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The study on biodegradation of methylene urea by activated sludge

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ABSTRACT

Methylene urea (MU) is a kind of slow-release fertilizer. The application of MU can increase nitrogen use efficiency, reduce environmental impacts and the labor intensity of farmers. This study investigated a rapid, simple and practical way to research MU biodegradation. The activated sludge after sterilization was used as the carrier for microbial growth and the soil with the MU-degrading microorganisms was added as inoculum. By adding glucose as the carbon source and MU as the nitrogen source, after 10–15 days of aerobic training at room temperature, the activated sludge with large amounts of MU-degrading microorganisms was used as research material. The hydrolysis of the urea released from dimethylenetriurea (DMTU) degradation was inhibited by N-(n-Butyl)thiophosphoric triamide (NBPT). The results showed that the nitrogen release process of DMTU is as follows: 1) the dissolved DMTU was firstly absorbed by microorganisms and then decomposed into urea, ammonium and formaldehyde by intracellular enzyme; 2) the urea and the ammonium nitrogen from DMTU degradation were released into the environment; 3) the urea released is further hydrolyzed into ammonium and carbon dioxide by urease in the extracellular environment. Because the dissolved DMTU can be rapidly mineralized by microorganisms and the degradation rate of DMTU is a constant, the degradation rate of DMTU is independent of the concentration of reactants and determined by other factors.

1. Introduction

MU is condensation products of urea and formaldehyde [1]. The general formula of MU can be expressed as $NH_2CONH(CH_2NHCONH)_nH$, n = 1 to 8–10 [2]. The degree of polymerization depends on the ratio of urea to formaldehyde but also condensation conditions [3]. When the mole ratio of urea to formaldehyde is in the range 1.2–1.5 to 1 [2], the polymeric products are suitable as slow-release fertilizer. MU are widely used as slow-release fertilizers in professional horticulture, nurseries, greenhouses, golf courses, household consumers, turf, landscape gardeners and public parks [1]. MU as a nitrogen fertilizer can increase nitrogen use efficiency by plants [4–6], reduce ammonia volatilization [7] and leaching losses of nitrate nitrogen [8,9], and prolonged availability of N through out the growing season [2,10–12]. Currently, the estimated use of urea reaction products is about 308,000 metric tons per year in the US, Western Europe, and Japan in 2004/2005 [13].

It is known that MU is degraded by microbial activity [14]. The mineralization of MU in soil has been described by a series of consecutive chemical processes [15]:

$$\text{Ureaform} \rightarrow \text{urea} \rightarrow NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$$

Only recently the specific information on the microorganisms capable of hydrolyzing MU has been published. Jahns et al. have isolated and identified two MU-degrading Gram-negative organisms: *Ochrobactrum anthropi* [16,17] and *Ralstonia paucula* [18]. Koivunen et al. purified a methylene urea-hydrolyzing enzyme from *Agrobacterium tumefaciens* [19]. From these microorganisms, they have purified and characterized enzymes (MUases) that hydrolyze MU to ammonium and urea. In *Ochrobactrum anthropi*, the MUase was shown to degrade MDU (methylenediurea), DMTU, and TMTU (trimethylenetetraurea) to ammonium, formaldehyde, and urea in a molar ratio of 2: 1: 1 (MDU), 4: 2: 1 (DMTU), and 6: 3: 1 (TMTU) [19,20].

The testing method of MU fertilizers in different countries is basically the same as AOAC 945.01 [21] and 955.05 [22]. Until

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1980s, the content of urea [23,24], MDU, DMTU and TMTU in MU fertilizers could be analyzed quantitatively by HPLC [25–28]. At present, the characteristics of the MU fertilizers are identified by AI (activity index) [29]. Although the AI value reflects the solubility of the MU fertilizers, the nitrogen release of MU also has a great relationship with microorganisms. So it is desirable to develop an analytical or biological method that more closely predicts biological activity and biological performance of Ureaforms than the current analytical method [2].

It is generally recognized that the mineralization of MU is carried out by microorganisms, but the detailed mechanism still remains unclear. The aim of this experiment is to establish a research method to study the mechanism of MU biodegradation and nitrogen release. According to the results of previous studies, the degradation process of DMTU was assumed to be: firstly, the dissolved DMTU is absorbed and decomposed into urea, ammonium and formaldehyde by microorganisms; and then the urea and the ammonium from DMTU degradation are released into the environment; finally, the urea is hydrolyzed into ammonium and carbon dioxide by urease in the environment. NBPT is an effective urease inhibitor [30]. In order to verify the above hypothesis, the hydrolysis of the urea derived from MU degradation is inhibited by adding a sufficient amount of NBPT, and the activated sludge after sterilization instead of the soil is used as the carrier of microbial growth to avoid the interference from soil to the analysis of ammonium nitrogen.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in this study were at minimum analytical grade. Urea was purchased from Aladdin Industrial Corporation, and NBPT (97%) was from J&K scientific LTD. DMTU was synthesized in the laboratory. 400 g urea and 400 ml water was added to a 1000 ml round bottom flask. When urea was completely dissolved, 1.5 ml phosphate (85%) and 125 g formaldehyde (40%) were added. After stirring at 45 °C for 1 h, the product was filtered and the precipitant was obtained and washed with about 40–50°Cwater a few times. The main ingredient of the precipitant is DMTU by HPLC. After drying at 80 °C, this precipitant is used as a raw material for the purification of DMTU. The preparation protocol of the purified DMTU is from the AOAC Official method [25]. The preparation conditions are as follows: AGLENT 1200; pump, P270; detector, UV230 UV Vis DETECTOR, column, SinoChrom ODS BP 9.4 × 250 mm, 5 μm, semi preparative column; flow rate, 5 ml/min. The purified DMTU was used as standard material and test materials. The remaining precipitant for the purification of DMTU was used as the nitrogen source of the sludge culture.

