Biodegradation of melamine formaldehyde by *Micrococcus* sp. strain MF-1 isolated from aminoplastic wastewater effluent

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Abstract

Novel bacterial strain MF-1, able to grow using melamine formaldehyde (MF) resin as main carbon and nitrogen source, is described and characterized. Strain MF-1 was isolated from wastewater effluent of an aminoplastic industrial plant. Growth rate kinetics of the strain on MF showed a maximum specific growth rate \( \mu_{\text{max}} \) of 0.83 cells ml\(^{-1}\) h\(^{-1}\) and \( K_s \) of 7.18 cells ml\(^{-1}\). Mineralization of MF by strain MF-1 was confirmed by the decrease in dissolved oxygen (DO), release of ammonia, and detection of intermediate metabolites during biodegradation. Melamine, cyanuric acid, and biuret were detected as intermediate metabolites in the culture filtrate, suggesting that biodegradation of MF by strain MF-1 proceeds via successive deamination reactions of melamine to cyanuric acid, which is hydrolyzed to biuret and finally to NH\(_3\) and CO\(_2\). Based on 16S-rDNA sequence analysis, strain MF-1 had a similarity of 97% to *Micrococcus* sp. MN 8.1d. However, the high bootstrap value obtained in the phylogenetic analyses suggests that this is a novel strain.

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1. Introduction

Melamine formaldehyde (MF) is a member of the amino resin family. Originally developed in the 1930s and prized for its toughness, chemical resistance, and relative ease of manufacture, MF is incorporated into a wide variety of products that are still in use today. Familiar products include Formica\textsuperscript{®} and melamine dinnerware. Commercial applications have included fabric impregnation, adhesives, paints, electrical mouldings, glass-reinforced substrates and engineered wood products. These condensed amino-plastic products are generally stable with low emitted formaldehyde ratio (Deppe, 1982).

MF belongs to the triazine family. Other members of this family have been used extensively as nitrogen fertilizers (Allan et al., 1989) and their accumulation and persistence in the environment is well known (Widmer and Spalding, 1995). Triazine residues have been detected in many soils and sediments (Erickson and Lee, 1989; Goodrich et al., 1991; Muller et al., 1997). MF is introduced to the environment from many industrial effluents. Both melamine and formaldehyde are known human health threats and MF releases monomers of both (Ishiwata et al., 1986; Bradley et al., 2005). Thus the biodegradation of MF in industrial effluents is important.

Bacteria and fungi known to dealkylate and dechlorinate \( s \)-triazine herbicides such as atrazine have been isolated (Kaufman and Blake, 1970; Donnelly et al., 1993; Mirgain et al., 1993; Nagy et al., 1995; Bouquard et al., 1997). A number of bacteria, including *Pseudomonas, Acinetobacter, Agrobacterium, Pseudaminobacter* and *Rhodococcus*, are able to mineralize atrazine to CO\(_2\) and NH\(_4\) in contaminated soils (Mandelbaum et al., 1995; Struthers et al., 1998;
Edward et al., 2000 a,b). Arthrobacter aurescens TC1 is known to metabolize diverse s-triazines (Lisa et al., 2002). However, some bacteria only initiate triazine biodegradation without complete mineralization. Strains of Nocardioides sp. able to biotransform atrazine to hydroxylated products and finally to N-ethylammelide have been isolated (Edward et al., 2000b). Streptomyces strain PS1/5 was able to co-metabolize several s-triazines (Shelton et al., 1997). The metabolic pathway for explosive triazine-derivatives by Phanerochaete chrysosporium has been elucidated (Diane et al., 2004).

