

Isolation and Identification of cellulase producing actinobacterium CAE 44 (KU519614) from the gut contents of the earthworm, *Perionyx excavatus* EM2 (KT716822).



Microbiology

KEYWORDS : actinobacterium, *Perionyx excavatus*, *Streptomyces microflavus*, Cellulase.

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ABSTRACT

In the present study, the individuals of the earthworm, *Perionyx excavatus* EM2 (GenBank accession No. KT716822) inhabiting the decomposing banana pseudo stem were collected from Patsoi, Imphal west, Manipur, India and actinobacteria were isolated from the gut contents of the worms by serial dilution technique by using three different media: Omeliansky's agar (OA) and Kenknight & Munaier's agar (K&M) medium supplemented with 0.1% cellulose and Nutrient agar medium. OA medium supplemented with 0.1% cellulose was found to be appropriate medium for isolation of cellulolytic actinobacterial isolates. A total of nine actinobacterial isolates were obtained from the gut contents of *Perionyx excavatus* earthworm and the isolates were screened for cellulase activity by Congo Red Assay. These isolates showed positive result in cellulase assay. The isolate CAE 44 which developed maximum zone of clearance was identified as actinobacterium by 16S rRNA gene sequence analysis. This actinobacterium was found to be gram positive and spore former. The identified actinobacterial sequence was submitted in GenBank under the accession number KU519614. The phylogenetic tree analysis by neighbor-joining method confirmed that the isolate CAE 44 belongs to *Streptomyces microflavus*.

1. INTRODUCTION

Earthworms are invertebrates which belong to the class Oligochaeta of phylum Annelida. They are well known for their contribution to lignocellulose decomposition in soil. The digestion of organic materials by earthworms is known as vermicomposting. Most earthworms depend on their resident gut microorganisms for degradation of organic matter. The substrates of composting are comprised mainly of plant material such as cellulose, hemicelluloses and lignin which are rather difficult to biodegrade (Ladisch et al. 1983). Lu et al. (2004) showed that increase of the microbial populations especially the lignocellulose-degrading microorganisms (LDM) in the compost accelerated the process of composting with different substrates. Thus, inoculation of LDM is a potentially successful strategy for improving the process of composting (El-Din et al. 2000).

Since cellulose is the major component of the agricultural wastes, many studies (like Lu et al. 2004) used extrinsic inocula to hydrolyse ligno-cellulose during waste composting. Bioconversion of cellulosic biomass into soluble sugars and glucose is carried out by a group of hydrolytic enzymes collectively known as cellulases. Many microorganisms including bacteria, actinobacteria, and fungi had been known to degrade cellulose (Immanuel et al. 2006). During the process of composting, actinobacteria are mainly responsible for decomposition of the organic matter at elevated temperatures since they can degrade natural substrates like chitin or cellulose effectively. Ljungdahl et al. (1985) showed that cellulose degrading microorganisms utilize cellulose as carbon and energy resource. Thus, providing cellulose as carbon source in the media will be useful to isolate cellulolytic actinobacteria selectively. The scope of this study is to find the appropriate medium for selective isolation of cellulolytic actinobacteria and to screen their ability to hydrolyze cellulose.

2. MATERIALS AND METHODS

2.1 COLLECTION OF EARTHWORMS

Individuals of the earthworm, *Perionyx excavatus* (Genbank Accession number - KT716822) inhabiting the decomposing banana pseudo stem were collected from Patsoi, Imphal west, Manipur, India.

2.2 DISSECTION OF EARTHWORMS

The earthworms were washed with tap water and then with sterile distilled water. They were anesthetized by keeping at -80°C for 30 minutes and their body surfaces were sterilized by a brief rinse with 1.2% sodium hypochlorite solution followed by the repeated washing with sterile distilled water. The worms were then dissected using sterile blades, pins, scissors and forceps in the dissecting board.

