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Isolation and characterization of *Pseudomonas syringae* for nitrate removal under aerobic conditions

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Abstract

This study describes the isolation and characterization of a novel bacterium for denitrification process under aerobic conditions. Twenty two denitrifying strains were isolated from sludge of secondary clarifier of waste water treatment plant of a local fertilizer industry. The isolated strains were initially screened using solid media for their denitrifying activities. Among them one of the bacterium displayed the highest reduction of nitrate without much accumulation of nitrite. The isolate was identified preliminarily by biochemical tests as *Pseudomonas syringae*, and further confirmed its identification by 16S rRNA sequencing analysis. This isolate strain showed 99.32% nitrate reduction under anoxic condition as well as 99.1% nitrate removal under aerobic condition.

Keywords: Denitrification, *Pseudomonas syringae,* Isolation, Characterization.

Introduction

Most of the industrial effluents often contain high concentrations of nutrients like nitrogen and phosphorus in the form of nitrates and phosphates. In the secondary biological treatment plants microorganisms require a small amount of nutrients for their growth. Excessive nutrients in receiving waters cause algal growth and also lead to oxygen sag. Excess nitrogen compounds discharged into the environment can cause problems such as the eutrophication of rivers, which leads to deterioration of water sources. In addition, excess nutrients in drinking water create health problems like "methemoglobinemia" in infants (USEPA, 1993). Nitrates cause cancer, hypertension and thyroid hypertrophy if consumed in excess (Naik et al. 2011). Further, nitrates can also form nitrosamines and nitrosamides that are potentially carcinogenic compounds.

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Conventional nitrogen removal systems in wastewater treatment plants consist of nitrification by autotrophs, such as *Nitrosomonas europia* and *Nitrobacter winogradskyi*, under aerobic conditions and denitrification by heterotrophs under anoxic condition. However, this system is expensive and difficult to operate due to slow nitrification. It is also necessary to operate in separate reactors. Denitrification and nitrate ammonification are to be considered as the highest-energy-yielding respiration systems in anoxic environments after oxygen has been consumed (Zumft et al. 1997). Recently, researchers have reported that aerobic denitrifying species isolated from canals, ponds, soils and activated sludge have the capacity to simultaneously utilize oxygen and nitrate as electron acceptors. These species are *Hyphomicrobium* (Timmermans et al. 1983), *Thiosphaera pantotropha* (Gupta et al. 1997), *Paracoccus denitrificans* (Su et al. 2001), *Alcaligenes faecalis* (Joo et al. 2005), *Citrobacter diversus* (Huang et al. 2001), *Pseudomonas stutzeri* (Su et al. 2001) and *Pseudomonas aeruginosa* (Chen et al. 2006), *Pseudomonas putida AD21* (Kim et al. 2008) etc. Aerobic denitrification has some advantages over anoxic denitrification. In this process simultaneous nitrification and denitrification can be carried out in the same reactor so as to reduce the construction cost and the operational complexity. It causes less acclimatization problems. The alkalinity is released during denitrification that can partly compensate for the base consumption in the nitrification process and also it can reduce the extra alkali costs and avoid the secondary pollution under aerobic condition (Kim et al. 2008).

Denitrifying bacteria have been isolated from diverse environments such as agricultural soils, deep sea sediments, waste water treatment plants, etc. A wide variety of bacteria belonging to diverse genera has been isolated and studied for their denitrifying activities by many investigators (Sora et al. 2010). *Pseudomonas* species are generally presumed to be the predominant bacteria used in denitrification processes (Abbas et al. 2010). However, most studies on denitrification using this species have been carried out under anoxic conditions. The strain isolated in the present study was able to denitrify both aerobic and anoxic conditions, which is the imperative motive

to carry out the further denitrification study employing this organism.

The present investigation was undertaken with the aim of isolating and screening of bacteria for denitrifying activities. The study also represents the identification of competent denitrifier based on biochemical and genomic characteristics. Further denitrifying efficiency of the isolated strain with respect to different initial medium pH, incubation temperature and agitation speed were also studied.

