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ORIGINAL PAPER

Degradation of pyridine and 4-methylpyridine by *Gordonia terrea* IIPN1

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Abstract *Gordonia terrea* IIPN1 was isolated and characterized from soils collected at petroleum drilling sites. The strain was able to catabolize pyridine and 4-methylpyridine as sole carbon and nitrogen source. The strain failed to catabolize other pyridine derivatives. Growing cells completely degraded 30 mM of pyridine in 120 h with growth yield of 0.29 g g^{-1}. Resting Cells grown on 5 mM pyridine degraded 4-methylpyridine without a lag time and vice versa. Supplementary carbon and nitrogen source did not significantly change the specific growth rate and degradation rate by the resting cells.

Keywords Biodegradation · *Gordonia* · 4-methylpyridine · Pyridine

Introduction

Pyridine and alkylpyridines are major environmental pollutants commonly found in locations associated with oil shale (Dobson et al. 1985; Leenheer et al. 1982) and coal mines and washeries (Pereira et al. 1983) and wood treatment sites (Pereira and Rostad 1985). A high water solubility and potential mobility of these compounds through soil (Leenheer and Stuber 1981) contribute to groundwater contamination. Traditional biological treatment processes can destroy a large fraction of biodegradable organic compounds existed in groundwater. However, many hazardous compounds mainly alkylpyridines are poorly removed in conventional biological treatment processes due to their toxicity, recalcitrance or inhibitions. Furthermore, they also have adverse impact on the composition and activities of microorganisms in activated sludge flocs. Therefore the removal of these compounds is a real challenge for waste treatment engineers and scientists. The microbial degradation of pyridine, quinoline, acridine, and their derivatives under aerobic and anaerobic conditions has been extensively studied (Kaiser et al. 1996). Degradation of alkylpyridine by *Arthrobacter* spp. (Shukla 1974, 1975), *Pseudomonas* sp. (Korosteleva et al. 1981) has been reported. 4-methylpyridine was found as a major contaminant (11–8 ppm) among total pyridine (13 ppm) near a tar processing site which is considered to be of potential public health concern (http://www.osti.gov/energycitation). However, degradation of pyridine and its alkyl derivatives specifically 4-methylpyridine by any individual microrganism has not yet been reported despite of their wide occurrence and toxicity (Kaiser et al. 1996; O'Loughlin et al. 1999).

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The actinomycete genus *Gordonia* has attracted much interest in recent years for their ability to degrade xenobiotic environmental pollutants, slowly biodegradable natural polymers as well as to synthesize possible useful compounds. Members of the *Gordonia* species are known to degrade natural and synthetic rubber, phthalic acid esters, *s*-Triazine, dibenzothiophene and other poorly assimilable carbon sources, such as *t*-butyl ether, methyl *t*-butyl ether, *t*-amyl methyl ether, cyclic alkanes, and polycyclic aromatic hydrocarbons. Due to its catabolic activity to degrade a wide variety of environmental pollutants, it has become a powerful candidate for bioremediation processes (Arenskötter et al. 2004).

Member of the genus *Gordonia* that are able to degrade 3-methylpyridine, 3-ethylpyridine (Yoon et al. 2000; Lee et al. 2001) and medium chain length alkanes (Kummer et al. 1999; Xue et al. 2003) has been reported. However, there is no report on degradation of pyridine and 4-methylpyridine by member of this genus. This paper describes the isolation and characterization of a *Gordonia* species capable of catabolizing pyridine and 4-methylpyridine as carbon and nitrogen source. The strain is also able to utilize hexadecane as sole carbon source.

Materials and methods

Enrichment and isolation

Environmental samples of soil were obtained from petroleum drilling sites located in Gujarat, India. One gram of soil sample was thoroughly mixed in 10 ml distilled water with intermitted shaking and filtered through Whatman No. 1 filter paper. Filtrate (1 ml) was used for inoculating 25 ml basal medium enriched with 5 mM pyridine as sole carbon and nitrogen source. The flasks were incubated at 30°C on a rotary shaker (150 rpm) for one week. Enrichment cultures showing growth as measured by absorbance at 540 nm were used as inoculum in fresh medium. Pure culture was isolated after three successive cycles of enrichment by streaking on solidified basal medium supplemented with 5 mM pyridine as sole nitrogen source.