2.3 ISOLATION OF ACTINOBACTERIA FROM EARTHWORM GUT CONTENTS

1.0g of the gut contents was suspended in 9 ml of sterile potassium phosphate buffer solution (PBS) with vortex mixing for 5 minutes on maximum speed. The resulting suspension was serially diluted with PBS and used as inoculums. After serial dilution, 100 μl of each dilution in the series was spread onto the surface of three mediums: Nutrient Agar, OA medium and K&M medium supplemented with 0.1% cellulose powder as carbon source. The morphologically different actinobacterial colonies were purified to obtain single colonies by streaking on OA plates. Stock cultures were made in Omeliansky's broth supplemented with cellulose containing 30% (w/v) glycerol and stored at -80°C .

2.4 SCREENING OF CELLULASE ACTIVITY

All the actinobacterial isolates were screened on Carboxy Methyl Cellulose agar plates for cellulase activity by Congo red Assay (Teather RM & Wood PJ, 1982).

2.5 16S rRNA GENE AMPLIFICATION AND PHYLOGENETIC ANALYSIS

Genomic DNA of the CAE 44 isolate was extracted and the 16S rRNA gene of the isolate was amplified by using the universal primer pair - 27F and 1492 R. The PCR product of 16S rRNA gene was sequenced. Additional sequences were downloaded from GenBank database after performing a search for similar sequences using the Blast tool (Altschul, 1990) in order to use as reference strains. Phylogenetic analysis was performed using the MEGA version 6 (Tamura et al. 2013). The nucleotide sequences were aligned using ClustalW software using the default settings. The neighbor-joining method (Saitou & Nei, 1987) was used following the Kimura-2-parameter

distance model (Kimura,1980) for the construction of evolutionary tree. A bootstrap analysis was performed with 1000 replications.

3. RESULTS

3.1 ISOLATION AND SCREENING OF CELLULASE PRODUCING Actinobacteria

In nutrient agar plates, after 48 hours incubation, fast growing bacteria dominated and thus overlapped the slow growing actinobacteria making difficult to isolate them. In modified K&M medium also, after 72 hours incubation, the bacterial population exceeded much more than actinobacterial population. Even though, the K&M medium was somewhat better than nutrient agar, it was still difficult to isolate pure actinobacterial colonies. In modified OA medium, the microorganisms took longer time to grow but the bacterial population did not interrupt the growth of actinobacterial population thereby allowing diverse actinobacterial colonies to grow properly. Thus the OA plates could be kept for more than five days to isolate the slow growing actinobacteria. Thus, out of the 3 mediums, OA medium supplemented with 0.1% cellulose was found to be appropriate medium for cellulolytic actinobacterial isolation as shown in Fig-1. A total of nine isolates were obtained from the gut of *Perionyx excavatus* EM2 and named as CAE 42 to CAE 50. All the isolates showed positive result in cellulase assay. The formation of a clear zone of hydrolysis around the inoculums indicated cellulose degradation .The colony morphology of the nine isolates and screening result are shown in Fig-2 and Fig-3 respectively.

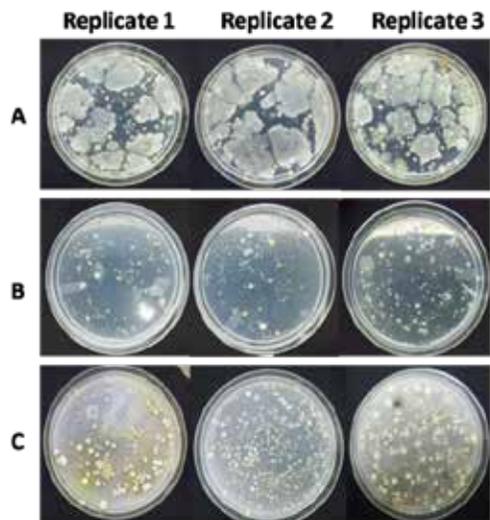


Fig-1 : Colony diversity in Nutrient agar (A), 0.1% cellulose supplemented Kenknight & Munaier's agar plates (B) and Omeliansky's agar plates (C) inoculated with 10³ dilutions of the gut contents.

