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Identification and characterization of microorganisms from earthworm viscera for the conversion of fish wastes into liquid fertilizer

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ABSTRACT

Five bacteria isolated from earthworm viscera and identified as *Brevibacillus agri*, *Bacillus cereus*, *Bacillus licheniformis*, and *Brevibacillus parabrevis* by 16S rRNA sequencing were employed in the conversion of fish wastes generated from a restaurant specializing in sliced raw fish into fertilizer. Within 120 h after inoculation of autoclaved fish waste with 5.15×10^5 CFU ml^{-1} mixed isolates, the amount of dry sludge decreased from 29.4 to 0.2 g, the pH changed from 7.05 to 5.70, and the cell number reached 6.45×10^5 CFU ml^{-1}. Analyses of an 84-h culture of inoculated fish waste indicated low phytotoxicity in a seed germination test, an amino acid content of 5.71 g 100 g^{-1}, a low concentration of heavy metals (Pb, As, Cd, Hg, Cr, Cu, Ni and Zn), and a N/P/K level of 2.33%. Therefore the converted fish waste has the potential for use as liquid fertilizer, although the low NPK level is a concern. This is the first demonstration of the reutilization of fish wastes as a liquid fertilizer.

1. Introduction

As fish consumption has increased, so has waste from processing of fish. Often, these wastes are not utilized further, but disposed off in landfills, by incineration, or dumping at sea. Therefore, there is an urgent need to find ecologically acceptable means for reutilization of these wastes. Conventional methods for reutilization are ensilation and production of high-protein meals used in animal feeds (Faid et al., 1997). Composting has also been suggested as a viable solution (Liao et al., 1997) and fermentation has been studied as a way of producing animal feed (Faid et al., 1997; Hassan and Heath, 1986). Yeasts and/or lactic acid bacteria were used to ferment fish wastes and to remove fish odors (Faid et al., 1994). Shrimp waste ensilation is also carried out as a preservation method and to allow the recovery of value-added by-products, such as chitin (Cira et al., 2002) and cartenoids (Sachindra et al., 2007). Recently, acid hydrolysis of fish wastes has been studied to produce low-cost nutrients for the production of lactic acid (Gao et al., 2006), and low-cost protein sources have been produced by ensiling hydrolysed fish viscera to obtain a suitable medium for lactic acid bacteria (Vazquez et al., 2008).

Some fish wastes are also reutilized for the fish meal production (Hall, 1992; Keller, 1990); however, this process is costly and heat required for drying makes the fish meal less digestible (Yamamoto et al., 2005). In addition, new wastes can be generated (Yano et al., 2008). Therefore, new biological-fermentation techniques have been proposed to process fish waste inexpensively (Yamamoto et al., 2004, 2005). Yamamoto et al. (2004) reported a new low-cost fermentation technique using *Aspergillus awamori* that could also be applicable to fish wastes, and Yano et al. (2008) described a fermentation technique that improves the quality of fish meal from fish wastes rich of lipids. Fast fermentation of squid processing by-products has been also reported for low-salt fish sauce production (Xu et al., 2008).

Organic wastes contain compounds, which are capable of promoting plant growth (Day and Katterman, 1992), and seafood processing wastewaters do not contain known toxic or carcinogenic materials unlike other types of municipal and industrial effluents (Afonso and Borquez, 2002). Therefore, fermented broth of fish wastes could be a valuable resource for agriculture. To date, only a few reports on reutilization of biodegraded waste products as liquid fertilizer are available. Algur and Kadioglu (1992) converted waste products from alcoholic fermentation of sugar beet, and Kalyuzhnyi et al. (1999) utilized diluted manure streams after biological treatment.

In Korea, there are a lot of restaurants specializing in sliced raw fish nationwide, and large amounts (approximately 2100 tons/day) of fish wastes are generated every day. This paper presents the results of a study aimed at developing a fermentation product from such wastes suitable as liquid fertilizer. For this purpose, useful microorganisms were isolated from the viscera of earthworm and employed in fermentation of fish wastes. The amino-acid composition, presence of noxious compounds, N/P/K content, and effect on seed germination of the fermentation product were analyzed to evaluate its fertilizing potential.

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2. Methods

2.1. Isolation of useful microorganisms

Bacteria were isolated from viscera of earthworms that inhabited areas polluted by fish wastes. Approximately 1 g of viscera was added into 5 ml of sterile 0.2% NaCl and agitated to obtain a homogeneous suspension. With continuous supply of oxygen, the suspension was transferred into various 150-ml flasks containing 0.8% nutrient broth (pH 6.8), yeast-maltose medium (3 g l^{-1} of yeast extract, 3 g l^{-1} of malt extract, 5 g l^{-1} of peptone, 10 g l^{-1} of glucose, and 0.05 g l^{-1} of ampicillin, pH 6.2) and Bennet's medium (1 g l^{-1} of yeast extract, 1 g l^{-1} of beef extract, and 10 g l^{-1} of glucose, pH 7.2), respectively. After 1 day of incubation at 45 °C with shaking at 180 rpm, cells in each flask were spread with a platinum loop on the same media solidified with 1.5% agar, respectively. A purified isolate was obtained by repeated streaking on fresh media. Each pure culture was maintained on the agar plate at 4 °C and transferred to a fresh plate every two weeks.

To screen for useful microorganisms, all isolates were spread both on 1% skim milk agar for detection of proteolytic microorganisms and 3.215% spirit blue agar for detection of lipolytic microorganisms. Plates were incubated at 45 °C until a change of color or a clear zone around each colony appeared explicitly.

