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Isolation and identification of microorganisms and their aerobic biodegradation of fish-meal wastewater for liquid-fertilization

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Abstract

To reutilize the wastewater generated during the process of fish-meal production (FMW), seven thermophilic microorganisms were newly isolated and their characteristics of aerobic biodegradation of FMW were examined in a 5 l-bioreactor. It clearly showed that the amino-acid composition (12.54 g 100 g sample^{-1}) in the final broth of the biodegradation using 8-folds diluted FMW was almost twice that of non-biodegraded FMW. The levels of amino acids in the final broth were also comparable to those in a commercial fertilizer. When more (32-folds) diluted FMW was used as a substrate, phytotoxicity of biodegraded final broth was further reduced with disappearance of a strong unpleasant smell in the end. The final broth of biodegradation using 32-folds diluted FMW required only 2-folds dilution to meet the minimum GI criterion of a phytotoxin-free fertilizer. All the results suggest the promising potential of biodegraded FMW for the production of fertilizer, which is expected to yield high economic value.

Keywords: Thermophilic microorganisms; Aerobic biodegradation; Fish-meal wastewater treatment; Fertilizer

1. Introduction

The amount of fisheries waste generated in Korea is expected to increase with a steady increase in population to enjoy taste of slices of raw fish. The fisheries waste is reduced and reutilized through the fish meal production. Depending on the raw material used, there are basically two types of fish-meal manufacturing processes: those that use fish wastes, such as heads, bones or other residues, and those that use the whole fish. The process, using fish wastes, is the commonest used in the Korean industries. The first step of the fish-meal manufacturing processes is the compression and crushing of the raw material, which is then cooked with steam, and the liquid effluent is filtered off in a filter press. The solids obtained are introduced to a rotating drier and finally cut and crumbled to obtain the commercial fish-meal product. The liquid stream contains oils and a high content of organic suspended solids. After oil separation, the fish-meal wastewater (FMW) is generated with stinky odor and shipped to wastewater treatment place.

FMW has been customarily disposed of by dumping into the sea, since direct discharge of FMW can cause serious environmental problems. Besides, bad smell, which is produced during fish-meal manufacturing processes, causes civil petition and stricter regulations for this problem come into force every year in Korea. Therefore, there is an urge to seek for an effective treatment to remove the organic load from the FMW; otherwise the fish meal factories will be forced to shut down.

Biological treatment technologies of fish-processing wastewater have been studied to improve effluent quality (Battistoni and Fava, 1995; Park et al., 2001). The common feature of the wastewaters from fish processing is their diluted protein content, which after concentration by a suitable method would enable the recovery and reuse of this valuable raw material, either by direct recycling to the process or subsequent use in animal feed, human food, seasoning, etc. (Afonso and Borquez, 2002). It has been reported that the organic wastes contain compounds, which are capable of promoting plant growth (Day and Katterman, 1992), and seafood processing wastewaters do

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not contain known toxic or carcinogenic materials unlike other types of municipal and industrial effluents (Afonso and Borquez, 2002). Therefore, FMW could be a valuable resource for agriculture. However, potential utilization of this fish wastes has been limited because of its bad smell (Martin, 1999). There is an increasing need to find ecologically acceptable alternatives to overcome this problem.

Aerobic biodegradation has been widely used in treatment of wastewaters, and recently references to the use of meso- and thermophilic microorganisms have become increasingly frequent (Cibis et al., 2006). During the biodegradation, the organic matter is biodegraded mainly through exothermic aerobic reactions, producing carbon dioxide, water, mineral salts, and a stable and humified organic material (Ferrer et al., 2001). There have been few reports that presented the reutilization of biodegraded waste products as liquid-fertilizer: a waste product of alcoholic fermentation of sugar beet (Algur and Kadioglu, 1992) and diluted manure streams after biological treatment (Kalyuzhnyi et al., 1999). Therefore, aerobic biodegradation is considered to be the most suitable alternative to treat FMW and realize a market for such a waste as fertilizer. Moreover, the reutilization of FMW can create the high additional value, since FMW is collected from the industry with security for cost of FMW treatment.

This paper presents the results of a study aimed to develop a biodegradation product of FMW and find its suitability as a fertilizer. For this purpose, microorganisms were newly isolated and identified. With the screened microorganisms, the first trial for the biodegradation of FMW was then carried out in a bioreactor, and both analysis of amino-acid composition and tests of seed germination and root length were accomplished to examine the fertilizing value of the biodegraded end-product.

