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Isolation, identification and removal of filamentous organism from SND based SBR degrading nitrophenols

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Abstract Four identical lab scale sequencing batch reactors R, R1, R2, and R3, were used to assess nitrophenol biodegradation using a single sludge biomass containing *Thiosphaera pantotropha.* Nitrophenols [4-Nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP) and 2,4,6-trinitrophenol (2,4,6-TNP)] were biotransformed by heterotrophic nitrification and aerobic denitrification (SND). Reactor R was used as background control, whereas R1, R2, and R3 were fed with 4-NP, 2,4-DNP, and 2,4,6-TNP, respectively. The concentration of each nitrophenol was gradually increased from 2.5 to 200 mg/l along with increase in COD, during acclimation studies**.** The final COD maintained was 4,500 mg/l with each nitrophenolic loading of 200 mg/l. During late phase of acclimation and HRT study, a filamentous organism started appearing in 2,4-DNP and 2,4,6-TNP bioreactors. Filaments were never found in 4-NP and background control reactor. Biochemistry and physiology behind filamentous organism development, was studied to obtain permanent solution for its removal. The effect of different input parameters such as COD loading, DO levels, SVI etc. were analyzed. The morphology and development of filamentous organism were examined extensively using microscopic techniques involving ESEM, oil immersion, phase contrast, and dark field microscopy. The organism was grown and isolated on selective agar plates and was identified as member of *Streptomyses* species.

Keywords Nitrophenols · *Thiosphaera pantotropha* · Heterotrophic nitrification · Aerobic denitrification · SND · SBR

Abbreviations

SND	Simultaneous nitrification &denitrification
SBR	Sequencing batch reactors
4N	4-Nitrophenol
2,4-DNP	2,4-Dinitrophenol
2,4,6-TNP	2,4,6-Trinitrophenol
HRT	Hydraulic retention time
COD	Chemical oxygen demand
NO_2–N	Nitrite nitrogen
NO_3–N	Nitrate nitrogen
HPLC	High performance liquid chromatogram
DO	Dissolved oxygen
SVI	Sludge volume index
ESEM	Environmental scanning electron microscope

Introduction

Nitro substituted aromatic compounds such as nitrophenols are important building blocks and intermediates

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for the large-scale synthesis of pesticides, pharmaceuticals, plastics, azo dyes, pigments, wood preservatives, rubber chemicals, and explosives (Kuscu and Sponza 2005; Karim and Gupta 2006; Bhatti et al. 2002). The annual production of 4-Nitrophenol (4-NP) alone is 20 million kg (Donlon et al. 1996). Since these compounds are frequently used in industrial, agriculture and defense purposes they find their way into effluents of these sources. 4-NP, 2,4-Dinitrophenol (2,4-DNP) and other nitrophenols are listed on US Environmental Protection agency's (USEPA's) Priority pollutant list (Karim and Gupta 2003).

Different types of filamentous bacteria have been identified in activated sludge in the wastewater treatment. They play important roles by directly affecting sludge settling properties. These organisms make provision for the rigid support network or backbone upon which floc forming bacteria can adhere and grow into suitable activated sludge flocs. Filamentous bacteria may be considered detrimental to wastewater treatment when they occur in excessive quantities (sludge bulking), but are just as important in the development of activated sludge flocs with proper settling and clarification properties Filamentous microorganisms are reported to be good indicators of conditions prevailing in an activated sludge system on a microbiological level. The indications given by the filamentous bacteria could be of low dissolved oxygen (DO) (e.g., *Sphaerotilus natans*), low food-to-microorganism (F/M) ratio (e.g., *Microthrix parvicella*, Type 0092), presence of septic waste (e.g., *Thiothrix* spp.), nutrient deficiency (e.g., *Haliscomenobacter hydrossis*) and low pH in the system (e.g., Fungi) (Gerardi et al. 1990).

