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Biomineralization of N,N-dimethylformamide by *Paracoccus* sp. strain DMF

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ABSTRACT

N,N-dimethylformamide (DMF) is a man-made compound that is widely used as a solvent for the synthesis of various organic compounds. In this study, a bacterial strain *Paracoccus* sp. DMF capable of using DMF as the sole carbon, nitrogen and energy source, was isolated from an enrichment culture developed using activated sludge from domestic waste water treatment unit as the source inoculum. The strain DMF was characterized by biochemical tests and 16S rDNA sequence analysis, to be belonging to the genus *Paracoccus*. Growth on DMF was accompanied with ammonia release and the total organic carbon (TOC) analysis indicated its extensive mineralization. Batch culture studies were conducted in the substrate range of 100–5000 mg L^{-1} to determine the biokinetic constants. Strain *Paracoccus* sp. DMF could tolerate very high concentrations of DMF as the growth was observed even at 15 000 mg L^{-1}. High (μ_{max}) and (K_i) showed the suitability of the strain for the treatment of DMF containing waste water. Transient accumulation of dimethylamine (DMA) in the medium during the growth on DMF and utilization of DMA and monomethylamine (MMA) as growth substrates by *Paracoccus* sp. strain DMF showed that the pathway of DMF degradation involves DMA and MMA as intermediates, ultimately leading to the formation of carbon dioxide (CO_2) and ammonia (NH_3).

1. Introduction

N,N-dimethylformamide (DMF) is a man-made industrial chemical with an estimated worldwide production of 2.7×10^5 tonnes per year [1]. As it is miscible with both water and organic solvents, DMF is extensively used solvents in chemical industries such as textile and pharmaceutical industries and in the production of very wide range of organic chemicals and polymers including polyurethane, rubber, dyes, wood, leather, films, paper, and pesticides. Consequently, it is commonly found in high concentrations in many industrial effluents even after its recovery. DMF is known to affect cellular differentiation, hepatotoxicity and causes gastric irritation and may be carcinogenic [2]. Due to these adverse effects of DMF, its removal from industrial effluents is gaining wide attention. Although there are physico-chemical methods available for the treatment of DMF containing waste water, biological methods are not only cost effective, but also environment-friendly as they lead to innocuous end products. Few reports are available on the isolation of the bacterial consortia and pure cultures, which can utilize DMF as the sole carbon and energy source [3–7].

In this study, we report the isolation and characterization of a bacterial strain *Paracoccus* sp. DMF from activated sludge of domestic waste water treatment unit, Kanpur, which can rapidly mineralize DMF. Studies on kinetic constants for the growth of strain on DMF as well as the pathway for its degradation are also carried out.

2. Materials and methods

2.1. Source of inoculum, medium, and growth conditions

Activated sludge obtained from a domestic wastewater treatment plant, located at Jajmau, Kanpur, was used as the inoculum for the development of enrichment culture.

The minimal medium (MM) used for the enrichment and growth of the strain consisted of following (in g L^{-1} distilled water): $Na_2HPO_4{\cdot}2H_2O$, 2.0; KH_2PO_4, 1.0; $MgSO_4$, 0.1; K_2SO_4, 0.06; $CaCl_2{\cdot}2H_2O$, 0.035; Yeast extract, 0.01. 45 mL of the medium was transferred to 150 mL Erlenmeyer flasks and after autoclaving, required amount of distilled DMF (having free amine content less than 0.2 ppm) was added. Flasks were inoculated with 5 mL culture grown on 400 mg L^{-1} of DMF and incubated at 37 °C with shaking on orbital shaking incubator at 120 rev min^{-1}.

2.2. Enrichment and isolation of bacterial strain

5 mL of 10-fold diluted activated sludge suspension was inoculated in 45 mL of minimal medium supplemented with 50 mg L^{-1}

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DMF, 5 mM sodium succinate and 20 mg L^{-1} yeast extract. Further enrichment was carried out by transferring 10% inoculum to fresh medium during which the DMF concentration was gradually increased to 400 mg L^{-1} while reducing the sodium succinate concentration to zero. After many transfers, the culture thus obtained, was serially diluted and appropriate dilutions were spread on MM agar (20 g L^{-1}). The purity of the culture was checked periodically by plating the liquid culture on nutrient agar plates.

2.3. Growth on DMF

Bacterial growth kinetics were monitored at different DMF concentrations from 100 to 15 000 mg L^{-1}. Aliquots were removed at different time intervals. Biomass buildup was determined by measuring the absorbance at 555 nm.

