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Isolation of the ε-caprolactam denitrifying bacteria from a wastewater treatment system manufactured with acrylonitrile–butadiene–styrene resin

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Abstract

ε-Caprolactam has high COD and toxicity, so its discharge to natural water and soil systems may lead to an adverse environmental effect on water quality, endangering public health and welfare. This investigation attempts to isolate ε-caprolactam denitrifying bacteria from a wastewater treatment system manufactured with acrylonitrile–butadiene–styrene (ABS) resin. The goal is to elucidate the effectiveness of isolated pure strain and ABS mixed strains in treating ε-caprolactam from synthetic wastewater. The results reveal that *Paracoccus versutus* MDC-3 was isolated from the wastewater treatment system manufactured with ABS resin. The ABS mixed strains and *P. versutus* MDC-3 can consume up to 1539 mg/l ε-caprolactam to denitrify from synthetic wastewater. Complete ε-caprolactam removal depended on the supply of sufficient electron acceptors (nitrate). Strain *P. versutus* MDC-3, *Hyphomicrobium* sp. HM, *Methylosinus pucelana* and *Magnetospirillum* sp. CC-26 are related closely, according to the phylogenetic analyses of 16S rDNA sequences.

Keywords: Biodegradation; ε-Caprolactam; Denitrification; *Paracoccus versutus*

1. Introduction

Global ε-caprolactam production amounted to 3.7 million tonnes in 2002, with more than 40% (1.6 million tonnes) produced in Europe and the Middle East [1]. Asia consumed 1.8 million tonnes of ε-caprolactam, yet produced less than 1.2 million tonnes, with most of the deficit being supplied from Europe [1]. In Taiwan, 236,000 t of ε-caprolactam is produced annually. ε-Caprolactam is adopted almost exclusively to produce nylon-6. ε-Caprolactam has high COD and toxicity so its discharge to natural water and soil systems may have an adverse impact on water quality, endangering public health and welfare.

Achromobacter cycloclastes W-2 and *Corynebacterium aurantiacum* B-2 (rough) could split ε-caprolactam, δ-valerolactam and γ-butyrolactam, and produced the ω-amino acids corresponding to them [2]. *Pseudomonas aeruginosa* MCM B-407 and *Pseudomonas putida* MCM B-408 could utilize ε-caprolactam as the sole source of carbon, nitrogen and energy [3,4]. Various yeasts (*Yarrowia lipolytica* DS-1, *Y. lipolytica* GSFC-5001, *Candida tropicalis* and strain Y-43) were used in the biodegradation of ε-caprolactam waste liquor from ε-caprolactam manufacture containing many mono- and di-carboxylic acid [5]. However, the availability of ε-caprolactam for denitrification had not been investigated. The aim of this investigation is to isolate ε-caprolactam denitrifying bacteria from the acrylonitrile–butadiene–styrene (ABS) resin-manufactured wastewater treatment system. The aim is also to understand the effectiveness of isolated pure strain and ABS mixed strains in treating ε-caprolactam from synthetic wastewater.

2. Materials and methods

2.1. Enrichment of ABS mixed strains

The ε-caprolactam denitrifying strains were enriched in the inorganic salt medium which comprised three solutions [6].

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The composition of solution A (pH 7.5) was (in grams per liter) K_2HPO_4, 0.66; KH_2PO_4, 0.54; KNO_3, 5; $(NH_4)_2SO_4$, 1. Solution B was 20 g of $MgSO_4{\cdot}7H_2O$ per 1 l distilled water. Solution C consisted of 2 g of $CaCl_2{\cdot}2H_2O$, 1 g of $FeSO_4{\cdot}7H_2O$, 0.5 g of $MnSO_4{\cdot}H_2O$, 0.1 g of $Na_2MoO_4{\cdot}2H_2O$ and 0.1 g of $CuSO_4{\cdot}5H_2O$ per 1 l of 0.1N HCl. These solutions were sterilized separately. Under aseptic conditions, 10 ml of solution B and 10 ml of solution C were added to 980 ml of solution A to yield 1 l of inorganic salt medium.