The basal medium contained (g/l) of 6.3 KH_2PO_4, 8.0 K_2HPO_4, 0.2 $MgSO_4$ and 10 ml of metal solution. The metal solution contained (g/l) of 2.0 $CaCl_2$, 1.0 NaCl, 0.5 $FeCl_2$, 0.5 $ZnCl_2$, 0.5 $MnCl_2$, 0.1 Na_2MoO_4, 0.05 $CuCl_2$, 0.05 $Na_2WO_4.2H_2O$ and 10 ml 10 M HCl per liter of deionized water. Pyridine as source of carbon and nitrogen was used at concentration ranging from 5 to 100 mM. For control experiments, 56.8 mM glucose and 50.1 mM $(NH_4)_2SO_4$ was used as carbon and nitrogen source respectively.

Identification and phylogenetic analysis of strain IIPN1

The organism was identified by substrate utilization test using Biolog™ GP-microplate identification system and 16S rDNA sequence analysis (Woese et al. 1983). Genomic DNA was isolated using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich Co; USA). PCR was performed using primers 5′ CAG GCC TAA CAC ATG CAA GTC 3′ and 5′ GGG CGG WGT GTA CAA GGC 3′. DNA sequencing was done and sequences were aligned using BLASTN 2.2.17 program available in NCBI website.

Pyridine degradation by growing cells

Exponentially growing cells were inoculated into conical flasks (250 ml) containing 40 ml basal medium at initial cell concentration of 200 mg dry cell weight (DCW) per liter. Pyridine (10–70 mM) was used as carbon and nitrogen source in the medium The culture was incubated in incubator shaker (Innova 4430, USA) at 170 rpm and 30°C. Medium without adding inoculum or pyridine served as control. The cell growth and pyridine utilization was estimated at regular time interval. Degradation of pyridine was monitored by scanning absorbance of the culture filtrates at wavelength from 200 to 400 nm using spectrophotometer (Chemito, India).

Pyridine and 4-methylpyridine degradation by resting cells

Bacterial cells were grown in basal medium with pyridine, 4-methylpyridine as carbon and nitrogen source, and glucose with ammonium sulphate as carbon and nitrogen source in different shake flask

culture up to exponential phase (OD_{540} = 1). Cells were harvested by centrifugation at 10,000*g* (Sorvall evolution RC, USA) and the cell pellet was washed three times with 50 mM potassium phosphate buffer (pH 7.0) before resuspending 20 mg cells in the same buffer (20 ml) supplemented with 1 mM pyridine or 1 mM 4-methylpyridine. The culture was incubated (Innova 4430, USA) at 170 rpm and 30̊C and tested for their ability to degrade pyridine and 4-methylpyridine in resting state.

Analytical methods

Cell growth was monitored by measuring the absorbance of culture broth at 540 nm and was expressed as dry cell weight, g/l measured from standard plot of OD versus dry cell weight. Pyridine and 4-methylpyridine concentration were determined by reversed-phase high pressure liquid chromatography (HPLC) in isocratic mode using C-18 column, Waters 2487 UV detector at 254 nm and a Waters 600 delivery system using methanol:water (4:1) as mobile phase at a flow rate of 1 ml/min. Cell free supernatant (1 ml) was extracted with ethyl acetate (1 ml) and 0.1 ml extracted sample was injected in the HPLC system to determine the concentration of pyridine and 4-methylpyridine.

The growth yield $Y_{x/s}$, specific growth rate (μ h^{-1}) and specific pyridine degradation rate ($q_{Pyr,}$ mM g $cells^{-1}$ h^{-1}) were calculated as described previously (Rhee et al. 1996).

Sequence submission

The 16S rDNA sequence of strain IIPN1 has been deposited in the GenBank database under accession number DQ139840.