2.2. Tests of antagonism and salt effect on growth

Since it was planned to utilize several strains simultaneously for cultivation on fish waste, it was necessary to determine if any of the isolates was antagonistic to the others. Screening for antagonisms was carried out by the perpendicular streak technique as described by Alippi and Reynaldi (2006). Each plate was incubated at 45 °C for 3 days to allow the production of antagonistic substances and then checked for any growth inhibition.

The effect of salt concentration on growth was investigated by spreading the isolates on 1% skim milk agar plates containing 1%, 2% and 3.5% NaCl. Plates were incubated at 45 °C for 3 days, and the effect of salt on protease production and secretion of each cell was verified by measuring difference in size of a clear zone around each colony.

2.3. Identification of useful isolates

Identification of the screened isolates was carried out using 16S-rDNA sequence analysis. DNA was extracted with AccuPrep® Genomic DNA extraction kit (Bioneer, Korea), according to the manufacturer's instructions. PCR amplification of the DNA using the 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were performed with a PCR thermal cycler DICE model TP600 (Takara, Japan). Reaction mixture contained primers (10 pmol μl^{-1}), 2.5 mM dNTPs, 10× reaction buffer, 2.5U Taq polymerase (TaKaRa, Japan), 1 μg DNA template and sterilized water to achieve a final volume of 50 μl. PCR was performed under the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension step at 72 °C for 10 min. Five microliters of amplification products were separated by electrophoresis on a 1% agarose in 0.5% TAE buffer at 100 V for 10 min. Gels were stained with ethidium bromide and photographed under UV light. Gel images were recorded using a digital camera.

Agarose pieces containing amplified DNA were excised from the gel and DNA recovered by AccuPrep® SV Gel and PCR Clean-up System (Promega, USA). The purified products were ligated into pGEM T-easy vector (Invitrogen) and then transformed into *Escherichia coli* DH5α MCR Competent Cells according to manufacturer's instructions (Promega). Colonies were blue/white screened on LB agar with ampicillin (Sigma), X-gal (Promega) and isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega). White colonies were randomly chosen, cultivated and stored in freezing medium (5 g l^{-1} of yeast extract, 10 g l^{-1} of NaCl, 10 g l^{-1} of Bacto tryptone, and 0.1 g l^{-1} of ampicillin) at −80 °C. Plasmid DNA was extracted with AccuPrep® Plasmid Extraction Kit (Bioneer, Korea), and sequencing was performed by Macrogen. Ltd. (Seoul, Korea). The 5′-end and 3′-ends of the constructs were sequenced using M13 primers flanking the cloning sites. These sequences were compared with entries in GenBank (National Center for Biotechnology Information, Rockville Pike, Bethesda, MD) using the Advanced Basic-Local-Alignment-Search-Tool (BLAST) similarity search option (Altschul et al., 1997) accessible from the homepage at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999) was used to check alignment and remove all positions with gaps before calculating distances with DNAdist programme in PHYLIP (version 3.5c; distributed by J. Felsenstein, University of Washington, Seattle).

2.4. Cultivation of bacteria on fish waste

Fish wastes (mixture of viscera, heads, tails and bones) were collected from a local restaurant specializing in sliced raw fish, cut to a size of 1 cm × 1 cm or less and autoclaved at 121 °C for 20 min. A 30 g (wet weight basis) portion was introduced into a 1-l five-neck flask (600 ml working volume). Five grams of bacteria paste (wet weight) containing equal amounts from each of the five isolates were added to the flasks. A thermometric sensor and a pH probe were inserted in two necks of the flask. On the other three necks, a sampling port, and inlet and outlet filters (0.2-μm pore size) were installed. Oxygen was supplied continuously into the flask from an oxygen tank (85% purity). The flask was placed in a hot-stirring bath system (Eyela, Japan) and maintained at 45 ± 0.2 °C. Mixing inside the flask was accomplished with the Variomag Telesystem (H+P Labortechnik AG, Germany) at a magnetic bar rotation rate of 500 rpm. Ten-fold diluted Antifoam 204 was used when foams was produced. Culture medium was sampled periodically from the flask by a peristaltic pump using Tygon tubing, and the amount of liquid removed (approximately 5 ml per sampling event) was replaced with an equal amount of sterile DW to prevent a decrease in moisture content of 45–60%.

2.5. Analytical methods

The concentrations of Na^+, NH_4^+, Ca^{2+}, Cl^-, NO_3^- and PO_4^{3-} were estimated by ion chromatography (IC) (Metrohm 792 Basic IC, Switzerland) on Metrosep C2-150 (150 × 4.0 mm) and Metrosep Supp 5–150 (150 × 4.0 mm) columns for cations and anions, respectively. Numbers of viable cells were measured after dilution of samples and plating on nutrient agar, which were expressed as colony forming units (CFU) per ml of sample. The dry-sludge weight (DSW) was determined after drying in an oven at 105 °C for 12 h. Sludge was obtained by centrifugation of a 5-ml sample of culture at 8000 rpm for 10 min. Amino-acid composition, N, P, and K content and concentration of the heavy metals Pb, As, Cd, Hg, Cr, Cu, Ni, and Zn were determined by Scientec Lab Center Co., Ltd. (Korea).

2.6. Seed germination test

To evaluate the phytotoxicity of the fish waste culture, a seed germination test was carried out according to Wong et al. (2001). Ten milliliters of culture was centrifuged at 8000 rpm for 10 min, filtered through a 0.45-μm membrane filter kept at 4 °C until