2. Materials and methods

2.1. Isolation of useful microorganisms

The potential aerobically degrading bacteria were isolated from commercial good-quality humus and from compost and leachate collected at three different sites of composting plants in the suburbs of Busan, Korea. The soil and compost samples (0.5 g each), and 0.5 ml of raw leachate sample were added into 5 ml of sterile 0.2% NaCl and agitated to obtain homogeneous suspension. One ml of each suspended liquid was pipetted into various 10 ml-tubes that contained 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). After 1 day of incubation at both of 45 and 55 °C and at agitation speed of 180 rpm, cells in each tube were spread with a platinum loop on each solid agar medium which contained the same liquid medium and 1.5% nutrient agar, respectively. A purified isolate was then obtained by repeated streaking on the fresh agar plates. Each pure culture was maintained on the agar plate at 4 °C until used and transferred to a fresh agar plate every 2 weeks.

To screen useful microorganisms among isolates, all isolates were spread on three different agar plates: 1% skim milk agar for detection of proteolytic microorganisms; 3.215% spirit blue agar for detection of lipolytic microorganisms; and starch hydrolysis agar (5 g l^{-1} of beef extract, 20 g l^{-1} of soluble starch, 10 g l^{-1} of tryptose, 5 g l^{-1} of NaCl, and 15 g l^{-1} of agar, pH 7.4) and cellulose agar (10 g l^{-1} of cellulose powder, 1 g l^{-1} of yeast extract, 0.1 g l^{-1} of NaCl, 2.5 g l^{-1} of $(NH_4)_2SO_4$, 0.25 g l^{-1} of K_2HPO_4, 0.125 g l^{-1} of $MgSO_4 \cdot 7H_2O$, 0.0025 g l^{-1} of $FeSO_4 \cdot 7H_2O$, 0.025 g l^{-1} of $MnSO_4 \cdot 4H_2O$, and 15 g l^{-1} of agar, pH 7.2) for detection of carbohydrate-degrading microorganisms, respectively. All agar plates were incubated under same conditions until change of color or a clear zone around each colony appeared.

2.2. Tests of antagonism and salt effect on growth

Screening of potential bacterial antagonists against other isolates was carried out by the use of perpendicular streak technique as described by Alippi and Reynaldi (2006). Of all 46 isolates, several isolates were arbitrarily selected and streaked on nutrient agar plates first. The surface of agar plate streaked by each isolate was divided into four sections, and then, four other isolates were streaked perpendicular to the original streak. Each plate was incubated at both of 45 and 55 °C for 3 days to allow the production of antagonistic substances and then checked for any growth inhibition of each isolate.

After the antagonism test, the effect of salt concentration on cellular growth was investigated with the screened microorganisms. Each screened cell was spread on three different agar plates (mentioned in Section 2.1) containing various concentrations of 1%, 2% and 3.5% NaCl additionally. All agar plates were incubated under the same conditions, and the effect of salt on the growth of each cell was verified by measuring difference in change of color or size of a clear zone around each colony.

2.3. Identification of useful isolates

Screened colonies after tests of antagonism and salt effect on cellular growth were primarily identified on the basis of Gram reaction, colony morphology, and microscopic examination of bacterial smear. All the isolates were cultured on nutrient agar plates for 1 day. Cells were then harvested, and stored in freezer until used.

Identification of the screened isolates was carried out using 16S-ribosomal deoxyribonucleic acid (16S-rDNA) sequence analysis. The deoxyribonucleic acid (DNA) was extracted from cells grown in the given medium 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 polymerase chain reaction (PCR) thermal cycler DICE model TP600 (Takara, Japan). Reaction mixture contained of each primers (10 pmoles μl^{-1}), 2.5 mM deoxy-nucleotide triphosphates (dNTPs), 10 × reaction buffer, 2.5 U 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.

The band 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 *E. 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 Isoprophyl-β-D-Thiogalactopyranoside (IPTG) (Promega). White colonies were randomly chosen, cultivated and stored in freezing medium at −80 °C. The plasmid DNA was extracted with AccuPrep® Plasmid Extraction Kit (Bioneer, Korea). The plasmid DNA was sequenced in the Macrogen. Ltd. (Seoul, Korea). The 5′-end and 3′-ends of the constructs were sequenced using M13 primers flanking the cloning sites. These partial sequences were searched against GenBank (National Center for Biotechnology