Filamentous bacteria constitute main cause of activated sludge bulking and foaming problem. Chemical control methods such as chlorination and the use of hydrogen peroxide are generally used to cure bulking and foaming but are only effective as temporary measures. More detailed understanding of the physiology and biochemistry of filamentous bacteria is still required for successful long-term control of bulking and foaming problems. Isolation and cultivation of filamentous bacteria in pure culture have been found as promising methods to gain better understanding of bulking and foaming and biochemistry behind filamentous bacteria development. An improved understanding of the phylogeny and physiology of filamentous microorganisms in BNR sludge is necessary for the formulation of effective bulking and foaming control measures (Bjornsson et al. 2002). Despite much research, bulking sludge seems to be a continuous problem in the wastewater treatment plants operation. This is likely caused by several facts. Many filamentous bacteria are not available in pure cultures, preventing a detailed study of these organisms. The condition of the plant operation under which bulking sludge occurs is usually only marginally documented (Martins et al. 2004).

Sequencing batch reactors (SBRs) offer both dynamic conditions and optimal substrate concentration and hence are the best solution for wastewater treatment plants treating mixed or industrial wastewater. They provide dynamic conditions that are typical of periodic system. These reactors are characterized by a large spectrum of operating conditions (easily obtainable on time scale) and high operation flexibility (Tomei et al. 2004; Tomei et al. 2003). However Steep substrate gradients occurring in highly dynamic activated sludge systems, such as SBRs and contact tank systems, stimulate substrate storage and regeneration phenomena in the sludge (Van den Eynde et al. 1984; Chiesa and Irvine 1985; Chiesa et al. 1985; Majone et al. 1996; Krishna and Van Loosdrecht 1999). In activated sludge systems with aerobic contact tanks, the relationship between dissolved oxygen concentration and storage polymers and their effects on sludge settleability is still ambiguous. Literature data are contradictory and bulking sludge is reported to occur under low (Houtmeyers 1978; Sezgin et al. 1978; Palm et al. 1980) and high (Houtmeyers 1978; Palm et al. 1980) bulk liquid dissolved oxygen concentration.

Ramothokang et al. (2003) assessed different techniques for effective isolation and cultivation of filamentous bacteria in pure culture. Liu and Liu (2006) reported that no work has been reported on identifying the types of filamentous microorganisms that develop under various stress situations in SBR. In the present study, a single sludge samples from sequencing batch reactor were screened microscopically to identify filamentous bacterial populations. The SBRs were used to biodegrade 4-NP, 2,4-DNP, and 2,4,6-TNP under heterotrophic nitrification and aerobic denitrification (SND) using a single sludge containing *Thiosphaera pantotropha.* The samples were serially diluted and plated onto a variety of different solid media; where after discrete bacterial

colonies were isolated and screened microscopically for filamentous morphology. The focus of present study is isolation and identification of filamentous organism.

Materials and methods

Experimental set up

The batch experiments were conducted in four identical SBR namely R, R1, R2, and R3, having working volume of 5 l. Reactor R was kept as control whereas R1, R2, and R3 were fed with 4-NP, 2,4-DNP, and 2,4,6-TNP, respectively. All reactors were maintained at room temperature (27 ± 4°C) throughout the study. The reactors were made up of plastic material with total capacity of 10 l. Air was supplied by variable flow compressor trough diffuser. Conventional aerobic/anoxic phase changing was not done in this SBR system due to specially designed mixed single sludge containing *T. pantotropha.* Experimentation was carried out at reduced aeration (DO = around 2 mg/l) due to the inherited property of mixed single sludge system for heterotrophic nitrification and aerobic denitrification (SND). Each SBR cycle included, fill phase 30 min, react phase with aerobic mode (HRT-1 h) with anoxic mode-nil, Settle phase 20 min, draw/decant phase 10 min, wastage 1–2 min. Each SBR cycle lasted 2 days i.e., 48 h (24 h cycle time with 50% volumetric exchange ratio). After settling, 2.5 l of the treated effluent was drawn and around 2.5 l sludge was maintained in the reactor for the next cycle, which gives 4d of hydraulic retention time (HRT) i.e., 96 h. 2.5 l of synthetic feed was fed to the reactor during each cycle i.e., 50% volumetric exchange ratio.

