon atoms, and is modified with three sugar radicals: mycarose, mycaminose and forosamine (Richardson et al., 1990). The clinically used spiramycin is a mixture of spiramycin I, spiramycin II (3-acetyl) and spiramycin III (3-propanoyl). The criterion of the ratio of these components varies in different countries. In France the major component is spiramycin I (>85%), while spiramycin II and III only account for <5% and 10%, respectively (Mourier and Brun, 1997). In contrast, the product in China mainly contains spiramycin II and III (>35%, respectively) with moderate spiramycin I (<10%) (Liu et al., 1997). Diffusion tests have demonstrated that the relative microbial activities of spiramycin II and III to spiramycin I are 57% and 72%, respectively (Liu et al., 1999). To date, seldom has been focused on the degradation of spiramycin under anaerobic condition. Castiglioni et al. (2006) have investigated the removal rate of spiramycin. However, the degradation pathways of spiramycin and the resultant metabolites await investigation.

Our previous studies showed that after spiramycin-containing waste was treated in the anaerobic digestion system, nearly no spiramycin could be detected by HPLC. This raised the questions: what and how spiramycin was changed to. To answer these, lab-scale anaerobic digestion tests were performed to verify the degradation results of spiramycin I, II, III, midecamycin and josamycin. Degradation products of spiramycin I were purified for structure analysis and degradation processes assay.

2. Methods

2.1. Reagents

Spiramycin I (SPM I, 95%) and spiramycins (SPM I <10%, II and III about 35%, respectively) were supported by Topfond (Henan, China). Spiramycin II (SPM II) and III (SPM III) were purified in lab with 93% purity. SPM I wastewater (COD 23,000 ± 3000 mg L^{-1}, SPM I concentration 65 ± 12 mg L^{-1}) and sludge (TS, 24%) were also supplied by Topfond. Midecamycin (MIM, 96%) and josamycin (JOM, 95%) were kindly supplied by NIFDC (Beijing, China). All other chemicals and solvents were of analytical or HPLC grade. All the above five macrolides were prepared in ethanol at the test solution (1024 mg L^{-1}) stored at −70 °C. Macroporous resin (NM100) was a gift from Nano-Micro (Suzhou, China). The matrix of the resin was polystyrene with a particle diameter of 40–100 μm.

2.2. Activation of sludge

The sludge was centrifuged and washed twice with distilled water in order to remove any metabolites and 12.5 L of the pretreated sludge was transferred to a 30 L jacketed and hermetic bioreactor with a 25-L working volume. Then, 12.5 L medium which containing 8 g L^{-1} starch, 5 g L^{-1} glucose, 4 g L^{-1} soybean meal, 2 g L^{-1} NaCl, 3 g L^{-1} NH_4NO_3 and 1 g L^{-1} KH_2PO_4 (pH 7.2) was added into the reactor to activate the sludge at 37 °C for two weeks. After that, 5 L activated sludge was withdrawn and same volume of fresh medium was added simultaneously everyday for three months with intermittent agitation (5 min twice a day) (Shimada et al., 2008).

2.3. Anaerobic degradation of five macrolides

In this study, 1 L hermetic jars with butyrate rubber septa were conducted for degradation tests of the five macrolides in triplicate. Each jar that contains 0.4 L activated sludge and 0.4 L medium was spiked with 160 mg of each of the five macrolides (final concentration 200 mg L^{-1}) (Table 1). The temperature was maintained at 37 °C throughout the 32-day digestion. Every four days, 1 mL sample from each fermentor was drawn and mixed with 1 mL ethanol for shaking two hours and then centrifuged to remove cell mass and other insoluble substances. Quantitative analysis was performed by HPLC (Waters, America; 2998 Photodiode Array Detector) with a Welchrom C18 column, 5 μm, 4.6 × 250 mm (Welch, America). The mobile phase was a 63:37 (v/v) mixture of 50 mM ammonium acetate water solution and acetonitrile. The column temperature was 35 °C and the effluent was monitored at 232 nm.

2.4. Anaerobic degradation process of SPM I

SPM I was chosen to further explore the degradation process in anaerobic digestion. The digestion was conducted in two 1-L jars with butyrate rubber septa. The activated sludge and medium used were same as above. In order to compare whether the nutrition af-