Results

Enrichment and identification

The bacteria that degrade pyridine were successfully enriched in shake flask culture. Bacterial strain IIPN1was isolated based on its ability to utilize pyridine as sole source of carbon and nitrogen. The strain formed orange-red colored circular colonies on solidified basal medium with pyridine as sole nitrogen source. The strain is Gram-positive, aerobic and showed catalase activity.

Near full-length 16S rDNA sequence of strain IIPN1 was compared with sequence database of the ribosomal database project (RDP-II). Sequence query showed 99% homology with *Gordonia terrea* DSM 43249. Nucleotide BLAST search of NCBI showed that IIPN1 shared 100% homology with *G. terrea* at the two hypervariable regions between nucleotide 136–229 and 996–1028 (numbering according to 16S rDNA of *E. coli* K-12). All *Gordonia* species differ from each other at these two regions (Arenskötter et al. 2004). The strain was also identified as *Gordonia terrea* (ID = 0.76) using Biolog^{TM} bacterial identification system. Based on the combined 16S rDNA sequence and substrate utilization tests, the strain was designated *Gordonia terrea* strain IIPN1. The phylogenetic position of the strain *Gordonia terrea* IIPN1constructed from evolutionary distance values by the neighbor-joining method shown in Fig. 1.

Growth associated degradation of pyridine

Pyridine degradation by strain *Gordonia terrea* IIPN1 was observed in mineral salt medium with pyridine as sole source of carbon and nitrogen. Medium containing 30 mM pyridine was completely degraded in 120 h with growth yield of 0.29 g g^{-1}. The growth response of the strain to pyridine as sole nitrogen source increased with increasing pyridine concentration reaching a maximum at 70 mM (Fig. 2). However exposure of the strain to pyridine concentration above 120 mM completely inhibited growth (data not shown). Increase in final cell concentration (2.56 g l^{-1}) was observed when hexadecane was supplied as supplementary carbon source in medium containing 30 mM pyridine as carbon and nitrogen source in comparison to cell concentration (0.71 g l^{-1}) when pyridine was used as sole carbon and nitrogen source.

Influence of supplementary nitrogen and carbon source on pyridine degradation

Degradation of pyridine (30 mM) was completed by the strain *Gordonia terrea* IIPN1 in 72 h in presence

Fig. 1 Phylogenetic tree based on 16S rDNA sequences showing the position of strain IIPTN

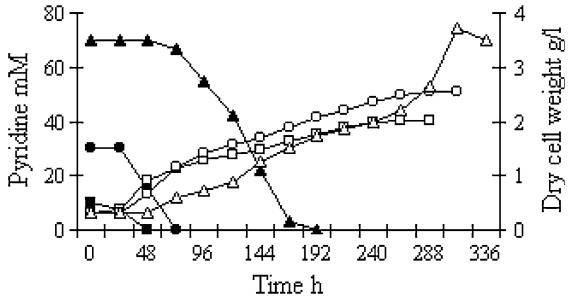

Fig. 2 Cell growth (◯ □ Δ) and pyridine degradation (● ■ ▲) at various concentration of pyridine (mM): 10 ■, 30 ●, 70 ▲)

of glucose but it required longer time (120 h) to degrade same quantity of pyridine when used a sole source of carbon and nitrogen. Supplementation with ammonium sulphate as nitrogen source had extended the degradation period further up to 144 h. Variation of pyridine degradation period due to presence of glucose and ammonium sulphate was observed due to variation of lag phase of the bacterial strain as shown in Fig. 3. The specific pyridine degradation rate (q_{pyr}) of the strain was maximum 0.71 mM g^{-1} l^{-1} in presence of glucose as compared to 0.69 mM g^{-1} l^{-1} with pyridine alone and 0.59 mM g^{-1} l^{-1} when substituted with ammonium sulphate. Thus presence of supplementary nitrogen source did not significantly contribute to the final cell concentration or the specific degradation rate; on the contrary glucose has enhanced the specific degradation rate marginally.

