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Molecular Detection of Bacteria in Activated Sludge from Municipal Wastewater of Cold Climate Regions

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In recent years wastewater treatment using microbial processes are very common. Activated sludge process using bacteria is the most widely used technology for municipal and industrial wastewater treatment. In present study we detected *Nitrosomonas sp.*, *Pseudomonas sp.*, *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* in activated sludge of municipal wastewater in cold climate regions of Iran. Total of 35 municipal wastewater specimens were collected from different regions of Chaharmahal Va Bakhtiari province in cold months and genomic DNA was extracted using kit. PCR reaction was performed using specific oligonucleotide primers for amplification of each genes and amplified products visualized on 1% agarose gel electrophoresis. From 35 municipal wastewater samples 22 (62.86%), 25 (71.43%), 9 (25.71%), 13 (37.14%), and 17 (48.57%) specimens were positive for *Nitrosomonas sp.*, *Pseudomonas sp.*, *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa*, respectively. These results showed that *Pseudomonas* sp. and *Nitrosomonas* sp. are more frequent bacterial species in activated sludge of municipal wastewater of cold climate regions. These findings demonstrated that growth controls of these bacteria are important in biological treatment processes of activated sludge and useful for decrease of biochemical oxygen demand (BOD) in municipal wastewater of cold regions.

Key words: Activated sludge, municipal wastewater, PCR, cold regions.

Municipal wastewater is the major source of pollution in the aquatic ecosystem of cold climate regions. A common procedure of aerobic wastewater treatment is activated sludge process. The aim of the process is to decrease amount of dissolved organic matter from wastewater, using bacteria[1,2]. Communities of microorganism play a basic role in wastewater treatment, since they are the ones responsible for the nutrient and carbon removal in wastewater[3].

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In activated sludge, bacteria are used for oxidation of organic materials and the change of nitrate and nitrite to nitrogen (nitrification and denitrification). The bacteria in activated sludge are commonly used to transfer ammonium to nitrate (nitrification)[2,4].

Both heterotrophic and autotrophic bacteria are puissant of denitrification. *Pseudomonas* sp. are the most common denitrifying bacteria, which can use hydrogen, methanol, carbohydrates, organic acids, alcohols, benzoates, and other aromatic compounds for denitrification[5]. *Pseudomonas fluorescens* was able to assimilate cyanide as a nitrogen source for growth via a novel pathway involving initial cyanide oxidation[6]. As raw wastewater is also a potential source of pathogenic microorganisms,

inhalation, contact and ingestion can endanger human health through water or air pathways[7]. *Pseudomonas aeruginosa is a bacterium that can cause disease in humans and many kinds of animals. It is found in soil, water, skin flora, and* wastewater. *Also, p. aeruginosa exist in lipid rich wastewater*[8].

Species of *Nitrosomonas* are gram negative, mostly rod-shaped and chemoautotrophic, microbes ranging between 0.6-4.0 microns in length[9]. This rare bacterium oxidizes ammonia into nitrite as a metabolic process and important players in wastewater treatment plants[7]. *Nitrosomonas* are utilizable in the process of bioremediation and in treatment of industrial and sewage. They are important in the nitrogen cycle by increasing the availability of nitrogen to plants while limiting carbon dioxide fixation [10,11,12].

Nitrosomonas exist in almost every ecosystem such as soil, freshwater, sewage, and on building surfaces, especially in polluted region that contains high levels of nitrogen compounds. Nitrifying bacteria play a critical role in the dynamic cycling of reduced nitrogen throughout our global ecosystem[1].

There are many methods such as bacterial cultures, serological tests like the microscopic agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA) technique, immuno-fluorescence assay (IFA), slide agglutination test (SAT), and in addition molecular methods such as PCR used for detection of bacteria in activated sludge from municipal wastewater. MAT is based on the use of live bacteria cultures and this method may take up to eight weeks with weekly inspection and examination[13,14]. Moreover, in others method such as ELISA or IFA many factors may cause false positive and negative results. PCR is the most sensitive, fast, and reproducible of the existing rapid methods to detect bacteria in activated sludge from municipal wastewater[15]. *16S ribosomal RNA* (rRNA) gene has been widely used to identify bacterial species in wastewater environments[15].

The aim of this study was to detection of *Nitrosomonas sp.*, *Pseudomonas sp.*, *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* in activated sludge from municipal wastewater of cold climate regions (Chaharmahal Va Bakhtiari province, Iran).