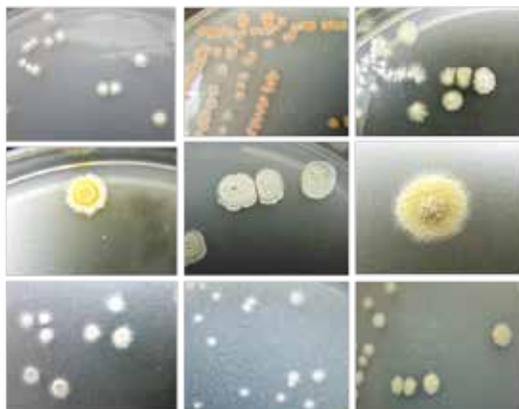


Fig-2: Colony morphology of the nine actinomycetes isolates.

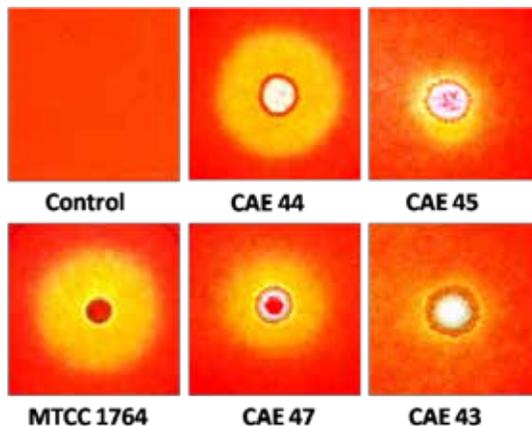


Fig-3: Screening for cellulase activity assay. Clear halos were detected in the positive control (MTCC 1764, *Streptomyces albiduncus*) and in the supernatants of strongly positive isolates (CAE 44) and (CAE 47) and poorly positive isolates (CAE 42) and (CAE 43).

3.2 IDENTIFICATION OF CELLULASE PRODUCING CAE 44 ISOLATE

The CAE 44 isolate produced maximum zone of clearance among the isolates. Hence, this isolate was further studied and identification of the isolate was further confirmed by 16S rRNA gene analysis. The sequence was submitted in NCBI GenBank under the accession number: KU519614. It was found to produce off-white, powdered, opaque colonies. It is Gram positive and spore former. It showed highest identity to *Streptomyces microflavus* (99.0%) in the NCBI blast search. Phylogenetic relationship of CAE 44 with reference strains is shown Fig-4. Based on the colonial morphology and comparison of its 16S rRNA gene sequences, it was identified and named as *Streptomyces microflavus* CAE 44.



Fig-4 : Comparative sequence analysis of 16S rRNA gene from Actinomycetes bacterium CAE44 with the reference strains of the genus Streptomyces and Micromonospora from Genbank using neighbor-joining method.

4. DISCUSSIONS

The detection of cellulolytic aerobes having the ability to degrade lignocelluloses in the earthworm gut is interesting as it is known that the earthworm gut is free of oxygen. In general, anaerobic organisms comprise a major population in the gut microbiota, and proliferation of aerobic microorganisms in the earthworm gut has been reported in earlier studies (Karsten G.R. & Drake H.L, 1995). The earthworm gut constitutes a unique microenvironment which leads to the proliferation of the microbial population inside the gut. The diversity of bacteria, fungi and actinobacteria were analyzed in the gut of *Eudrilus eugeniae* (Esakkiammal B & Lakshmbai L, 2013) and *Glyphodrilus tuberosus* (Chhotaray et al. 2011). Parthasarathi et al. (2007) also reported the diversity of fungi, bacteria, yeast, actinobacteria and protozoa in the gut and casts of *Eudrilus eugeniae*, *Lampito mauritii*, *Eisenia fetida* and *Perionyx excavatus*. Both the studies dealt only with the general diversity of the microorganisms while Fujii et al. (2012) and Kim et al. 2004 reported the diversity of cellulose

lytic bacterial and fungal genera from two earthworm species *Amyntas heteropoda* and *Eisenia fetida*. Diverse actinobacteria having antimicrobial activity as well as amylase, xylanase and lipase activity were also obtained from the castings of *Pheretima posthuma* by Kumar et al. (2012).

In the present study, a gram positive, spore forming cellulolytic *Streptomyces microflavus* CAE 44 was isolated from the gut of *Perionyx excavatus* earthworm which can be used as an external inoculum along with earthworm to enhance composting process.

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