Effect of pyridine concentration

The cell growth and pyridine degradation with the increase of pyridine concentration from 10 mM to 70 mM as sole nitrogen source is shown in Fig. 3. The strain showed high growth response at higher pyridine concentration. The specific growth rate (μ) and specific pyridine degradation rate (q_{Pyr}) at 10 mM pyridine were 0.12 h^{-1} and 0.33 mM g^{-1} h^{-1}. Increase in pyridine concentration from 10 mM to 30 mM, the μ as well as q_{Pyr} increased to 0.17 h^{-1} and 0.69 mM g^{-1} h^{-1} respectively. However, at 70 mM pyridine, the μ further increased to 0.22 h^{-1} but the q_{Pyr} decreased to 0.64 mM g^{-1} h^{-1}. Increase in pyridine concentration, however, extended the lag phase before growth. The growth of the cells continued till 300 h while the degradation of pyridine was completed in 200 h.

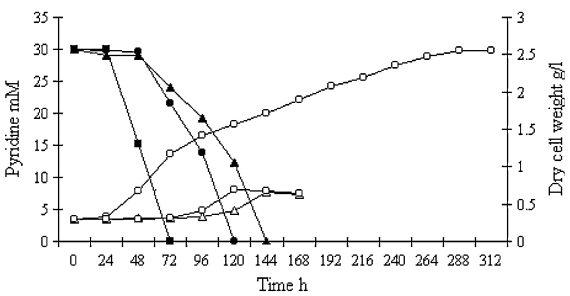

Fig. 3 Effect of supplementary nitrogen (50.1 mM ammonium sulphate Δ) and carbon source (56.8 mM glucose □) on cell growth (◯ □ Δ) and pyridine degradation (● ■ ▲) in medium containing 30 mM pyridine (◯)

Degradation of pyridine and 4-methylpyridine by resting cell

Induction of the biodegradation enzymes in the cells grown on pyridine and 4-methylpyridine was compared with cells grown on glucose and $(NH_4)_2SO_4$. Cells grown on pyridine degraded 4-methylpyridine without a lag time and vice versa. On the other hand cultures grown on glucose and ammonium sulphate showed very slow degradation of pyridine and 4-methylpyridie (Fig. 4). The degradation of 4-methylpyridine or pyridine was much faster (16 mM g^{-1} h^{-1}) by cells grown in pyridine or 4-methylpyridine respectively as compared to pyridine grown cells degrading pyridine (12 mM g^{-1} h^{-1}). Nevertheless overall degradation rate of pyridine and 4-methylpyridine by the resting cells of *Gordonia terrea* IIPN1 was much faster than that of by the growing cells.

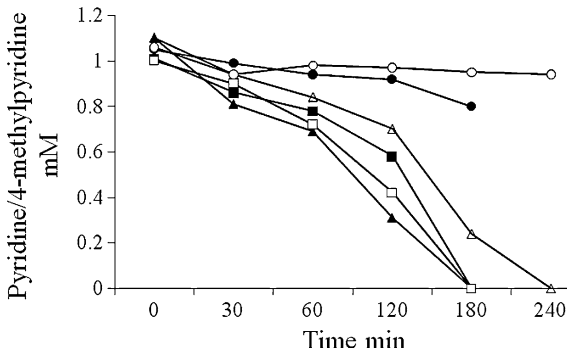

Fig. 4 Degradation of pyridine (◯ □ Δ) and 4-methylpyridine (● ■ ▲) by resting cell grown on pyridine (Δ ▲), 4-methylpyridine (□ ■) and ammonium sulphate and glucose (◯ ●). Initial cell concentration was 1.0 g (dry cell weight) per liter

Discussion

The biodegradation of pyridine, 4-methylpyridine and hexadecane by *Gordonia terrea* IIPN1 was investigated in this study. Strain IIPN1 produces colonies with reddish pigmentation, indicating their capacity to synthesize significant amount of carotinoids which is common characteristics in many *Gordonia* species. *Gordonia jacobaea* MV-1 was widely studied because of its property of producing large amount of carotenoids (De Miguel et al. 2000).

