

## Isolation and Characterization of Filamentous Bacteria from A Full-Scale Municipal Wastewater Treatment Plant in Dubai

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**Abstract.** This study aimed at isolating filamentous bacteria from the activated sludge samples of a full-scale municipal wastewater treatment plant in Dubai and studying their physiological and biochemical characteristics. Fluorescent in situ hybridization technique (FISH) was applied to identify filamentous bacteria pure culture isolates. In total, sixteen filamentous bacteria were successfully isolated and maintained on various culture media. Ten gram positive filamentous bacteria were successfully identified as nocardioform actinomycete using nocardioform group specific probes in FISH analysis. Most of the isolates were shown to utilize different carbon sources like glycerol, maltose, fructose etc. The optimum growth of most of the isolates was found to be at 27 °C and neutral pH, however a few isolates were able to grow at 47 °C and acidic pH. Overall results indicate that filamentous bacteria isolated in this study were quite diverse in their physiological and biochemical characteristics and could be of significance for further studies to establish their taxonomic status and possible role in foam formation and its control in the activated sludge process.

**Keywords:** Activated sludge, foaming, filamentous bacteria, nocardioform actinomycete

### 1. Introduction

Understanding the relationship between microbial community and wastewater treatment efficiency is crucial to operating biological wastewater treatment plants [1, 2, 3, 5]. Over the last few years several studies had been conducted on characterization of important microorganisms responsible of the treatment of wastewater in various formats [3, 4, 5]. Most of these studies relied on the use of culture independent techniques to understand microbial community structure, function and their in situ metabolic activities. Dubai sewage treatment plant (DSTP) is one of the major wastewater treatment plants in Dubai, United Arab Emirates (UAE). The successful operation and maintenance of DSTP is of critical importance to the city of Dubai. Like several other sewage treatment plants worldwide [6, 7, 8], DSTP's activated sludge system very often faces bulking and foaming problems which are related to over growth of filamentous bacteria [9]. Filamentous foaming, which develops on the surface of activated sludge aeration basins and secondary clarifiers, prevents sludge separation, thus reducing effluent quality [10]. A past study in the UAE indicated that foaming and bulking was very common in DSTP [9].

There have been several studies in other countries related to filamentous bacteria growth and physiological characteristics in their pure culture form [6, 8, 11]. Nevertheless, very little or no efforts have been made to correlate the problem of foaming with their causative microorganisms in sewage treatment plants in the UAE region. For effective long-term control of foaming, basic understanding of the microbial population specific to the treatment plant is required. Therefore, the main objectives of this study were

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isolation; cultivation of filamentous bacteria from the foaming activated sludge of DSTP and studying their physiological and biochemical features. Furthermore, filamentous bacteria isolates were screened into the nocardioform actinomycete and non-actinomycete by Fluorescence In Situ Hybridization (FISH) technique using published group specific 16S rRNA targeted oligonucleotide probes.

## **2. Materials and Methods**

### **2.1. Sampling**

Grab samples of foaming mixed liquor were collected, personally every fortnight, in sterile plastic bottles from Dubai Sewage treatment plant (DSTP) at various points (e.g. secondary sedimentation tank, aeration tank and distribution tank) over a period of one year beginning April 2011. The samples were taken to the lab within 1 hour and stored at 4 °C before isolation of filamentous bacteria.

### **2.2. Isolation and Characterization of Filamentous Bacteria**

The mixed liquor samples were centrifuged and diluted before isolation of filamentous bacteria. For the isolation of filamentous bacteria, the following media were used: R2A, CGYA, I-media and TYGA [12]. Briefly, pre-treated mixed liquor samples were serially diluted ( $10^1$ – $10^7$ ), homogenized by a vortex mixer and sample aliquots from the dilutions were plated on various solid media as described by Ramothokang et al. 2003 [12]. Streak plate and spread plate techniques were employed all through the isolation procedure [8, 11]. All plates were incubated for 10-15 days at 27 °C followed by purification and maintenance in the stock culture. During this incubation period, culture media plates were observed under the microscope for the selection of colonies with filamentous morphology. Well defined and morphologically unique colonies were isolated onto agar plates and pure cultures of isolates were obtained by continuous sub-culturing and incubation at 27 °C. The plates were incubated and observed for growth between 2-4 weeks.

