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Effect of alkaline protease-producing *Exiguobacterium* sp. YS1 inoculation on the solubilization and bacterial community of waste activated sludge

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ABSTRACT

A new approach to the solubilization of waste activated sludge (WAS) using an alkaline protease-producing bacterial isolate, *Exiguobacterium* sp. YS1, was investigated under controlled mild alkaline conditions at pH 10. Compared with the noninoculated experiment, the inoculated experiment in an anaerobic bioreactor increased soluble chemical oxygen demand concentration and alkaline protease activity by more than 40%, indicating a synergistic effect could be achieved when both bacterial inoculation and alkaline treatment were combined. Indeed, this combination led to 56.6% COD solubilization after 5 days of reaction time. However, the inoculant was not effective in the aerobic bioreactor. Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA fragments revealed that the inoculated *Exiguobacterium* sp. YS1 became the predominant population in the bacterial community during the anaerobic solubilization processes. These results suggest that bioaugmentation of the organism might be useful for enhancing the solubilization of WAS at mild alkaline pH.

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1. Introduction

Although the conventional activated sludge process is the most widely used biological process for the treatment of domestic and industrial wastewater, this process generates a large amount of waste activated sludge (WAS) as the main by-product. The cost of WAS processing and disposal is as high as 50% of the total operating costs (Vlyssides and Karlis, 2004), and environmental regulations on sludge disposal are becoming more and more stringent. Therefore, the management of WAS is considered one of the most serious issues in wastewater treatment plants (Elliott and Mahmood, 2007).

Anaerobic digestion is the most commonly applied process for reducing the volume of WAS. However, conventional anaerobic digestion systems require long residence times (about 20 days) due to the slow rate of WAS degradation. Four stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) are involved in the anaerobic digestion of WAS, and the biological hydrolysis stage is the rate-limiting step (Tiehm et al., 2001). To reduce the impact of the rate-limiting step, pretreatment of WAS by mechanical, thermal, chemical, ultrasonic and thermochemical methods (Elliott and Mahmood, 2006) has been applied to disrupt microbial cells in WAS. Cell disruption by these pretreatments can accelerate the solubilization (hydrolysis) of WAS and reduce the particle size, which ultimately improves the anaerobic digestion (Kim et al., 2003; Feng et al., 2009). Alternatively, the solubilized products can be recirculated to the aeration tank for conventional activated sludge processing or used as an external organic carbon source for denitrification in advanced activated sludge processing.

Among the various pretreatment methods, alkaline pretreatment, which normally uses sodium hydroxide at ambient temperature, is efficient and cost-effective for inducing cell lysis and solubilization of WAS (Li et al., 2008). It significantly increases both chemical oxygen demand (COD) solubilization and total solid elimination rates as the dose of NaOH increases. Increasing concentrations of NaOH increase the pH, which is the most important pretreatment parameter for COD solubilization. However, extreme reactor pH conditions lead to low biodegradability performance, probably due to the formation of refractory compounds through intermolecular interactions between solubilized compounds (Penaud et al., 1999). In addition, high concentrations of sodium cations are known to inhibit methanogens (Chen et al., 2008). Corrosion of equipment is another potential problem associated with pretreatment with high NaOH. To circumvent these problems, combinations of NaOH treatment with other methods, such as thermal treatment, have been recommended to break up WAS under gentler operating conditions (Wei et al., 2003; Valo et al., 2004; Vlyssides and Karlis, 2004).

Proteins are the main organic components of WAS, representing approximately 50%; thus microbial proteases, particularly alkaline proteases, have been shown to reduce the solid content of WAS and accelerate the removal of pathogens through their lytic action toward microbial cells (Parmar et al., 2001). As an alternative to conventional alkaline treatment, it is therefore hypothesized that inoculation of alkaline protease-producing microorganisms into a

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reactor under alkaline conditions could improve the solubilization of WAS. To test this hypothesis, we isolated an alkaline protease-producing bacterial strain and evaluated its ability to solubilize WAS under mild alkaline conditions (pH 10.0). To monitor the changes in the bacterial community structure and to assess the survival of the inoculated strain, molecular analysis of bacterial community composition during WAS solubilization was carried out by means of denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene segments. To the best of our knowledge, this is the first report describing a combined system of bacterial inoculation and alkaline treatment for effective solubilization of WAS.

2. Methods

2.1. Isolation and identification of bacterial strain

Bacterial strains capable of producing alkaline protease were isolated from soil samples by enrichment culture using a basal medium (pH 10.0) containing 10 g/L of skim milk. Each liter of basal medium contained 3.0 g yeast extract, 6.7 g $(NH_4)_2SO_4$, 0.5 g NaCl, 1.2 g K_2HPO_4, 0.7 g KH_2PO_4, and 0.5 g $MgSO_4{\cdot}7H_2O$. The enriched culture broth was successively diluted and streaked on basal agar medium (pH 10.0) containing 10 g/L of skim milk and incubated at 28 °C for 3 days under aerobic conditions. Several colonies producing a clear zone were streaked on nutrient agar until a pure culture was established. Among the isolates, one strain, designated YS1, exhibited good growth and produced the largest clear zone around the colony; this strain was used in this study.