Strain IIPN1 appears to be different among the reported pyridine and alkylpyridine degrading bacteria. Complete mineralization of 2-methylpyridine, 2-ethylpyridine, 2,4-dimethylpyridine and 2,4,6-trimethylpyridine by several *Arthrobacter* spp. (Shukla 1974, 1975), 3-methylpyridine by *Pseudomonas* sp. (Korosteleva et al. 1981), 2-ethylpyridine, 3-ethylpyridine, and 4-ethylpyridine by a mixed culture (Feng et al. 1994) have been reported. However, to our knowledge, *Gordonia terrea* IIPN1 is the only well-characterized bacterial strain degrading pyridine, 4-methylpyridine and alkane. Ronen et al. (1998) reported degradation of pyridine and 4-methylpyridine by an uncharacterized strain but there is no further report.

Different members of the genus *Gordonia* able to degrade alkylpyridine and alkanes are known. *G. nitida* LE31 degraded 3-methylpyridine and 3-ethylpyridine but failed to degrade pyridine and 4-methylpyridine (Lee et al. 2001). Degradation of alkane by the strain is not known. Similarly, *G. alkanivorans* (Kummer et al. 1999) and *G. paraffinivorans* (Xue et al. 2003) are known to degrade alkanes but there is no report on degradation of pyridine and alkylpyridine by the two species. Attempts to amplify the alkane monooxygenase gene in Gordonia *terrea* IIPN1 using *alkB* specific primers of *P. aeruginosa* PAO1 (Belhaj et al. 2002) failed to yield any amplification products. This suggested that the strain *Gordonia terrea* IIPN1 does not have the same alkane degrading genes as that of *P. aeruginosa.*

The strain has the ability to degrade high concentration of pyridine and 4-methyl pyridine. Growth of *G. nitida* LE31 was found to be significantly inhibited at 3 mM 3-methylpyridine and 4 mM 3-ethylpyridine (Lee et al. 2001). Similarly, the maximum growth response of *Arthrobacter* sp. in 2-ethylpyridine was observed at 10 mM (O'Loughlin et al. 1999). Growth of the cells took longer time (160–288 h) to reach stationary phase as compared to the degradation time of pyridine (72–144 h) may be explained by the fact that pyridine and 4-methyl pyridine was degraded through intermediate products which were assimilated by the strain at a slower rate than that of the substrate (Fig. 3). However, the strain *Gordonia terrea* IIPN1 could completely degrade 70 mM pyridine at 0.22 h^{-1} specific growth rate and 0.69 mg g^{-1} h^{-1} specific degradation rate. Higher rate (16 mg g^{-1} h^{-1}) of degradation of 4-methyl pyridine and pyridine could be achieved by using resting cells of *Gordonia terrea* IIPN. Therefore the strain is favorable candidate to be used for bioremediation of sites heavily contaminated with pyridine and pyridine derivatives. Supplementation of carbon and nitrogen also does not required for degradation of pyridine and 4-methyl pyridine by the resting cells of *Gordonia terrea* IIPN1. Such characteristics are desirable in microorganisms to be used for bioremediation purpose (Rhee et al. 1996).

Throughout the course of degradation experiments, culture experiments were examined to detect metabolic intermediate. Detection of cyclic intermediate by UV scanning (200–400 nm) was unsuccessful. Similar results were also reported with pyridine and 2-Methylpyridine degrading bacteria (Lee et al. 2001; Rhee et al. 1997; Watson and Cain 1975).

Cells grown in pyridine degraded 4-methylpyridine without a lag time and vice versa. Shukla (1974) reported similar results with 2-methylpyridine or 2-ethylpyridine grown cells of an *Arthrobacter* sp. Cells grown in pyridine degraded pyridine at a faster rate than 4-methylpyridine grown cells and vice versa. Lee et al. (2001) also reported similar results with cell of G. *nitida* LE31 grown on 3-methylpyridine and 3-ethylpyridine. Such phenomena indicate that some of the enzymes in the pathway of pyridine and 4-methylpyridine degradation are shared.

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