2.2. The cultivation of the activated sludge with MU-degrading microorganisms

The activated sludge from SBR (Sequencing Batch Reactor) of laboratory was precipitated for 15 min and the supernatant was discarded. After high pressure sterilization, the sludge was used as the carrier for microbial growth. The soil once applied MU fertilizer was used as a microbial source. With a 5 L container, the activated sludge was cultured under aerobic conditions. The mineral nutrient medium was prepared in accordance with EPA 712–C–98–076 [31], but with MU (the raw material for the purification of DMTU) instead of ammonium chloride as nitrogen source. 5 g glucose per time was added as carbon source. Every morning and evening to stop aeration for 30 min and discard the supernatant, the mineral nutrient medium, MU, glucose and some water were added.

2.3. Experiment design

2.3.1. The inhibition of urea hydrolysis by NBPT

The experiment consisted of three treatments: 1) Urea (0 mg NBPT), 2) Urea + NBPT (50 mg and 100 mg NBPT) and 3) Control (blank control group, No urea or NBPT added), with three replicates. The urea content in the solution was determined by HPLC and the higher mineral content would interfere with the determination of urea, so the sludge was washed with distilled water. Add a certain amount of activated sludge to several 1000 ml cylinders and let it settle for 15 min. After discarding the supernatant, add a proper amount of distilled water, let it settle for 15 min and discard the supernatant again. Repeat the above operation. When the content of the total dissolved solids in supernatant is about 30 ppm with TDS meter (hold), the supernatant is discarded and the sludge after mixing is used as test materials. Then 200 ml sludge is added to every test container with aeration device. After drying at 105 °C and weighing, 200 ml activated sludge contains 4.64 g dry matter. The content of MLSS, mixed liquor suspended solids, is equivalent to 23.21 g/l. In order to inhibit urease activity, 25 mg and 50 mg NBPT were added to the sludge of Urea + NBPT50 and Urea + NBPT100 treatment for pretreatment. After 20 min of aerobic training, 100 ml solution containing 50 mg urea and 200 ml water was added to the containers of Urea, Urea + NBPT50 and Urea + NBPT100 treatments. In the experiment, the mineral medium was not added to avoid interfering with urea test by HPLC. The containers of Control treatment was only added 200 ml sludge and 300 ml distilled water to 500 ml. After adding urea, the samples was taken at 0, 0.5, 1, 2, 3, 5, 7 h and filtrated through 0.22 μm filter membrane, and the filtrate was used to measure the content of urea and ammonium nitrogen. The content of urea was determined by HPLC, and the content of ammonium nitrogen was by the indophenol blue colorimetric method [32].

2.3.2. The effect of the sterilized sludge on DMTU degradation

After washing in accordance with 2.3.1, part of the sludge was sterilized (DMTU-S treatment, with three replicates). 200 ml sterilized sludge was added to every test container respectively, and then the solution containing 50 mg DMTU was added. The volume was adjusted to 500 ml by supplementing the distilled water. After adding DMTU, the samples was taken at 0, 0.5, 1, 2, 3, 5, 7 h and filtrated through 0.22 μm filter membrane to obtain the filtrate used as DMTU test. According to the method of 2.4, DMTU was measured by HPLC.

2.3.3. Microbial degradation of DMTU

The washing method of the activated sludge is the same as 2.3.1. Before adding DMTU, the sludge of DMTU + NBPT treatment was treated with 50 mg NBPT for 20 min, then the solution containing 50 mg DMTU and water were added to every test container, and the total volume was 500 ml. The Control treatment is the same as 2.3.1. The method of sampling, filtering and test is the same as 2.3.2. The content of urea and DMTU was determined by HPLC, and the content of ammonium nitrogen was by the indophenol blue colorimetric method.

In order to study the effect of the sludge activity on the test results, the sludge of the normal cultivation was used as the sludge of the higher activity (DMTU1, DMTU + NBPT1), and the sludge, after 3 days of prolonged cultivation without adding any carbon source and nitrogen source, was used as the sludge of the lower activity (DMTU2, DMTU + NBPT2).

2.4. HPLC analysis

The qualitative analysis of DMTU and urea was carried out by