The sludge for strain enrichment was obtained from the denitrification tank of a wastewater treatment system manufactured using ABS resin. Each of four glass bottles (volume: 50 ml) contained 3 ml of the sludge, 30 ml inorganic salt medium and various ε-caprolactam concentrations (125, 251, 502 and 1003 mg/l, respectively) at the beginning of microbial enrichment. After the aforementioned steps were completed, each glass bottle was filled with inorganic salt medium and sealed with a glass stopper, before being placed in a cultivation room at 30 °C for 7 days. When the medium became turbid and generated gas bubbles, 3 ml of liquid was taken out and put in another, new sterile glass bottle. Then, the aforementioned steps continue were repeated to complete microbial enrichment.

2.2. Isolation of pure strains from ABS mixed strains

After the aforementioned steps were performed three times, 5 ml of liquid was removed from each glass bottle. The liquids that had already been removed were mixed in a tube and appropriate dilutions were spread-plated on yeast extract agar medium (YA) to yield individual colonies. (The plates were incubated at 30 °C for 24 h.) The loopfuls of the single colony from each plate were streaked onto other fresh YA plates to check for purity. The composition of YA was (in grams per liter) yeast extract, 3; peptone, 5; glycerol, 10; KNO_3, 9.8; agar, 16.5.

2.3. Test of ε-caprolactam degradation potential

Test tubes that contained 10 ml phosphate-buffered medium (PBM) [7,8] and pure strain were used. The pure strain was initially at a cell concentration that yielded 0.1 OD_{600} units. After various ε-caprolactam concentrations were added, the tubes were shaken at 120 rpm in the dark at 30 °C, and the OD_{600} value measured growth. The PBM composition was (in grams per liter): $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.02; NH_4Cl, 0.1; K_2HPO_4, 1.0; KH_2PO_4, 1.0; trace element solution, 10 ml/l. The trace element solution in the PBM comprised (in milligrams per liter) $FeSO_4{\cdot}7H_2O$, 300; $MgCl_2{\cdot}4H_2O$, 180; $CoCl_2{\cdot}6H_2O$, 106; $Na_2MoO_4{\cdot}2H_2O$, 34; $ZnSO_4{\cdot}7H_2O$, 40. The pH of PBM was adjusted to 7.5.

2.4. Identification of ε-caprolactam denitrifying bacteria

The DNA of ε-caprolactam denitrifying strains was extracted from 1.5 ml of enriched pure culture after pelleting by centrifugation and the resuspension of the pellet in 150 μl of sterile water. Ten microliters of lysozyme (5 mg/ml) was added to break down the cell walls; then 40 μl of SDS (10%) was added to the tube and incubated at 65 °C for 30 min. After 30 min, 20 μl of protease K (10 mg/ml) was added to and incubated at 37 °C for 4 h; then 400 μl of STE buffer was added to the tube. The composition of the STE buffer was NaCl, 0.1 M; Tris–HCl, 10;mM (pH 8.0), and EDTA, 1 mM. To extract total genomic DNA, equal volumes of phenol and chloroform were added to the tube which was centrifuged at 14,100 × *g* for 5 min; then the supernatant was added to the new microtube. The extraction procedure was performed three times until the amount of impurities was minimized. After extraction, 50 μl of sodium acetate (3 M, pH 5.2) and 1 ml of 95% ethanol were added to supernatant to precipitate for 10 min, and then the tip was applied to roll up the bacterial genomic DNA from the tube. Finally, the bacterial genomic DNA on the tip was transferred into 70 μl of H_2O and used in a PCR reaction.

The 16S ribosomal genes were amplified from the bacterial total genomic DNA in using a polymerase chain reaction (PCR). Purified extracted DNA (5 μl) was the template in a PCR reaction mixture with the modified amplification primer 27f (5′-GAGTTTGATC(AC)TGGCTCAG-3′) and 1492r (5′-TACGG(CT)TACCTTGTTACGACTT-3′) [9]. Each 200 μl microtube contained 5 μl purified extracted DNA, 2 μl of dNTP at 2.5 mM, 2.5 μl of 10× *Taq* DNA polymerase buffer, 0.2 μl of 2.5 unit *Taq* DNA polymerase, 11.3 μl of sterile water and 2 μl of the modified amplification primers (total amount: 10 pmol). The PCR was performed in a GeneAmp PCR system 2400 thermal cycler with a hot start at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

The 16S rDNA was purified using Viogene's Gel Extraction System (gel extraction miniprep kit) and sequencing was conducted at National Chung Hsing University Biotechnology Center (Taichung, Taiwan). The 16S rDNA sequence was compared to the GenBank database using the NCBI Blast program.