The phenotypic characteristics of the isolate were analyzed using VITEK® 2 Compact (bioMérieux, France). The nearly complete sequence of the 16S rDNA was determined using the Silver Sequence DNA Sequencing System (Promega Co., USA) as previously described (Kang et al., 2001) and then submitted to the Advanced BLAST program to identify the sequences of any closely related organisms. The 16S rDNA sequence of strain YS1 was deposited in the GenBank database under accession number FJ013096.

2.2. Cultivation of the isolate

The isolate YS1 was cultivated aerobically in a 5-L jar fermentor (Biotron Co., Korea) containing 2 L of skim milk basal medium. The temperature and pH were automatically controlled at optimal values, 28 °C and 9.0, respectively. The air flow rate was 0.5 vvm and the agitation speed was 200 rpm. After cultivation for 24 h, the cells were harvested by centrifugation, washed twice with distilled water, and then concentrated by centrifugation. The cell pellet was used as the inoculum in batch experiments for WAS solubilization.

2.3. Batch experiments for WAS solubilization

The WAS was drawn from the municipal wastewater treatment plant in Daejeon, Korea, and concentrated by gravity settling at 4 °C for 12 h. The main characteristics of the WAS are as follows: pH 6.7, total chemical oxygen demand (TCOD) 14,300 mg/L, soluble chemical oxygen demand (SCOD) 110 mg/L, total suspended solids (TSSs) 14,130 mg/L, and volatile suspended solids (VSS) 10,800 mg/L.

The batch experiments for WAS solubilization were conducted anaerobically or aerobically in a 5-L jar fermentor containing 2 L of WAS. After adjustment of initial pH at 10.0 by addition of 3 M NaOH, the reactor was inoculated with the cell pellet (equivalent to 2 g dry cell weight/L) of YS1 and operated at 28 °C for 10 days with a stirring rate of 100 rpm. During the batch experiments, the pH was automatically controlled at 10.0. Under these experimental conditions, the dissolved oxygen concentration and oxidation reduction potential in the mixed liquor were maintained below than 0.05 mg/L and −300 mV, respectively. In the aerobic batch experiment, the dissolved oxygen tension in the mixed liquor was maintained above 20% of air saturation by controlling the agitation speed and air flow rate. Control experiments were performed under the same operating conditions as above but were not inoculated with the isolate YS1.

2.4. Enzyme assay

The activated sludge mixed liquor was centrifuged at 13,000 rpm for 5 min and the supernatant served as the crude enzyme source. The protease activity was determined with casein as a substrate using modification of the method of Tobe et al. (2005). The reaction mixture of 0.5 mL enzyme and 2 mL of 1.0% (w/v) casein in 0.2 M glycine–NaOH buffer (pH 10.0) was incubated at 30 °C for 10 min. The reaction was terminated by addition of 3 mL of 10% trichloroacetic acid solution, and then the reaction mixture was filtered through a syringe membrane filter with 0.45-μm pore size. The amount of free amino acids was estimated from the absorbance at 275 nm using a UV/VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of tyrosine per 1 min per 1 mL of enzyme solution. All experiments were carried out in triplicate.

2.5. Analytical methods

The mixed liquor in the reactor was sampled at regular intervals and filtered through a Whatman GF/C glass microfiber filter with 1.2-μm pore size. The filtrate was immediately analyzed for SCOD, soluble protein, carbohydrate, and volatile fatty acids (VFAs). The analyses of TSS and VSS were conducted in accordance with standard methods (APHA, 1995). SCOD and TCOD were estimated using a colorimetric method after digestion of the samples in the COD analyzer (HACH DR/3000 spectrophotometer). The degree of COD solubilization was calculated by the ratio of SCOD/TCOD (Kim et al., 2003). Carbohydrate was determined by the Anthrone–sulfuric acid method with glucose as the standard (Dreywood, 1946). Soluble protein was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. VFAs were measured by injecting supernatants into a HP6890 plus gas chromatograph equipped with a flame ionization detector and a SUPELCO simplicity-wax column (30 m × 0.25 mm × 0.25 μm). The oven temperature was initially maintained at 60 °C for 4 min and then was raised to 280 °C at a rate of 8 °C/min.

2.6. PCR-DGGE

Total community DNA from the activated sludge mixed liquor collected at different times was extracted with the Fast DNA Spin Kit (Q-BIO Gene, USA) according to the manufacturer's instructions. The V-3 region (*E. coli* position 341–534) of bacterial 16S rDNA was amplified by PCR using primers GC341F/518R (Muyzer et al., 1993). The expected size of the PCR product with these primers was 218 bp. The PCR products were separated on 10% (w/v) polyacrylamide gels (acrylamide: bisacrylamide = 37.5:1) with a 35–65% linear gradient of denaturant, where 100% was defined as 7 M urea with 40% (v/v) formamide. Gels were run for 16 h at 60 V in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 50 mM EDTA, pH 8.0) at 60 °C. Denaturing gels were run using the DCode Universal Mutation Detection System (Bio-Rad, USA). The gels were then stained for 30 min in Gel-red (100 μL/L) in 1× TAE buffer and visualized by UV illumination. Gel images were acquired with the ChemDoc (Bio-Rad, USA) gel documentation system. Individual bands were selected for sequencing analysis,

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