Materials and Methods

Nitrate rich (NR) medium: NH_4Cl-0.3g/L, KH_2PO_4-1.5g/L, $Na_2HPO_4.7H_2O$- 7.9g/L, KNO_3-2 g/L, $C_4H_40_4Na_2.6H_2O$-27g/L. $MgSO_4.7H_2O$ Solution (5 mL/L): 20g/L.
Trace element solution (5mL/L): EDTA-50.0 g/L; $ZnSO_4$-2.2 g/L; $CaCl_2$-5.5 g/L; $MnCl_2.4H_2O$-5.06 g/L; $FeSO_4.7H_2O$-5.0 g/L; $(NH_4)_6Mo_7O_{24}.4H_2O$-1.1 g/L; $CuSO_4.5H_2O$-1.57 g/L and $CoCl_2.6H_2O$-1.61 g/L.
Bromothymol blue (BTB) medium: L-asparagine-1 g/L; KNO_3-2 g/L; KH_2PO_4-1 g/L; $FeCl_2$–P_6H_2O-0.05 g/L; $CaCl_2$–$2H_2O$- 0.2 g/L; $MgSO_4$–$7H_2O$-1 g/L; BTB reagent, 1ml/L [1% in ethanol]; and agar - 20 g/L; pH 7.0–7.3. (Su et al. 2001; Kim et al. 2008).
All the reagents used were of analytical grade

Enrichment and Isolation of bacteria

The nitrate rich sludge sample was collected from waste water treatment plant (secondary clarifier) of a nearby fertilizer industry. The sludge obtained from the treatment plant was inoculated into an NR liquid medium, so called enrichment. 5 mL of each sample was aseptically transferred into 250 ml Erlenmeyer flask containing 100 mL of sterile NR medium. The Erlenmeyer flasks were incubated at 30°C in a rotary shaker (150 rpm) for two days. The similar sets of flasks were maintained without aeration. After 2 days of incubation, 5mL of sample mixture was transferred into fresh NR medium and incubated further for two days. This procedure was repeated one more time with fresh NR medium.

Screening of Bacteria

The primary screening method was carried out using Bromothymol blue (BTB) agar medium plates. After six days of incubation, 1mL of the sample mixture was serially diluted under aseptic condition. The resulting bacterial suspensions were plated onto BTB medium plates using spread plate technique and incubated at 30°C for 1–3 days in order to isolate pure culture of bacteria. The colonies forming blue color / halo zones in the solid agar medium indicates the denitrifying activities. Such colonies were further purified and separated on nutrient agar media and maintained as a single strain before subjecting them into further denitrification studies.

Secondary Screening for potential denitrifier in liquid culture

Though initial solid agar screening was very efficient in selecting of suitable denitrifier, additional confirmation of denitrification process in liquid culture had to be carried out. To study the denitrification process by the selected strains under liquid condition, defined NR liquid medium (Kim et al. 2010) was used. During the study, 3-4 loop full individual stains were inoculated into 100 mL of NR medium in 250 mL Erlenmeyer flask and incubated one set under aerobic and another set under anoxic (BOD bottles) conditions. After 48h of incubation, sample was withdrawn and centrifuged at 10000 rpm for 10 min in 4°C to separate supernatant and residue of bacterial biomass.

Denitrification studies by P. syringae

The effect of pH on denitrification was studied by preparing the NR media with initial medium pH of 5, 6, 6.5, 7, 7.2, 7.5, and 8.0. The flasks containing 100 mL of respective medium was inoculated with fresh *P. syringae* cultures. The flasks were incubated at 30°C under 150 rpm. To study the effect of agitation speed on denitrification process, the NR media is inoculated with *P. syringae* culture and then incubated at 30°C at different agitation conditions i.e., from 0 rpm (static culture), 50 rpm, 100 rpm, 120 rpm, 150 rpm, 180 rpm and 240 rpm. The effect of temperature on denitrification by *P. syringae* was investigated by incubating inoculated NR media at different temperatures such as 26, 28, 30, 35 and 37 and 40°C. In each case sample cultures were withdrawn in every 6h of incubation for biomass, residual nitrate and nitrite analysis.

Analytical Methods

Growth of the bacterium was monitored by measuring the optical density at 610 nm using UV-Visible Spectrophotometer (Shimadzu) (Shu-Cheng et al. 2011). The culture samples were centrifuged at 10,000 rpm for 10 min (REMI, India) and supernatant was used for nitrate and nitrite analysis by Ion exchange chromatography (DIONEX, USA) with Na_2CO_3 (12 mM) and $NaHCO_3$ (8 mM) as eluents. H_2SO_4 was used as regenerent (Dhamole et al. 2011).

Results and Discussion

Aerobic denitrifying bacteria were isolated from waste water sludge collected from a nearby fertilizer industry. Twenty two strains were obtained as denitrifier from the initial solid agar screening techniques. Here the colonies formed blue color and/or halos zones on BTB agar medium due to increase in pH. These strains were individually separated and further employed in liquid cultures for confirming the denitrification process. In liquid culture of the strain coded with kjdr2 (NITK) was found as efficient denitrifier. This isolate denitrified 99.1% under aerobic condition with minute nitrite accumulation, which is highest in comparison to other isolates (Table 1). The isolate kjdr3 denitrified 99% but with a high accumulation of nitrite *i e.,* 453.56 mg/L as an intermediate compound. This shows incomplete denitrification and hence kjdr2 isolate was selected for further studies.