MATERIALS AND METHODS

Sampling and DNA purification

35 municipal wastewater samples were collected in new and clean bottles between September 2011 and April 2012. Bacterial cells were harvested from homogenized samples by centrifugation and washed twice. After re-suspending them in 100 µL of 10 mM Tris-EDTA buffer, bacterial DNA was extracted from each wastewater sample using DNA extraction Kit (DNP™ Kit, Cinnagen Inc., Iran) according to manufacturer's instructions. The concentration of DNA was determined by measuring the absorbance of each sample at A260/280 using a nanodrop spectrophotometer (Nanodrop ND 1000, Nanodrop Technologies, Wilmington, DE). Only DNA with A260/280 ratios was kept for PCR analysis. Extracted DNA was immediately used or stored in a freezer at -20°C until needed.

PCR procedures

The PCR procedures were performed using 5 pair of oligonucleotide primers that shown in Table 1. Primers for *16S rRNA* gene of *Nitrosomonas* sp. and *gl06 phenol hydroxylase* gene of *Pseudomonas* sp. were designed according to the published sequence and primers for detection of *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* were used from published articles. Each PCR was carried out in 25 µL total reaction volumes, each containing 2.5 µL of PCR buffer 10X (50 mM KCl, 10 mM Tris-HCl [pH=8.3], 0.1% Triton X-100), 100 ng of target DNA, 0.2 pM of each primer, 1.5 mM $MgCl_2$, 200 mM dNTPs and 1 unit of *Taq* DNA polymerase (Roche Applied Science, Germany). PCR were performed in a Gradient Palm Cycler (Corbett Research, Australia). In PCR negative control (no DNA) was performed by adding 1 µL of sterile ultra-pure deionized water. PCR amplification reaction consisted *pre-denaturation* step of *5 min* at *94°C,* followed by 35 cycles of *amplification* (1 min denaturation at 94°C, 1 min annealing according to Table 1, and 1 min extension at 72°C), and a final extension of 5 min at 72°C.

Analysis of PCR products

Amplified products were analyzed by agarose gel electrophoresis (1%) and ethidium bromide staining. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM,

EDTA (pH=8.0) 4 ml of 0.5 M EDTA (pH=8.0), combine all components in sufficient H_2O and stirred to dissolve). 10 µL of PCR products with 2 µL gel loading buffer were loaded in the gel. Constant voltage of 80 V for 30 min was used for products separation. The DNA fragment size was compared with a standard molecular weight (100 bp DNA ladder of Fermentas, Germany). After electrophoresis, the amplicons were stained with ethidium bromide (5 µg/mL) and visualized by ultraviolet light. Then, photographed were obtained in UVIdoc gel documentation systems (UK).

RESULTS

DNA was successfully extracted with high quality from activated sludge of municipal wastewater samples using the commercial kit. For detection of bacteria in activated sludge PCR method were performed and analysis of PCR products for presence of *Nitrosomonas* sp., *Pseudomonas* sp., *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* on 1% agarose gel revealed a 306, 243, 595, 349, and 827 bp (base pairs) fragments (Fig. 1.).

From 35 municipal wastewater samples in 22 (62.86%) specimens contain *Nitrosomonas* sp. and *Pseudomonas* sp. were detected in 25 (71.43%) samples. Furthermore, *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* were investigated from 9 (25.71%), 13 (37.14%), and 17 (48.57%) wastewater samples, respectively (Table 2).

Table 1. The sequence of primers that used for detection of bacteria in activated sludge from municipal wastewater of cold climate regions

Bacterial species	Primer name	Nucleotide sequence	Accession Number (GenBank)	Annea-ling	Ampl-icon size (bp)
Nitrosomonas sp	Nitro-F	5'-TGAGAGGACGACCAACCACAC-3'	AJ621029	62°C	306
	Nitro-R	5'-CTGACTTACAAAACCGCCTGC-3'			
Pseudomonas sp.	Phenol-F	5'-GCAAGTCCACGCCATGAGC-3'	HQ915639	62°C	243
	Phenol-R	5'-AAGGTGACCGTGGCCATATC-3'			
Proteus inconstans	Incons-F	5'-GACTCAGGCACTACGCGATATG-3'	AB019704	60°C	595
	Incons-R	5'-GAATGATCTCTTTAATGGTCGC-3'			
Pseudomonas fluorescence	Pfluo-F	5'-TAGAGATAGATTGGTGCCTTCGG-3'	HE586392	63°C	349
	Pfluo-R	5'-ATTCACGATTACTAGCGATTCCGAC-3'			
Pseudomonas aeruginosa	Paero-F	5'-ATATTCAATCGCTTCAGCAGAGTC-3'	X99471	63°C	827
	Paero-R	5'-GTAGTGAATGCCGGTGTAGAGAC-3'			