All isolates were screened microscopically for cellular morphology and staining characteristics. Gram staining and Neisser staining in combination with phase contrast microscopy was carried out according to procedures described by Jenkins et al. 2003[10]. Parameters that were determined included cell shape and size ( $\mu\text{m}$ ), trichome shape and diameter ( $\mu\text{m}$ ), presence and absence of sheath. Various biochemical tests were performed as described by Goodfellow et al.1982 [17]. Biochemical tests conducted were Indole test, MR (methyl red), VP (Voges-Proskauer), Nitrate reduction, Oxidase, Catalase, Urease, H<sub>2</sub>S production, Citrate utilization, Nitrite and Nitrate tests. To observe the effect of temperature and pH on the filamentous bacteria growth, all isolates were grown in casitone glycerol yeast autolysate (CGYA) broth up to 7-10 days at various temperatures (27 °, 37 ° and 47 °C) and pH (4, 7 and 8). The effect of temperature and pH on the growth was evaluated in terms of turbidity developed upto 96hours by measuring absorbance at 600nm using a UV-Vis Spectrophotometer (Jenway 6305 U.K). Growth in case of floc/pellets readings were taken after subjecting a sample to brief vortexing with 0.2 mm glass beads. All the isolates were characterized for their ability to utilize various sugars and other substrate groups as sole carbon source. Isolates were grown in CGYA broth substituted with various carbon sources at 0.5g/L concentration (initial pH 7) for 6 days of incubation at 27 °C. Visible growth of the isolates was recorded by scoring the acid reaction (from pH values).

### **2.3. FISH analysis**

Fluorescence In Situ Hybridization (FISH) analysis on the isolated pure cultures was carried out to identify nocardioform actinomycetes members as described in an earlier study [13]. For FISH analysis, filamentous bacteria pure cultures were fixed by the addition of ethanol and 4% (w/v) paraformaldehyde as described by Daims et al. 2005[14]. Briefly, In situ hybridization of fixed isolates was carried out at a constant temperature of 46 °C in 50 ml polypropylene centrifuge tubes as a hybridization chamber. On each well of the microscope slide, 9  $\mu\text{l}$  hybridization buffer (5 M NaCl; 1 M Tris-HCl, pH 7 $\pm$ 2; 10%, w/v, SDS) and 1  $\mu\text{l}$  of probe (50 nanogram) was applied and incubated for 90-120 minutes. Stringent hybridization conditions for the different oligonucleotide probes were adjusted by different formamide concentrations in the hybridization buffer. Washing buffer(5 M NaCl; 1 M Tris-HCl, pH 7 $\pm$ 2; 0.5 M EDTA) with varying sodium chloride corresponding to the formamide concentrations was prepared according to the method described by Daims et al. 2005 [14].For microscopic examination the slides were mounted in Citifluor AF1

(Citifluor Ltd., London, UK) and examined with an epifluorescent microscope (NIKON eclipse 80i, JAPAN) fitted with filter sets G-2E/C (for TRITC) and B-2E/C (for FITC) and a 100W high pressure mercury lamp. FISH images were captured and analyzed using the ProgRes digital camera system (JENOPTIK, Germany).

### 3. Results

#### 3.1. Isolation and Cultivation of Filamentous Bacteria

Attempts to isolate filamentous bacteria from mixed liquor and foam samples by direct plating methods were largely unsuccessful. But dilution and centrifugation of mixed liquor samples prior to plating on solid media prevented overgrowth of fast growing microorganisms and facilitated recognition and isolation of the desired filamentous bacteria. A total number of 16 (Coded: FB01 to FB16) cultures were isolated by microscopic screening of morphologically unique colonies and employing the direct plating technique. Isolates were maintained either on R2A or CGYA agar. These isolates were re-cultured onto fresh media every two weeks. Gram stained samples were examined throughout sub-culturing in order to ensure purity of cultures.

#### 3.2. Identification of Filamentous Bacteria Pure Cultures by FISH

Identification of pure cultures of filamentous bacteria was performed using HGC69a, MNP1, Myc657, S-G-Gor- 0596-a-A-22 and S-S-G.am 0192-a-A-18 oligonucleotide probes [13,15]. In total out of the 16 isolates, 10 isolates were hybridized by both HGC69a and MNP1 confirming that these isolates belong to nocardioform actinomycetes group. Eight of these isolates hybridized with the Myc657 probe indicating that these isolates were mycolic acid containing actinomycetes. However, all sixteen isolates failed to hybridize with *Gordona amarae* and *Gordona* genus specific probe indicating that none of these isolates belong to *Gordona* genus. The FISH analysis results are described in figure 1.

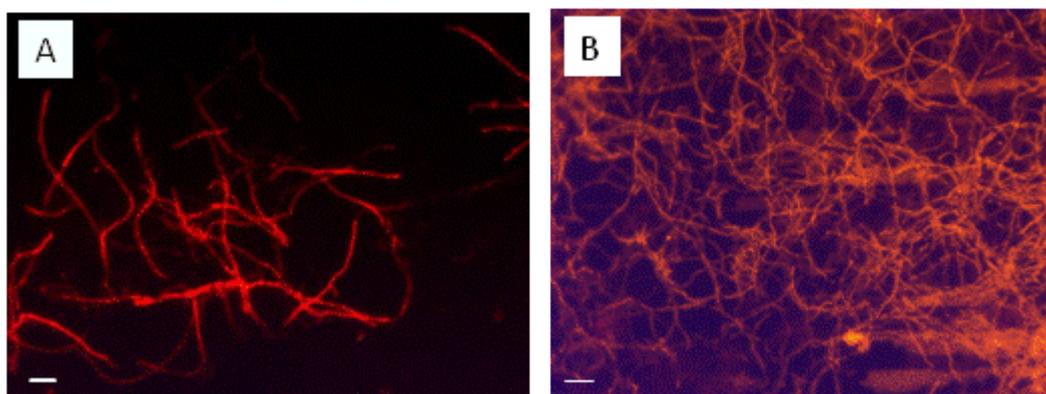


Fig. 1: Identification of filamentous bacteria pure culture. Nocardioform actinomycete pure culture isolates hybridized by TRITC-labelled MNP1 probe. A) FB15; B) FB16. Bar =10  $\mu$ m and applies to all photomicrographs. Original magnification: 1000X