Composition of synthetic feed and inocula

The synthetic feed designed for the study contained $MgSO_4$ (300 mg/l), $CaCl_2$ (62 mg/l), K_2HPO_4 (800 mg/l), KH_2PO_4 (200 mg/l), $FeCl_3{\cdot}6H_2O$ (16 mg/l). Nitrophenols, and acetone were used nitrogen and carbon source. For Control reactor, the media composition was same as for nitrophenol bioreactors. The Carbon source for control reactor was sodium acetate and nitrogen source was KNO_2. The single sludge was prepared specifically with dairy sludge (aerobic) and cow dung (1:1) along with biomass of *T. pantotropha.* Activated sludge (5 l) was obtained from Mahananda dairy effluent plant at Goregaon, Mumbai. *T. pantotropha* culture was obtained from Dr. L. A. Robertson Laboratory, University of Delft, Netherlands.

Study to evaluate effect of COD loading

To study the effect of substrate loading, reactor were operated with and without external carbon source i.e., acetone. During the study, acetone was used as carbon source and nitrophenols as nitrogen source. Studies were carried out for nitrophenol as sole source of carbon and nitrogen. The SVI value with and without high COD (i.e., with and without acetone) loading were compared to evaluate its effect on sludge bulking.

Media used for isolation and growth of filamentous microorganism

Different media such as R2A agar, PDA agar, Nutrient agar, Specific medium for *T. pantotropha* etc. were used to grow and isolate filamentous microorganism and find out its type.

Microscopic examination

Microscopic examination was routinely carried out to check the presence of filamentous organism and its proportion in sludge. The Microscope used was Ziess optical microscope with image analysis software. After isolation and purification of filamentous organism, Gram staining and morphological characteristics were observed microscopically under 10, 40, and 100×— magnifications using oil immersions, phase contrast dark field microscopy modes. SEM examination of filamentous organism was carried out to study structure and morphological features of using ESEM. During ESEM examination, filamentous organism from the reactors and plates was taken directly without processing, dehydrated using solvents and directly put on stab of ESEM.

Analytical procedures

The analytical procedures for all tests were as outlined in standard methods for examination of water and wastewater (Standard Methods 1998), unless specified otherwise. Daily measurements were taken for the

influent and effluent pH, COD, NO_3–N, NO_2–N. The nitrophenol estimation was done with HPLC. The sludge samples were analyzed for suspended solids (SS) and volatile suspended solids (VSS) on biweekly basis. The pH of sample was measured within 5 min of withdrawal in order to minimize pH changes; a pH meter (control dynamics, India) with glass electrode was used to measure pH. Nitrophenols were analyzed by injecting 50 µl filtered liquid samples to a high-pressure liquid chromatography. (Shimadzu, LC_6A, Japan) equipped with UV–Vis detector (SPD-6A), and C18 reverse-phase column (250 mm × 4.6 mm, 5n µl ODS, Hypersil, UK). The detection wavelength used were 280 nm for 4-NP, 2,4-DNP, and 2,4,6-trinitrophenol (2,4,6-TNP). Mobile phase was 1:1 deionised water and HPLC grade methanol at flow rate of 1 l/min. Minimum detection limit was 0.5 mg/l for each nitrophenol.

Results and discussions

Sequencing batch reactors were operated at high COD and nitrophenol loading, (200 mg/l of nitrophenol and around 4,500 mg/l COD) during acclimation and HRT study. COD was maintained with acetone as carbon source. Nitrophenols were used as the sole source of nitrogen. Sludge bulking was critical problem during present study showing high SVI values. Extensive filaments started appearing in 2,4-DNP and 2,4,6-TNP bioreactors, and were absent in 4-NP and background control bioreactor. The parameters that might be responsible for filament formation were identified. High COD loading was found to be critical factor, at the same time increase in NO_2–N in 2,4-DNP and 2,4,6-TNP affected COD/NO_3 ratio favoring filamentous condition. DO was found to be critical factor for filament development as DO less than 2 mg/l led filaments formation. Higher values of SVI in 2,4-DNP and 2,4,6-TNP bioreactor were indication of sludge bulking.