Table 1: Percentage denitrification by different isolates under aerobic condition.

Isolate	Initial Nitrate Conc. mg/L	Final Nitrate conc. mg/L	Final Nitrite conc. mg/L	Percentage denitrification
kjdr1	860	227.91	328.44	73.49
kjdr2	**860**	**7.74**	**13.88**	**99.1**
kjdr3	860	5.41	453.56	99.37
kjdr4	860	851	13.84	1.04
kjdr5	860	555.21	97.1	35.44
kjdr6	860	797.5	49.9	7.26
kjdr7	860	797.6	44.53	7.25
kjdr8	860	424.19	211.63	50.67
kjdr9	860	251.69	315.6	70.73
kjdr10	860	136.22	359.1	84.16
kjdr11	860	634	116.4	26.27
kjdr12	860	648.55	112.12	24.58
kjdr13	860	754.23	64.02	12.29
kjdr14	860	732.17	78.86	14.86
kjdr15	860	614.61	119.42	28.53

kjdr16	860	659.67	99.16	23.29
kjdr18	860	563.14	125.1	34.51
kjdr19	860	855.76	4.64	0.49
kjdr20	860	222.18	312.88	74.16
kjdr21	860	773.26	62.89	10.08
kjdr22	860	443.87	226.24	48.38

Identification of the microorganism

Based on the morphology and microscopic studies, the bacterial isolate kjdr2 was found to be Gram-negative, rod shaped and creamish color. As per Bergey's Manual of Determinative Bacteriology (2000), the isolate was characterized as *Pseudomonas. sp.* based on the biochemical tests as shown in Table 2.

Table 2: Morphological and biochemical characteristics of the bacterial isolate kjdr2**.**

Tests/ Characteristics	Results
Colony morphology	Round
Margin	Irregular
Elevation	Raised
Surface	Smooth
Pigment	Creamish
Gram staining	Gram negative
Shape	Rod
Catalase test	Positive
Starch hydrolysis	Negative
Methyl red test	Negative
Indole test	Negative
Urease test	Positive
Hydrolysis of gelatin	Negative
Casein hydrolysis	Positive
Oxidase test	Positive
Citrate utilization	Positive

Further, the identification of the isolate was confirmed by 16S rRNA partial genome sequencing method, which was done in Agarkar Research Institute, Pune (India). The culture sample was processed for isolation and purification of genomic RNA. The isolated RNA was amplified using polymerase chain reaction **(**PCR) with combination of primers FDD2 – RPP2. After amplification process, the 16S rRNA genomic sequence was identified using Basic Local Alignment Search Tool **(**BLAST). The genomic sequence is presented in Fig. 1. The genomic sequence of the bacterial isolate was compared with the library genomic sequences.

GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
GGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT
CGGATTGTAAAGCACTNTAAGTTGGGANGAAGGGCAGTTACCTAA
TACGTRTCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACT
CTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGAA
TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGC
AAGCTAGAGTATGGTAGAGNGTGGTGGAATTTCCTGTGTAGCGGT
GAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACC
ACNTGGACTGATACTGACACTGAGGTGCGAAAGNGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAA
CTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCAT
TAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
TGAATTGACGGGGGCCNGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGAAGC

Figure 1: 16S rRNA gene sequence of the bacterial isolate

A phylogenetic tree was constructed by the neighbour joining method as shown in Fig 2 (Kim et al. 2008; Saitou et al. 1987). With closest phylogenetic identification, the isolate was identified as *Pseudomonas syringae JQ317219.1*.

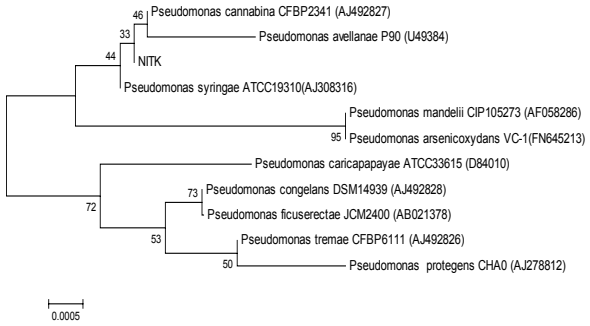

Figure 2: Phylogenetic tree based on a comparison of the 16S rRNA gene sequence. Bar shows two nucleotide substitutions per 1000 nucleotides.