DMF degradation and the ammonia release were monitored at a specific concentration of 400 mg L^{-1}. Samples withdrawn at different time intervals were centrifuged at 1100 × *g* and filtered through 0.45 μm filter membrane. DMF concentration was determined in the culture filtrate by HPLC, whereas ammonia was estimated colorimetrically. Total organic carbon (TOC) analysis was also carried out with the culture filtrates taken just after inoculation and at the end of the growth phase.

2.4. Determination of the kinetic constants

The kinetic constants for DMF degradation were determined by performing a number of batch runs in the concentration range of 100–5000 mg L^{-1}. Biomass increase was monitored periodically by measuring the optical density (OD) at 555 nm.

2.5. Utilization of other possible intermediates as growth substrates

To study the response of the strain DMF to utilize possible intermediates of DMF degradation pathway, dimethylamine, monomethylamine, formamide, and formate were individually used as the sole carbon source in minimal medium at an initial concentration of 400 mg L^{-1}. Flasks were inoculated with DMF grown cells and the growth response was monitored periodically.

2.6. Characterization and identification of isolated bacterial strain

Gram staining and other biochemical tests were carried out for the characterization of the strain as per standard procedures [8]. Identification of the isolated bacterial strain was done by the 16S rRNA gene sequence analysis. Genomic DNA extraction and purification as well as PCR-mediated amplification of the 16 rRNA gene were carried out as described by Bhattacharya et al. [9]. Sequencing of the 16 s rDNA gene was carried out using MicroSeq 16S rDNA Full Gene Identification Kit with ABI Prism 310 genetic analyzer (Applied Biosystems, Foster city, CA) as per the manufacturer's instructions. 1385 base pair rDNA sequence was used to search for the similar sequences using the BLAST program of NCBI [10] and a multiple sequence alignment was done using ClustalX program [11]. Phylogenetic analysis was done using the neighbour-joining method [12]. Sequence has been deposited in the GenBank with accession number DQ851168.

2.7. Detection of the plasmid

The presence of the plasmid was detected in the bacterial strain *Paracoccus* sp. DMF. The plasmid isolation was carried out by alkaline lysis method as described in Molecular cloning by Sambrook and Rusell [13].

2.8. Analytical procedures

Biomass growth was monitored by measuring the OD in a UV–visible spectrophotometer (Shimadzu, Japan, model 160A) at 555 nm against distilled water. Ammonia was estimated colorimetrically by Nesslerization [14]. Dimethylamine concentration in culture filtrate was determined colorimetrically using carbon-disulphide reagent [15]. DMF was quantified by HPLC. For HPLC analysis, culture filtrate, 100 μL, taken at different time intervals of growth phase, were injected to 4.6 mm × 250 mm column (Hypersil MOS2 SU C-8). A 50 mM sodium dihydrogen phosphate solution containing 0.5% acetonitrile was used as the solvent and flow rate of 1 mL min^{-1}. Analysis was carried out at 210 nm using UV–visible detector (Amersham Pharmacia biotech-900, UV-900, PH/L-900). Under these experimental conditions, the retention time of DMF was 7.1 min. Known standards of DMF were used to obtain a calibration curve by the area under the curve before running the unknown samples.

3. Results

3.1. Enrichment, isolation and identification of the strain

A strain capable of utilizing DMF as its sole carbon and nitrogen was isolated from batch culture enrichments. Strain *Paracoccus* sp. DMF was aerobic, gram negative and coccoid or small rod shaped organism. Individual colonies were small to moderate (0.5–1 mm) in size. They were white, opaque and circular in nature with smooth ridges. The strain was tested positive for catalase, nitrate reduction, carbohydrate fermentation, and oxidase tests while indole and urease tests were negative. Sequencing of the 1385 nucleotides of PCR-amplified 16S rRNA gene of strain DMF was carried out. Sequence comparison showed that the strain belonged to the genus *Paracoccus* (Fig. 1) and was closely related to *Paracoccus* sp. R-24652 strain which is a denitrifying bacterium (99.78% similarity).

3.2. Growth of strain Paracoccus sp. DMF on DMF

Fig. 2 shows the growth curve for the culture of *Paracoccus* strain DMF along with substrate consumption at an initial DMF concentration of 400 mg L^{-1}. In inoculated controls, no growth was observed in the absence of DMF. After 15 h of incubation, DMF could not be detected in the culture filtrate. The mean doubling time in the exponential growth phase was calculated to be 3.7 h. Release of ammonia–nitrogen was equivalent to 93% of initial DMF concentration. Rest was probably incorporated into the cell, as DMF was also the sole nitrogen source. Initial TOC was 233 mg L^{-1} and was reduced to 12 mg L^{-1} after 18 h. This, along with near stoichiometric release of ammonia showed that the *Paracoccus* sp. strain DMF completely mineralized DMF.