Effect of high COD loading

To study the effect of high substrate loading, reactors were operated with and without external carbon source. As previously mentioned acetone was used as a carbon source and nitrophenol as a nitrogen source during acclimation and HRT study. Studies were carried out for nitrophenol as a sole source of carbon and nitrogen (Figs. 1, 2). During HRT study, COD loading was around 4,500 mg/l and nitrophenol loading was 200 mg/l. Whereas when bioreactors operated without external carbon source (acetone), COD value dropped down to 300 mg/l from 4,500 mg/l. The COD offered by 200 mg/l of nitrophenol was around 300 mg/l for all the three. Nitrophenolic concentration was kept same i.e., 200 mg/l for with and without external carbon source study.

The problem of filaments was found to be immediately solved when nitrophenols were used as a sole source of carbon and nitrogen and energy with 200 mg/l concentration. Within 4–5 days filaments were completely removed from the reactors. The nitrophenol removal rates were also high. NO_3–N was in the range of 15–30 mg/l. SVI was in the range of 45–65 ml/g for all three nitrophenol reactors i.e., 4-NP, 2,4-DNP, and 2,4,6-TNP. The process became cost effective as no addition of external carbon source needed. The COD offered by nitrophenol (around 300 mg/l) was found to be sufficient for both nitrification and denitrification as NO_2–N was never appeared and NO_3–N was 15–30 mg/l as for all three nitrophenol reactors.

Liu and Liu (2006) have reported the causes and control of filamentous growth in aerobic granular sludge SBRs. Granules in aerobic granular sludge SBR experience multiple stresses that can induce progressive development of filamentous growth, reduced settleability and eventual washout of biomass. Controlling overgrowth of filamentous bacteria requires attention to the effects of combined stresses. Because the substrate, nutrients and DO have different

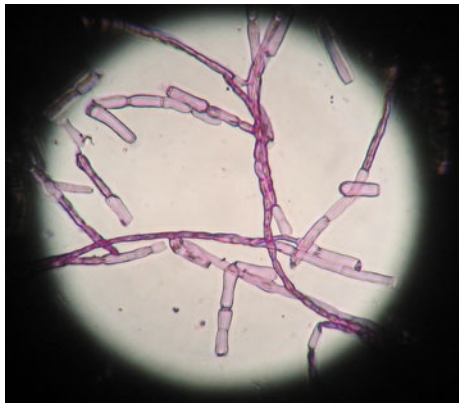

Fig. 1 Gram staining of Isolate

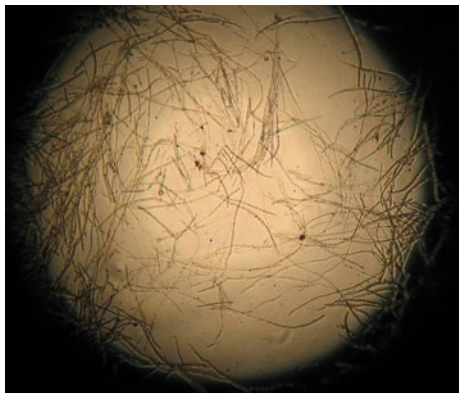

Fig. 2 Low power appearance of isolate (×100 magnifications)

diffusivities in the granule, bulk concentrations of nutrients and DO need to be increased in differing proportions so as to prevent development of dominant filamentous growth (Liu and Liu 2006).

When acetone was used as external carbon source, COD loading was high around 4,500 mg/l. Even though promising COD removal was obtained (more than 98% for 4-NP, 2,4-DNP, and 2,4,6-TNP such high COD loading imposed steep substrate gradient. It has been reported that substrate gradient is important for filament formation (Martins et al. 2003) when high substrate loading (COD loading due to external carbon source) was applied. The multiple substrate diffusion in sludge flocs, play a more important role in biological flocs. The transport of substrates (e.g., organic substrate, oxygen and nutrients) is expected to be mainly diffusional. Substrate diffusion limitation is key parameter in development of filaments. It is assumed that, in the presence of substrate diffusion limitation inside the flocs, filamentous bacteria have a higher outgrowth velocity due to the way they grow. Filamentous structures grow preferentially in one direction and floc structures in three directions.