Metabolism of melamine is based mainly on enzyme-catalyzed hydrolytic reactions (Cook, 1987; Eaton and Karns, 1991 a,b; Wackett et al., 2002). Melamine, as an s-triazine, was metabolized mainly by soil pseudomonads. Biodegradation of melamine by most pseudomonads proceeds via a stepwise deamination reaction producing the intermediates, ammeline, ammelide and cyanuric acid (Cook and Hutter, 1981). Klebsiella terragena strain DRS-1 was able to deaminate melamine successively in a similar way to other pseudomonads (Shelton et al., 1997). In general, cyanuric acid is produced, a compound metabolized by several soil bacteria.

While many bacteria and fungi known to degrade different s-triazines have been isolated and characterized, no reports have been found regarding biodegradation of MF resin. In this study, we have isolated and characterized a new MF-1-degrading bacterium from wastewater effluent of an aminoplastic industrial plant.

2. Materials and methods

2.1. Chemicals

MF resin was provided by the aminoplastic processing company Ceria Misr Co. Ltd. (Cairo, Egypt). Melamine, cyanuric acid and biuret authentic standards were purchased from Wako chemicals.

2.2. Enrichment and isolation of MF-degrading bacteria

Wastewater effluent from the Ceria Misr Co. aminoplastic plant was used as a source of inoculum for isolation of MF-degrading bacteria. Samples were filtered to remove large particles and 50 ml of effluent was used to inoculate 150 ml sterile basal mineral medium (Farrell and Quilty, 1999) that had been modified by removing (NH4)2SO4 and supplementing with 100 mg l−1 MF resin as an main carbon and nitrogen source. Liquid medium was sterilized by autoclaving at 121 °C for use or lyophilized for long term preservation. MF was prepared, inoculated, and incubated at 30 °C in a shaking incubator at 150 rpm. Samples were taken periodically and cell numbers determined using a counting chamber. Growth kinetics and MF biodegradation were determined using different initial MF concentrations (10−100 mg l−1) in the basal mineral medium. Cultures were incubated at 30 °C and growth rate was monitored at different time intervals. It was fitted to the Monod model. The Lineweaver–Burk double reciprocal plot was used for plotting kinetic data to estimate \( K_s \) (half-saturation constant) and \( \mu_{max} \) (maximum growth rate).

Dissolved oxygen (DO) during the course of biodegradation was determined with resting cells under non-growth conditions. Cells were grown in minimal medium with 100 mg l−1 MF as sole source of carbon and nitrogen. Cells were harvested by centrifugation, washed twice with distilled water, and resuspended in basal mineral medium at OD_{600~3} MF was added at a final concentration of 100 mg l−1. After equilibration, the system was monitored for reduction in DO using a meter (Lutron DO-5509, USA).

2.4. Characterization of intermediate metabolites

Intermediate metabolites of MF biodegradation by resting cells were determined. A heavy inoculum of cells (OD_{600} = 3) was made in basal mineral medium with MF as previously described. Cells were aerated by continuous air flushing at 30 °C. MF was added at a final concentration of 100 mg l−1. One milliliter samples were taken at time intervals and analyzed by HPLC (Elite-LaChrom, Hitachi, Japan) equipped with a photodiode array. Samples were centrifuged at 14,000 rpm for 5 min and supernatants were filtered through 0.2 μm filter. Filtrates were mixed with an equal volume of methanol and applied to a C18-reverse phase Hiber-Purospher RP-18.5 μm HPLC (150 by 4.6 mm) column (Cica-Merck). Column oven temperature was 40 °C and the mobile phase was 0.1 M phosphate buffer, pH 7, at a flow rate of 1 ml min−1. The melamine (1,3,5-triazine-2,4,6-triamine) standard (99% purity) was detected at 220 nm after 3.2 min, the cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine) standard (98% purity) at 220 nm after 2 min and the biuret (imidocarbonic diamide) standard (97% purity) at 200 nm after 4 min. Concentrations of intermediate metabolites were determined by comparing peak areas with those of the authentic standards analyzed by the same protocol. Ammonia was detected testing. The purified bacterial strain was kept on agar slants at 4 °C for use or lyophilized for long term preservation.
2.5. TOF-mass spectrometric analyses