Yield of biomass was calculated to be 0.296 mg/mg DMF degraded. Significant growth and ammonia release were observed only after the 70% DMF removal (Fig. 2). This suggested a transient accumulation of an intermediate. This lead to the detection and quantification of DMA. It was observed that DMA concentration increased with time up to 9 h. Maximum accumulation of DMA was equivalent to 62% of initial DMF concentration. Its utilization was observed beyond 12 h and was associated with the further biomass increase.

3.3. Growth on different DMF concentrations and determination of kinetic constants

Growth of *Paracoccus* sp. DMF was investigated at a range of DMF concentration and the results are presented in Fig. 3. There was a linear relationship between the absorbance at 555 nm (biomass) and

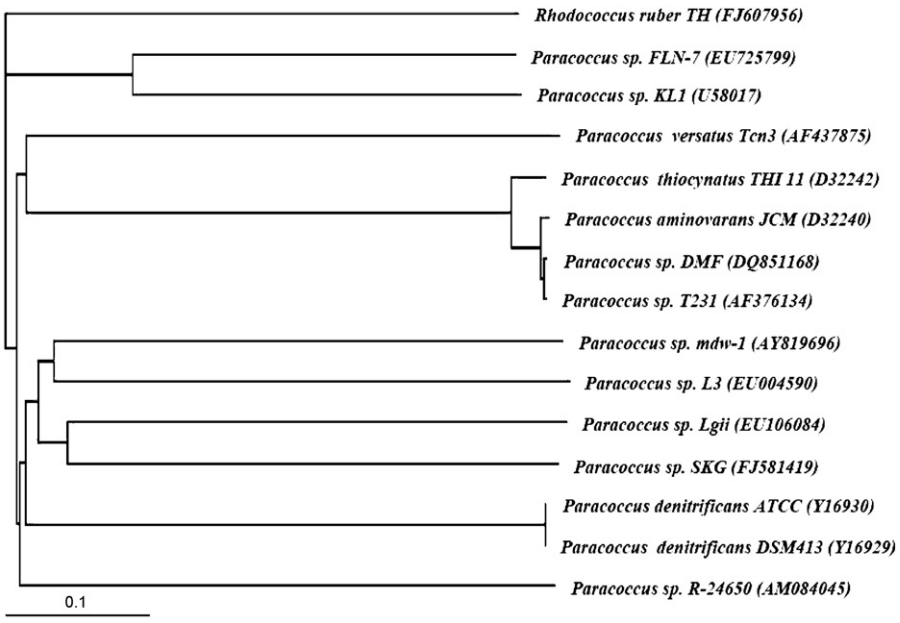

Fig. 1. Neighbour-joining tree showing phylogenetic position of *Paracoccus* sp. DMF. All the 16S rRNA gene sequences have been retrieved from NCBI database. The *Rhodococcus ruber* strain TH was taken as out-group and was also used to root the tree. GenBank accession numbers are shown in parentheses. 0.1 denotes the genetic distance.

the initial DMF concentration up to 400 mg L^{-1}. Growth decreased marginally beyond 1000 mg L^{-1}. The data on growth kinetics at different concentrations was used to determine biokinetic constants for its degradation, as described by Yadav et al. [16]. A semi-logarithmic plot of absorbance at 555 nm (biomass growth) against time was prepared and specific growth rate (μ) was determined from the slope of the linear portion (exponential growth phase). It was observed that highest μ value of 0.295 h^{-1} was observed at 1000 mg L^{-1} DMF and decreased beyond this substrate concentration (Fig. 4). Hence, Haldane's relationship (Eq. (1)) was used for the determination of maximum specific growth rate (μ_{max}), saturation constant (K_S) and inhibition constant (K_i):

$$\mu = \frac{\mu_{max}}{(1 + (K_s/S) + (S/K_i))} \quad (1)$$

The above equation can be rearranged as follows:

$$\frac{S}{\mu} = \left(\frac{1}{\mu_{max}K_i}\right)S^2 + \left(\frac{1}{\mu_{max}}\right)S + \frac{K_S}{\mu_{max}} \quad (2)$$

Defining $a = 1/\mu_{max}K_i$, $b = 1/\mu_{max}$ and $c = K_S/\mu_{max}$.