Table 2. Bacterial detected in activated sludge of municipal wastewater samples in cold climate regions (Chaharmahal Va Bakhtiari province, Iran) after genes amplification

Bacterial species	Number (percent)
Nitrosomonas sp.	22 (62.86%)
Pseudomonas sp.	25 (71.43%)
Proteus inconstans	9 (25.71%)
Pseudomonas fluorescence	13 (37.14%)
Pseudomonas aeruginosa	17 (48.57%)

DISCUSSION

Today one of environmental concerns is the contamination of aquatic ecosystem with municipal and industrial wastewater, agricultural runoff, pesticide discharges from manufacturing plant, leaching, equipment washing operations, accidental spills and other sources[16]. Activated sludge in municipal wastewater is probably the most versatile of the chemical and biological treatment processes capable of producing an effluent with any desired BOD[17]. These processes have wide application among domestic, municipal, and industrial wastewaters treatment. The bacteria are the most important group of microorganisms that responsible for the structural and functional activity of the activated sludge of municipal wastewater[3].

Activated sludge play an important roles in wastewater treatment and the isolation and

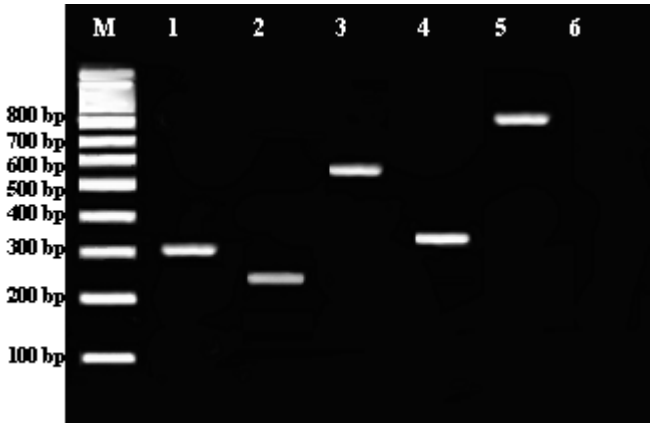

Fig. 1. Agarose gel electrophoresis of the PCR products for detection of bacteria in activated sludge of municipal wastewater samples (Line M: 100 bp DNA ladder (Fermentas, Germany), lines 1-5: *Nitrosomonas* sp., *Pseudomonas* sp., *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* respectively, and line 6: negative control without DNA)

identification of the microorganisms such as *Nitrosomonas* sp., *Pseudomonas* sp., *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* are more significant in physical and chemical process of wastewater[18]. The purpose of present study was to isolation and detection of *Nitrosomonas sp*., *Pseudomonas sp*., *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* in activated sludge from municipal wastewater of cold climate areas in southwest Iran (Chaharmahal Va Bakhtiari province) using molecular technique. In this research 35 municipal wastewater samples were examined and *Nitrosomonas sp*., *Pseudomonas sp*., *Proteus inconstans*, *Pseudomonas fluorescence*, and *Pseudomonas aeruginosa* were detected in 22 (62.86%), 25 (71.43%), 9 (25.71%), 13 (37.14%), and 17 (48.57%) wastewater samples, respectively. There are many studies performed using molecular technique for detection of bacteria in activated sludge of wastewater. Dionisi et al. were detected *Nitrosomonas* and *Nitrospira* spp. in full-scale wastewater treatment plants by competitive PCR[1]. Hikuma and co-workers performed rapid method for detection of ammonia-oxidizing bacteria such as *Nitrosomonas europaea* and *Pseudomonas putida* in activated sludge based on *16S-rRNA* gene by using PCR and fluorometry[15].

Jilani and Altaf Khan isolated *Pseudomonas* in a batch activated sludge process and investigated the biodegradation of cypermethrin by these bacteria[16]. Movahedyan *et al*. in Iran identified the phenol-degrading bacteria and *Pseudomonas putida* in activated sludge by PCR and 6 of 10 isolated bacteria were *Pseudomonas putida* and positive for methyl phenol operon (DmpN) gene[19]. Their study used molecular technique for detection of *Pseudomonas* and in this respects same to present research.

In conclusion, the results of present study demonstrated that *Pseudomonas* sp. and *Nitrosomonas* sp. are the most population in activated sludge from municipal wastewater of cold climate regions and plays an important role in the wastewater of these areas. Control and prevention of these bacteria in activated sludge is important and necessary for biological and chemical treatment processes and decrease of biochemical oxygen demand (BOD) in municipal wastewater of cold climate regions.

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