Effect of initial medium pH on denitrification

The effect of pH on denitrification was studied by preparing the NR media with initial medium pH 5, 6, 6.5, 7, 7.2, 7.5, and 8.0. The pH of the medium was altered by using 1N HCl /NaOH solution. In the present study at a pH 7.2 a maximum nitrate removal by *Pseudomonas syringae* was observed. It is evident from the Fig. 3 that, at pH 7.2 nitrate removal of 96.37% was achieved and maximum biomass quantity produced was 0.942 g/L. Adequate nitrate removal of 85.67% at pH 6.5; 89.916% at pH 7.0; 84.65% at pH 7.5 with respective biomass quantity of 0.51g/L, 0.734g/L, 0.74g/L was produced. At acidic pH, both nitrate removal and biomass growth was found to be less. At pH 5, 5.5 and 6 the nitrate removal efficiency was found to be 38.33%, 43.56% and 61.612% respectively and the corresponding biomass quantity produced was 0.21g/L, 0.32 g/L and 0.402g/L. At alkaline pH, it is observed that, there is decrease in nitrate removal as well as biomass growth. At pH 8, the biomass quantity produced was 0.34 g/L and 68.46% nitrate removal was observed. The isolate *P. syringae* was found to be more effective in nitrate removal at pH 7.2 to 7.5.

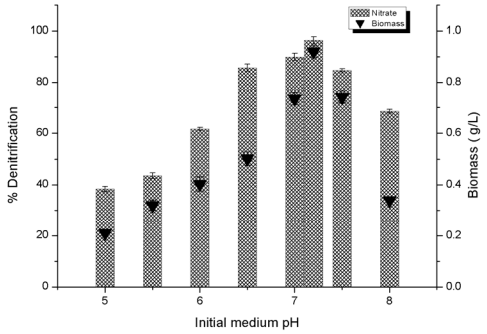

Figure 3: Effect of initial medium pH on percentage denitrification by *P. Syringae.*

Wang et al (1995); Urbain et al (1997) have reported similar denitrification process occurring optimally at pH 7.5. Wang et al (1995) reported that, cultures of *Pseudomonas denitrificans*, when grown at 30°C reduced nitrate optimally at a pH between 7.4 and 7.6 and nitrite at a pH between 7.2 and 7.3.

Effect of agitation speed on denitrification

The effect of agitation speed on denitrification was studied by varying the agitation speed of the flasks containing the NR medium with initial supplement of 300 mg/L of nitrate concentration. The flasks were inoculated with the *P. Syringae* and incubated at 30º C at different agitation speeds 0 rpm (static culture), 50 rpm, 120 rpm, 150 rpm, 180 rpm and 240 rpm. With increase in agitation speed, there is corresponding increase in oxygen transfer into the media which enhances the dissolved oxygen (DO) level in the medium (Taylor et al. 2009; Shu-Cheng et al. 2011). It is evident from the Fig. 4 that, with increase in agitation speed, there was no significant change in nitrate removal observed till 180 rpm. A maximum of 97.9 % denitrification was achieved in the culture incubated at 150 rpm. The corresponding biomass quantity produced was 0.97 g/L. At 0 (static culture) 50 and 100 rpm, the nitrate removal was 92.1%, 89.37% and 94.3% respectively. The corresponding biomass quantity produced at 0, 50, 100 rpm was 0.73 g/L, 0.81g/L and 0.86 g/L respectively. At agitation speed of 240 rpm, a decrease in nitrate removal as well as biomass growth was observed. The percentage denitrification was 56.21% and the amount of biomass produced was 0.53 g/L. It can be observed from the Fig. 4 that, at agitation speed of 240 rpm, the biomass growth is less which may be due to rupture of the cells at higher agitation speed which resulted in lesser nitrate removal.

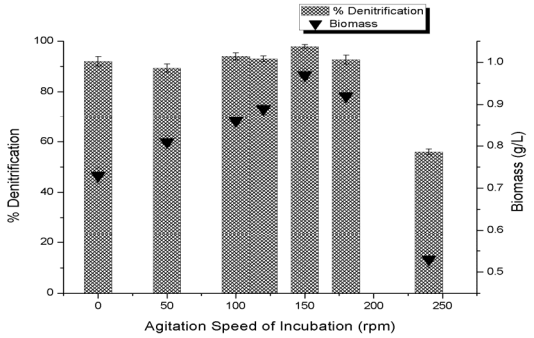

Figure 4: Effect agitation speed on percentage denitrification by *P. Syringae.* Results are represented as means ± SEM (n=2)

Shu-Cheng et al (2011) reported similar results, in which 420 mg/L NO_3^- N was completely removed within 30 h at 160 rpm and 200 rpm.