Eq. (2) can be written as

$$\frac{S}{\mu} = aS^2 + bS + c$$

Derived μ values at specific substrate concentrations were approximated by quadratic polynomial using Sigma plot 5.0 to determine the values of coefficient a, b and c. From these, μ_{max}, K_S and K_i were calculated to be 0.21 h^{-1}, 30 and 1188 mg L^{-1}, respectively.

The strain DMF showed growth even at 15 000 mg L^{-1} although the specific growth rate was significantly decreased (data not shown).

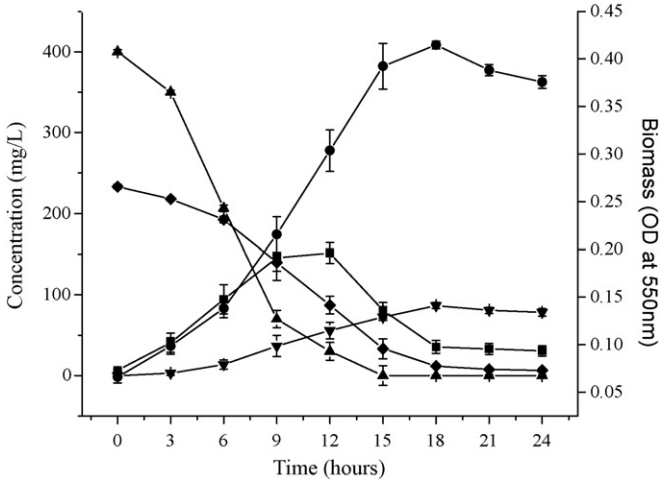

Fig. 2. Degradation of DMF along with the accumulation of DMA and ammonia, increase in the biomass and decrease in TOC with respect to time for 400 mg L^{-1} of initial DMF concentration. (-▲-) DMF; (-▼-) ammonia; (-■-) DMA; (-●-) biomass; (-♦) TOC.

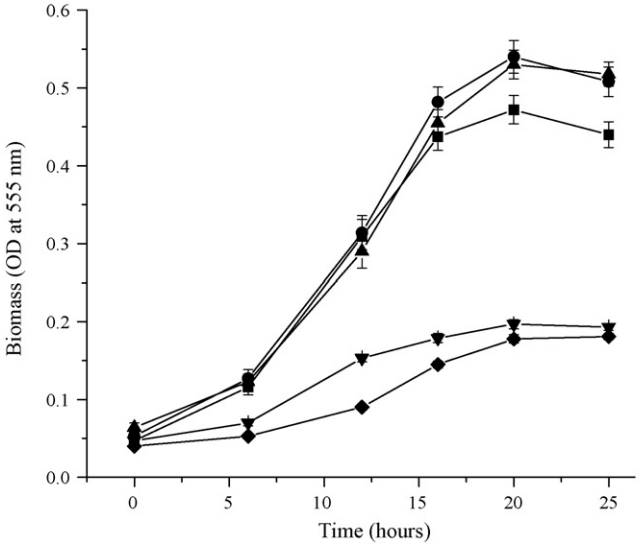

Fig. 3. Growth of *Paracoccus* sp. DMF on different initial concentrations of DMF. (-■-) 400 mg L^{-1}; (-●-) 2000 mg L^{-1}; (-▲-) 5000 mg L^{-1}; (-▼-) 10 000 mg L^{-1}; (-♦-) 15 000 mg L^{-1}.

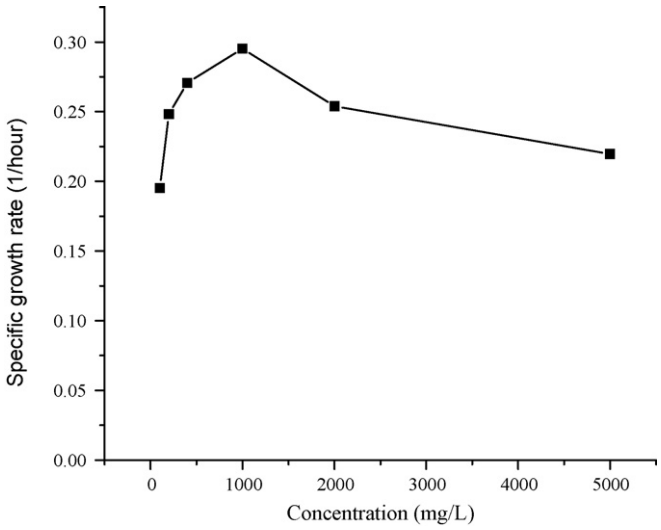

Fig. 4. Plot of specific growth rate v/s initial substrate concentration.