Metabolites were further identified by time-of-flight (TOF)-MS spectrometric analyses. Metabolites were isolated and purified from the filtrates by HPLC as previously described except that the solvent used was an acetonitrile gradient in water (de Souza et al., 1996). The program was set as follows: 3 min of 3% acetonitrile, 5 min linear gradient to 50% acetonitrile, 5 min linear gradient to 100% acetonitrile, 3 min linear gradient to 3% acetonitrile, and 3% acetonitrile for 2 min. Fractions were taken, evaporated, and finally taken up in water:methanol:formic acid (1:1:0.002) for TOF-MS analyses. Samples were applied to the Applied Biosystem MS/MS spectrometer model (QSTAR-XL) running in positive electrospray ionization mode.

2.6. Isolation, cloning, sequencing and analysis of 16S rDNA

Extraction of genomic DNA from the bacterial isolate was performed according to Ausubel et al. (1999) and the 16S rDNA amplified as described previously (Arturo et al., 1995). A pair of forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-ACGGCTACCTTGTACGACT-3') primers was used for the PCR. Thermal cycling parameters were 95°C for 5 min, 30 s, 52°C for 30 s, 72°C for 30 s, 1 min, and 4°C forever. 16S rDNA-containing plasmid vectors were constructed using pGEM-T Easy vector (Promega, USA) and used to transform Escherichia coli JM109 cells according to the instruction manual. The nucleotide sequence analysis of the selected clones were determined by automated fluorescent dye terminator sequencing (Sanger et al., 1977) with a model ABI 310 genetic sequence analyzer (Applied Biosystems, CA, USA) according to the user manual.

2.7. Nucleotide sequence accession number

The 16S rDNA sequence of strain MF-1 has been deposited in DDBJ (DNA Data Bank of Japan) under accession number AB213661.

3. Results and discussion

3.1. Growth kinetics and biodegradation of MF

After inoculation of MF-enhanced agar plates with effluent, three colonies surrounded by clear zones resulting from MF degradation were identified. One bacterium, designated MF-1, was able to grow efficiently on MF as sole carbon and nitrogen source, and was selected for further investigation. MF-1 was able to couple MF degradation to growth, as shown by the increase in cell density in cultures containing only MF as a sole source of carbon and nitrogen. In the MF concentration range tested (10–100 mg l⁻¹), the growth kinetics were well described by the Monod model (Fig. 1A). Increasing initial MF concentration in the culture medium resulted in a concomitant increase in specific growth rate. When MF concentration reached the point of saturation, growth rate was steady. Fig. 1B represents the Lineweaver–Burk double reciprocal plot. A maximum growth rate (μmax) of 0.83 cells ml⁻¹ h⁻¹ and Ks of 7.18 cells ml⁻¹ were determined.

Fig. 1. Cell growth kinetics of Micrococcus sp. MF-1 coupled to MF biodegradation. (A) Kinetics are well described by the Monod model (μ = μmax.S/(S+Ks)). (B) The Lineweaver–Burk double reciprocal plot (1/μ = (Ks/μmax).1/(S)+1/μmax).
3.2. MF biodegradation pathway