At low aerobic contact time (Martins et al. 2003), or at a combination of high COD loading rate and low dissolved oxygen concentration (Palm et al. 1980; Pernelle et al. 2001), the inner floc layers can experience depletion of substrate due to diffusion resistance. These substrate diffusion gradients inside the floc are, most likely, the determining factors for the growth of filamentous structures. Bacteria will compete for the limiting substrate and those with a higher outward growth velocity (most probably the filamentous bacterial structures) will win the competition. Once the filamentous bacteria have grown beyond the floc surface they will experience higher substrate concentrations and will grow even more. A network of filamentous bacteria will then be formed in the bulk liquid and a state of bulking sludge will be reached. Similarly, finger and filamentous type structures are commonly observed and described in biofilms growing under substrate transport limited regimes (Ben-Jacob et al. 1994; Van Loosdrecht et al. 1995; Wimpenny and Colasanti 1997), as supported by modelling results (Picioreanu et al. 1998).

Comparison of SVI with and without high COD loading

The comparison of SVI with and without external carbon source, acetone (COD loading), was carried out for 2,4-DNP and 2,4,6-TNP bioreactors. It was found that at low COD loading without external carbon source, SVI values were less than half of SVI values at high COD loading with acetone. This clearly indicates filamentous organism formation was governed by high COD loading due to external carbon source (acetone) as sludge bulking was found directly related to COD loading (Table 1). High COD loading was giving advantage for filamentous microorganism to proliferate over floc forming organisms due to formation of diffusion gradient.

A proper SVI value, especially below 100 ml/g, is of great importance in the activated sludge process. Better organic compounds removal by well-settleable sludge (of low SVI) has been demonstrated Rensink and Donker (1991). However, in the conventional secondary clarifier, activated sludge with low SVI contributed the SS being carried over to the effluent. SVI values of activated sludge as low as 30–60 ml/g in a bench-scale sequencing batch reactor was obtained

Table 1 Comparison of SVI versus COD for 2,4-DNP and 2,4,6-TNP

SVI vs COD	2,4 Dinitrophenol (ml/g)	2,4,6-trinitrophenol (ml/g)
SVI at high COD loading with external carbon source	≥150	≥150
SVI at low COD loading without carbon source	45–65	45–65

without any turbid effluent (Janczukowicz et al. 2001). Hait and Mazumder (2011) studied high-rate wastewater treatment by a shaft-type activated sludge reactor. The sludge bulking or filamentous bulking problem was not observed during both batch as well continuous studies since sufficient DO concentration (>2 mg/l) was present in the reactor. The sludge from the shaft-type reactor was highly flocculated in nature showing a good settleability (Hait and Mazumder 2011).

Effects of DO at high and low COD loading

At high COD loading with external carbon source (acetone), DO was found to decrease during initial COD loading due to high substrate gradient. At the same time dominance of filaments could be one of reasons to decrease DO. Combination of high COD loading due to external carbon source and low DO was found to be ideal condition for filament development. This led to development of steep diffusion gradient of both COD and DO where filamentous organisms were found to take advantage over floc forming bacteria due to one dimensional growth capacity. However at low COD loading without external carbon source, diffusion gradient was not that steep and hence filament formation was prevented. However for *T. pantotropha,* optimal DO has been reported to be 2. For experimental purpose DO used was around 2 mg/l as being special property of *T. pantotropha* which makes nitrification and denitrification highly cost effective due to reduced aeration. Previous literature also suggests that substrate diffusion is an important factor for the growth of filamentous microorganisms (Sezgin et al. 1978; Kappeler and Gujer 1994). As a general principle substrate diffusion is hypothesized to dictate the settling characteristics of the activated sludge and SBR. DO is reported to be critical factor for filamentous organism development. DO was maintained slightly (around 2 mg/l) due to inheretent property *T. pantotropha.*

Increased filamentous bacteria growth in activated sludge reactors is a well-known consequence of plants that operate at long (>10 days) SRTs and sustained low (<2 mg/l) DO concentrations. The development of excess growth of filamentous bacteria has been associated with poor settling characteristics or bulking of sludge, as defined by a sludge volume index (SVI) in excess of 150 ml/g (Jenkins et al. 2003). In general, the conditions for bulking sludge require sustained periods of low DO operation in treatment plants, in the order of days or weeks. However, a recent survey of wastewater treatment plants in the Mediterranean region found that sustained intermittent aeration conditions can also favour the growth of *M. parvicella* (Noutsopoulos et al. 2007). In the present study, at combination of high COD and nitrite loading and low DO, mixing was limited which limits mass transfer of oxygen and substrate transport to biomass leading to the filament organism development.