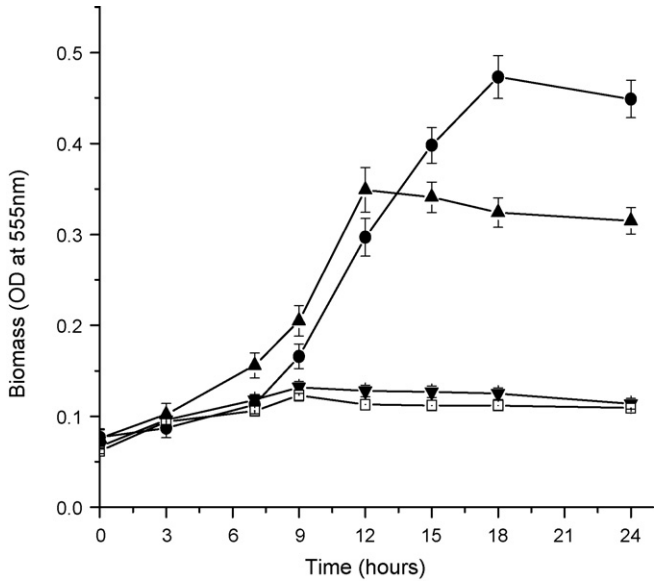

Fig. 5. Growth of *Paracoccus* sp. DMF on possible intermediates (DMA, MA, formamide and formate) as growth substrates. (-●-) DMA 400 mg L^{-1}; (-▲-) MA 400 mg L^{-1}; (-▼-) formamide 400 mg L^{-1}; (-□-) formate 200 mg L^{-1}.

3.4. Growth on other possible intermediates

Excellent growth was observed when strain DMF was grown on 400 mg L^{-1} concentration of DMA and MMA, respectively (Fig. 5). Before inoculation, the pH was adjusted to 7.0 using 0.5 N HCl as the addition of DMA and MMA resulted in an increase in pH. The strain showed faint growth in medium having formamide (400 mg L^{-1}) and formate (200 mg L^{-1}).

4. Discussion

DMF is extensively used as versatile solvent in various processes and thus released in large quantities in many industrial effluents. Due to its toxic effects on human health and slow rate of chemical degradation, attention is focused on the bacterial degradation of DMF as a method for treating these effluents. In this study, a new bacterial strain with a high potential for DMF degradation is reported.

Determination and comparison of the 16S rDNA sequence shows that this strain belongs to the *Paracoccus* genus and closely related to the thiocyanate utilizing *Paracoccus* sp. strain R-24650 with 99.78% similarity [17] and trimethylamine degrading *Paracoccus* sp. strain T231 [18]. Strains of *Paracoccus aminophilus* and *Paracoccus aminovorans* have been earlier reported to be utilizing DMF [4,5].

Growth of strain DMF on 400 mg L^{-1} of DMF was accompanied with near stoichiometric release of ammonia. More than 90% dissolved organic carbon removal after DMF consumption indicated complete mineralization. Observed specific growth rate of strain DMF was higher than those reported for DMF degrading bacterial consortium [19].

Two different pathways have been proposed for the bacterial degradation of DMF [3]. Few bacterial strains hydrolyze DMF to dimethylamine and formate, which is catalyzed by dimethylformamidase (pathway-1). DMA is further converted to MMA. These products are further metabolized to NH_3 and CO_2. The other pathway involved repeated oxidative demethylations to formamide, which is hydrolyzed by a formamidase to ammonia and formate. Thus formate is an intermediate in both pathways and ammonia is the end product in both pathways.

As per available literature, pathway-1 appears to be the preferred one. DMA accumulation at very high DMF concentrations has been reported by BromLey-Challenor et al. [19] and Veeranagouda et al. [7]. Further it was observed that this intermediate was not detected if the pH was in the range of 7.0–8.6.

In this study, transient accumulation of DMA was observed even at 400 mg L^{-1} of DMF when pH was near 7.8, and was completely degraded within 18 h. Further both DMA and MMA were used as growth substrates. These observations indicate that strain DMF utilizes pathway-1 for degrading DMF.

DMFase is the key enzyme in the degradation of DMF. There are reports on the isolation and purification of DMFase from *Pseudomonas* DMF 3/3 [20] and *Alcaligenes* KUFA-1 [6]. The gene for the enzyme was shown to be chromosomally encoded in *Alcaligenes* KUFA-1 and it was cloned and expressed in *E. coli*. DMFase activity was detected in the transformed *E. coli* cells [21]. In the present study, plasmid has been detected in *Paracoccus* sp. strain DMF.

It will be interesting to know whether the catabolic genes for DMF degradation is plasmid encoded. These studies are presently underway.

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