Additional evidence for MF degradation by strain MF-1 was provided by the detection of intermediate metabolites obtained during the biodegradation process. Metabolites were detected and quantified by HPLC, and further identified by TOF-Mass analyses. The HPLC elution profiles for intermediates were consistent with authentic standard of melamine, cyanuric acid and biuret. Fig. 2 shows the HPLC profile for the time-dependent production of melamine and cyanuric acid as biodegradation products of MF by strain MF-1. After purification of intermediate metabolites, they were further identified by TOF-MS spectrometry. TOF-MS spectra of metabolite (A) showed a molecular weight of 127 (M + 1), corresponding to the empirical formula (C₃H₆N₆) of melamine. Metabolite (B) gave a molecular weight of 140 (M + 1), which corresponds to the empirical formula (C₃H₃N₃O₃) of cyanuric acid. Metabolite (C) was identified as biuret with the empirical formula (C₂H₅N₃O₂) and molecular weight of 104 (M + 1). Fig. 3 shows the TOF-MS spectra of intermediate metabolites obtained during the biodegradation of MF by strain MF1. Based on the metabolites detected, it was concluded that MF-1 was able to split melamine moieties and remove formaldehyde prior to melamine degradation. Further metabolism of melamine was found to be similar to general triazine catabolism (Cook, 1987; Eaton and Karns, 1991 a,b; Wackett et al., 2002). The proposed biodegradation pathway of MF by strain MF-1 is given in Fig. 4. Successive deamination of melamine results in the formation of cyanuric acid. Decarboxylation of cyanuric acid produces biuret, the hydrolysis of which produces urea and finally ammonia. Release of ammonia during the biodegradation course was consistent with melamine degradation

![HPLC elution profile for the intermediates produced during biodegradation of MF by strain MF-1. The time-dependent production of melamine, as well as its biotransformation to cyanuric acid, is shown. The increase in absorption spectra after 5 min (a) and 10 min (b) incubation correspond to the production of melamine. After 15 min (c) and 20 min (d), melamine concentration is decreased and after 25 min (e), cyanuric acid is produced, reaching a maximum after 30 min (f) incubation.](image_url)

![TOF-MS spectra of metabolites obtained during the biodegradation of MF by dense cell suspension. The feature at m/z 127 (M + 1) corresponds to melamine (A). The feature at m/z 130 (M + 1) corresponds to cyanuric acid (B). The feature at m/z 104 (M + 1) corresponds to biuret (C).](image_url)
The increase of ammonia is due to the deamination of melamine and further metabolites to produce finally cyanuric acid. When cyanuric acid was depleted from the medium, ammonia levels fell below the detection limit. This pattern was consistent with the mode of metabolism by *Klebsiella terragena* strain DRS-1 (Shelton et al., 1997).

In a resting cell experiment, biodegradation of MF was accompanied by a marked decrease in DO and concomitant release of ammonia. Fig. 5 shows the time course of biodegradation of MF by strain MF1. At the beginning of the biodegradation process, melamine is the major intermediate. As biodegradation of melamine proceeds, it undergoes successive deaminations, indicated by the release...
of ammonia, which finally lead to cyanuric acid, detected after complete elimination of melamine.

3.3. Phylogenetic analyses

16S rRNA gene sequence of strain MF-1 (1380 b) was determined and aligned with related bacteria and known triazine-degraders. Strain MF-1 was closely related to Micrococcus sp. MN 8.1d. However, the relatively low sequence similarity (97%) and high bootstrap value support the novelty of strain MF-1. It has been demonstrated recently that strains with 16S rRNA evolutionary distances of 2.5–3 have a low DNA–DNA homology and therefore would be considered as different species (Stackebrandt and Goebel, 1994). Based on such a concept it is probable that strain MF-1 represents a new strain with a close relationship to strain MN 8.1d. Fig. 6 shows the phylogenetic relationships of strain MF-1 with those of the Micrococcus group and other triazine-degrading bacteria. Micrococcus sp. MF-1 is a Gram-positive bacterium, phylogenetically different from triazine-degrading Gram negative bacteria such as pseudomonads (Edward et al., 2000a). Although Micrococcus is known for its ability to biodegrade a variety of aromatic compounds (Wright et al., 1993), degradation of MF has not yet been reported. Such diversity in triazine-ring degrading bacteria may have a great relevance in nature. These bacteria may acquire and exchange genetic information from each other in the community by horizontal gene transfer (HGT) (Top and Springael, 2003). It is well established that HGT plays an important role in bacterial adaptation to xenobiotics and therefore to the evolutionary trend of these bacteria in nature.

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References