Gaval and Pernelle (2003) found that filamentous bulking develops as a result of short term but repeated events with low DO concentrations. Filamentous bacteria growth was encouraged in various pilot reactors with low DO operation, only when air shutoff was repeated at 11 different 24 h events, the SVI increased from 151 to 295 ml/g. After resuming aeration of the reactors, the number of filamentous organisms reduced significantly. However, the abundance of filamentous bacteria did not return to its baseline level (Dotro et al. 2011). These results suggest that the repetition of oxygen deficiency can lead to an amplification of the filamentous bacteria response, indicating a cumulative effect. Filamentous bulking also occurs during the normal operation of BNR plants, especially by *M. parvicella* and *Nostocoida limicola* II (Seviour et al. 1990).

Effect of NO_2 release

As filaments were observed in 2,4-DNP and 2,4,6-TNP and not in 4-NP, high nitrite release in 2,4-DNP (twice) and 2,4,6-TNP (thrice) to 4-NP could be one of reasons of filament dominance. However, nitrite was not detected at the end of cycle in any of these nitro phenol bioreactors due to high nitrification rates of *T. pantotropha* with no intermediary accumulation of nitrite. But there could be steep gradient of NO_2 in the beginning of feeding phase during acclimation or HRT cycle just like substrate gradient (COD, DO) favoring growth of filamentous organism. High NO_2 release due to breaking of nitrophenols played important role in the present study in the filament development. High nitrite contents in 2,4-DNP and 2,4,6-TNP bioreactors disturbed nitrogen to carbon equilibrium.

Tsai et al. (2003) studied the effects of intermittent aeration on the proliferation of *M. parvicella* in a laboratory-scale nitrifying–denitrifying reactor. The

authors found that, under incomplete nitrification conditions, the residual ammonia was partially responsible for the increased growth of *M. parvicella*, effectively enabling their proliferation during the typical long SRT conditions to cause a bulking sludge. This is in agreement with the findings of other researchers at full scale where BNR plants that incorporated a final aerobic stage (DO $>$ 1.5 mg/l) and maintained the ammonium concentrations below 1 mg/l were reported to discourage *M. parvicella* growth (Kruit et al. 2002). Similarly in the present study, high COD loading due to external carbon along with high NO_2 content and low DO found to be ideal condition for filament growth. When equilibrium among these three factors was changed by reducing COD loading (using nitrophenol as sole source of carbon, nitrogen and energy with COD around 300 mg/l), filamentous organism development stopped immediately and they never reappeared in bioreactors.

The isolation and identification of filamentous organism from bioreactors

The isolation and identification of filamentous organism from bioreactors was carried out using different selective and general purpose media. Frequent microscopic examinations were done to identify species of filamentous organism. The media used for isolation and identification of filamentous bacteria are given in Table 2. As study was mainly consisted of *T. pantotropha,* the media for its growth was also used to see effect of filamentous organism on the growth of *T. pantotropha.*

Table 2 Media used for inoculation of single sludge biomass to identify filamentous colony

Media	Organisms reported to grow
R2A agar	Filamentous bacteria like *M. parvicella, S. natans,* types 1701, 0803, 1863, 0092, 0411 and *Leptothrix* spp.
PDA Agar	Fungal filaments
Media for *T. pantotropha* (Robertson et al. 1988)	For *T. pantotropha* our basic culture.
Nutrient agar	Bacterial isolates

Direct inoculation

Untreated mixed liquor samples were serially diluted (10^{-8}–10^{-10}), homogenized with a vortex mixer for approximately 10 s, and then directly inoculated onto solid media using surface spread technique. Media that were used for isolation (e.g., R2A agar) have been reported to be successful in supporting the growth of a wide range of filamentous bacteria.

The techniques employed for the inoculation of agar plates were the streak-plate and spread-plate techniques. Plates were incubated at 30°C for 4–5 d followed by isolation of well-defined colonies onto fresh agar plates. Pure cultures of isolates were obtained with continuous sub-culturing and incubation at 30°C.