Effect of incubation temperature on percentage denitrification

Temperature is one of the important parameters which significantly affect the growth of the microorganism as well as its metabolic activities. To study the effect of temperature on growth of *P. syringae* and its nitrate removal, the organism was inoculated into NR medium then the flasks were incubated at different temperature with the initial nitrate supplement of 300 ppm. The growth and percentage denitrification by *P. Syringae* is shown in Fig. 5. The organism showed its maximum growth at incubation temperature of 35°C. At this temperature biomass yield of 0.931g/L was obtained and 96%, denitrification was achieved, which was the highest among other incubation temperatures. At lower temperatures i.e., 20, 25, 27 & 30°C the biomass quantity produced was 0.210 g/L, 0.234 g/L, 0.502g/L and 0.824g/L respectively. The corresponding percentage nitrate removal was found to be 47.1%, 50.6%, 66.7%, and 79.6% respectively. At higher temperatures for 37°C and for 40°C the percentage denitrification was found to be decreasing and biomass yield obtained for the these temperature was 0.512 g/L and 0.496 g/L respectively. The corresponding percentage denitrification 86.11% and 78.72% was obtained. Wen et al. (2010) reported that the maximum denitrification was achieved 81.6% at 32°C and 79% at 37°C. Wang et al. (2011) reported in their study that, around 90% nitrate removal occurred in the temperature range 30-35°C by the isolate X31 *Pseudomonas* sp. under aerobic conditions.

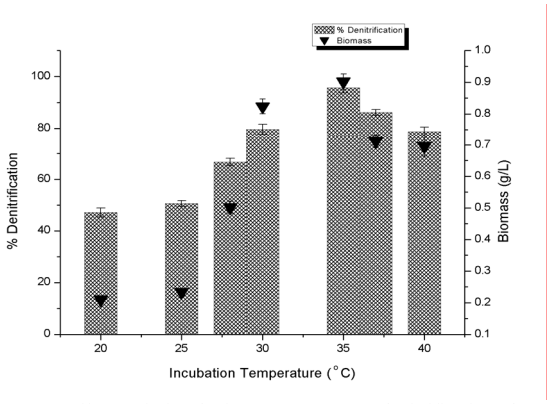

Figure 5: Effect of incubation temperature denitrification by *P. syringae* Results are represented as means ± SEM (n=2)

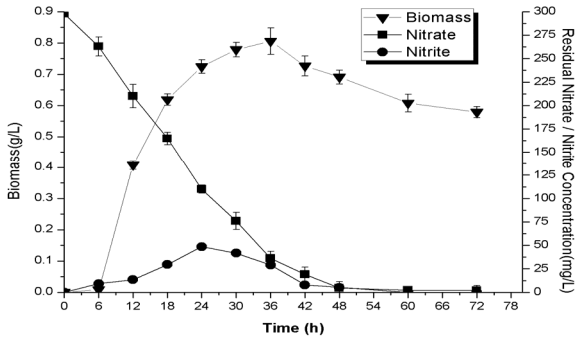

Figure 6: Time course profile of nitrate and nitrite consumption, cell growth, NR medium by *P. Syringae.* Results are represented as means ± SEM (n=2).

Time-course data for the consumption of nitrate and biomass growth for *Pseudomonas syringae* is shown in Fig. 6. The strain *P. syringae* has the capability of mixotrophic metabolism that can grow better by respiring simultaneously nitrate and oxygen when both are present in the media. Su et al (2001) reported that in a highly oxygenated atmosphere, *P. stutzeri SU2* rapidly reduced nitrate to nitrogen gas without any nitrite accumulation.

Conclusions

The bacterium, *Pseudomonas syringae* (kjdr2) isolated in the present study was found be an efficient denitrifier in aerobic conditions. It has very good denitrification ability which denitrified 98.01% of initial 300ppm of nitrate within 48th h under aerobic conditions. It also showed significant nitrate removal at pH 7.2, at 150 rpm agitation speed and at temperature of 35°C. Moreover, the denitrification activity of the bacterium was not much affected by with the increase in agitation speed up to 180 rpm, which indicated the aerobic denitrification process of the bacterium. These results suggest that *Pseudomonas syringae* (kjdr2) may be a prospective candidate for aerobic wastewater treatment. However, further studies on effect of carbon, nitrogen and other growth

parameters has to be studied for the additional evaluation of the efficiency of aerobic denitrification process.

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