#### 3.3. Biochemical and Physiological Characteristics of Isolates

Comparison of the biochemical and physiological properties of the isolated strains revealed important fundamental differences among bacterial groups with respect to their overall biochemical activity, temperature and pH tolerance and their ability to degrade a variety of carbon sources. All isolates were negative to IMVC test which means that they do not belong to the Enterobacteriaceae group. FB01, FB12, FB14 and FB15 were able to utilize citrate. Except for FB04, FB05 and FB13 all the strains were catalase positive. Strains FB05, FB10 and FB16 were able to hydrolyze urea. Most of the isolates, except FB06, FB08, FB14 and FB15, produced hydrogen sulfide and hence were able to reduce organic sulfur. Most of the isolates were found to reduce nitrate.

Apart from glycerol, most of the isolates were able to utilize glucose, fructose, and maltose as carbon substrates. Out of all 16 isolates, FB01, FB14 and FB15 were able to utilize mannitol as carbon source. FB03, FB06 and FB10 could not utilize fructose while FB05, FB08 and FB13 were able to grow on simple sugars

and substrates only and did not grow on both fructose and maltose based media. As indicated in table 4, most of the isolates were able to tolerate higher temperatures up to 37 °C. All the cultures were best grown at 27 °C and pH7. Four isolates (FB06, FB08, FB10 and FB12) were found to have a wider range of temperature and pH tolerance and FB02, FB03 and FB09 did not grow at 37 °C and above, while FB14, FB15 and FB16 showed slight growth at 37 °C. Except FB06, FB08, FB10 and FB12, all strains showed little or no growth at 47 °C. FB03, FB04, FB11 and FB12 were very sensitive to pH and able to grow only at neutral pH. FB05, FB09, FB14, FB15 and FB16 were able to grow at acidic pH while FB02, FB06, FB08 and FB13 were tolerant to basic pH.

#### **4. Discussion**

The major aim of this study was to isolate and study the characteristics of dominant filamentous bacteria in the foaming activated sludge samples from a sewage treatment plant in Dubai. In our previous study [9] we observed a dominant gram positive filamentous bacterium with a true branching pattern as described earlier [10]. Classical microscopy based classification and identification of filamentous organisms in mixed liquor samples is not considered reliable method as most of the nocardioform actinomycetes group members are known to change their morphotypes in the activated sludge processes [8, 13]. A truly branched filamentous bacterium (*Nocardia* like organism) was consistently observed in mixed liquor and foam samples of DSTP since 2007. We particularly targeted this and other related bacteria in the mixed liquor and foam samples in DSTP for isolation and characterization.

A morphological shift, from filamentous to single cell form was observed for eleven isolates upon repeated sub-culturing. This is in accordance with a previous study on filamentous bacteria pure cultures [16]. The nocardioform actinomycete group members are generally known to exhibit pleomorphic features during their growth [17] and our observations strongly support these earlier reports. With regard to biochemical features, thirteen isolates were found to be nitrate reducers and twelve isolates produced hydrogen sulphide. Growth studies at different temperatures revealed excellent growth of most of the isolates at 27 °C and 37 °C. However, at least four isolates (FB-06, FB-08, FB-10, and FB-12) showed significant growth at 47 °C. It was reported in a previous study that some *Nocardia* species within nocardioform actinomycete group could grow at higher temperatures. This result indicates that some of our isolates were probably *Nocardia* species. Ten filamentous isolates, which according to staining and morphological characteristics were presumed to belong to the nocardioform group were successfully hybridized with a nocardioform group specific probe (MNP1) and HGC69a probe for gram positive bacteria with a high G+C content during FISH analysis [13]. None of our isolates hybridized with the *Gordona* genus and *Gordona amarae* specific probes [15], indicating that members of *Gordona* genus were probably not dominant in the foaming activated sludge of DSTP. Furthermore, direct hybridization of these probes onto the foaming and mixed liquor samples failed to detect any of *Gordona* genus members in DSTP.

#### **5. Conclusion**

The findings of this study throw light on the diversity of filamentous bacteria occurring in the foaming samples of activated sludge system of Dubai sewage treatment plant (DSTP). Sixteen filamentous bacteria present in foaming sludge samples were successfully isolated and cultivated for characterization and identification. The results of this study indicated that filamentous pure culture isolates from foaming activated sludge were quite diverse in their physiological and biochemical characteristics. It is recommended to verify their correct taxonomic status through 16S rRNA sequencing, clone library and polyphasic approaches.

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