Isolation, characterization and identification

After 4–5 d incubation well-defined bacterial colonies were isolated onto fresh agar plates. Pure cultures of isolates were obtained with continuous sub-culturing and incubation at 30°C. All isolates were screened microscopically for filamentous morphology using gram staining as well as a simple crystal violet staining procedure. Phase contrast view of isolate was also observed through image analysis microscope under 40× magnification. Morphological aspects of isolates were studied under dark background during dark field microscopy. Characterization of cellular morphology of filamentous bacterial isolates was conducted in conjunction with assessment of colonial morphology as seen on agar plates. R2A agar was used specifically used for isolation and characterization of filamentous organism along with PDA agar. Different agar media used during inoculations are given in Table 2. Broth cultures of the presumptive filamentous bacteria were then prepared so as to assess the difference in growth on both solid and liquid media.

Microscopic examination of isolate

Microscopic examination was routinely done to check the presence of filaments and its proportion in the sludge. After isolation and purification of filamentous organism, gram staining and morphological characteristics were observed microscopically. Figure 1 shows gram nature of isolate. The isolate showed gram-positive nature with distinct morphology

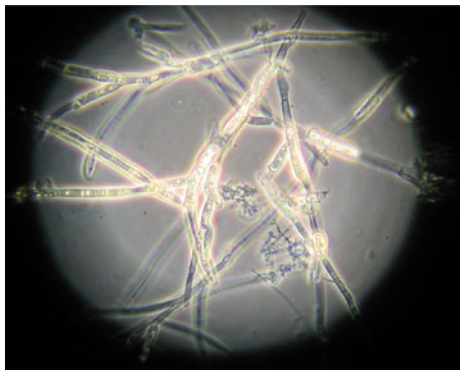

Fig. 3 Filamentous biomass in 2,4-DNP bioreactor (phase contrast microscopy ×40)

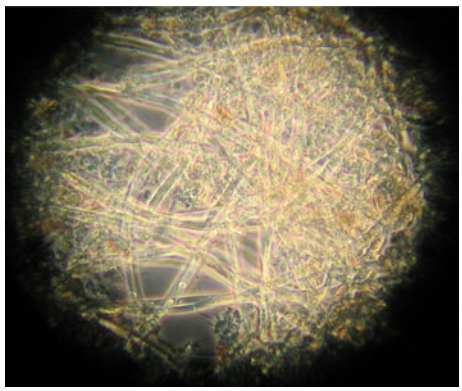

Fig. 4 Filamentous biomass in 2,4,6-TNP bioreactor (phase contrast microscopy ×40)

showing two types of filamentous structures. It showed thick cells i.e., basal filaments and some arial filaments showing conidial type morphology, which is typical characteristic of *Actinomycetes* Species. The isolate belongs to *Actinimycetes* spps showing similarity to *Streptomyces*. Figure 2 shows low power appearance of isolate where typical filamentous morphology can be seen. Figures 3, 4 shows phase contrast appearance of filamentous organism under 40× magnification where bright filaments can be clearly seen against background with sharp contrast.

Conclusions

The problem of filamentous organism development was found to be governed by three critical factors viz. high COD loading, DO and nitrite content. High COD loading was observed as major factor for filamentous growth, during present study. Increase in NO_2 concentration in 2,4 and 2,4,6-TNP affected COD/NO_2 ratio favoring conditions for filamentous organism development. Substrate diffusion across the flocs is an important factor for the growth of filamentous microorganisms due to substrate diffusion limitation. One more factor important for filament development was DO. At combination of High COD and nitrite loading and low DO, mixing was limited in the present study which limits mass transfer of oxygen and substrate transport to biomass and leading to filament development. Due to specific property of *T. pantotropha*, DO was kept constant around 2 mg/l. NO_2 content is chemical property of each nitrophenol which can not be changed. The only changeable factor was high COD loading which could alter conditions favoring filamentous organism development. When equilibrium among high nitrite, high COD and low DO was changed by deceasing COD, filamentous organism lost advantage of substrate gradient development over floc forming organisms. This immediately stopped its growth and development. The filamentous organism was isolated and identified and found to be belonging to *Actinimycetes* species with similarity to *Streptomyces* species via extensive microscopic studies.

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