2.5. Denitrification using ε-caprolactam by mixed and pure strain

The experiments were performed using a series of 120 ml batch reactors. Each reactor contained 40 ml of PBM with mixed or pure strain. The initial amount of mixed and pure strain began at a cell concentration of 0.1 OD_{600} units. After the cultures were gassed using argon gas for 3 min (flow: 5 ml/s) and sealed with Teflon/silicon stoppers, and various ε-caprolactam concentrations (784–1631 mg/l) were added. The reactors were shaken at 120 rpm in the dark at 30 °C to observe ε-caprolactam removal under anoxic conditions. The mixed bacteria culture was taken from the denitrification tank of a wastewater treatment system manufactured using ABS resin.

2.6. Analytical methods

Samples were extracted directly from the reactors using a syringe. After filtration through the membrane, NH_4^+ was measured by the indophenol blue approach [10]. The ε-caprolactam concentration was measured using HPLC and a UV–vis detector. HPLC was performed using a Hitachi system equipped with

a Merck Lichrospher 100 PR-18-endcapped (5 μm) column at a flow rate of 0.6 ml/min. The acetonitrile:water ration of the eluent was 3:7. The UV detector absorbency wavelength was fixed at 210 nm.

The concentrations of NO_3^- and NO_2^- were measured using HPLC and a conductivity detector in a Hitachi system equipped with a Hamilton PRP-X100 (10 μm) column at a flow rate of 2.0 ml/min. The eluent comprised 4 mM *p*-hydroxybenzoic acid and 2.5% methanol. The pH of eluent was adjusted to 8.5. A gas sample for N_2 analysis was injected into GC equipped with a thermal conductivity detector (TCD). GC was performed using an HP 6890 system equipped with a 5 Å molecular sieve column. The temperatures of the oven, the injector and the detector were 80, 150 and 200 °C, respectively. Gaseous argon was supplied as the carrier gas at a flow rate of 20 ml/min.

The pH, DO and OD_{600} were measured using a pH meter, a DO meter and a spectrophotometer at 600 nm, respectively.

2.7. Close relationship between ε-caprolactam denitrifying bacteria and other substrate denitrifying bacteria

The NCBI-research system or the SDSC Biology Workbench–ndjinn multiple database search system was used to identify the 16S rDNA gene sequence of other substrate (not ε-caprolactam) denitrifying bacteria. The 16S rDNA gene sequences of denitrifying bacteria were aligned using the SDSC Biology Workbench–CLUSTALW (multiple sequence alignment) program. The aligned 16S rDNA sequences were analyzed using the SDSC Biology Workbench–CLUSTALTREE (phylogenetic analysis with clustal w) program to produce the phylogenetic trees. The intimate relationship between the ε-caprolactam denitrifying bacteria and other substrate denitrifying bacteria was elucidated by performing phylogenetic analyses of 16S rDNA sequences.

3. Results and discussion

3.1. Denitrification with ε-caprolactam by ABS mixed strains

Two ε-caprolactam concentrations were used to understand the denitrification performance of ABS mixed strains. Fig. 1 shows the results for the denitrification of the ABS mixed strains with 880 mg/l of ε-caprolactam and 1201 mg/l of NO_3^-. At 23 and 34 h, 182 and 496 mg/l of ε-caprolactam was removed by the ABS mixed strains; the residual concentration was 214 mg/l at 44 h. The amount of NO_3^- decreased as the reaction proceeded. At 23 and 34 h, 369 and 1085 mg/l of NO_3^- were removed, respectively. It was completely removed at 44 h. The amount of NO_2^- increased and declined as the reaction proceeded and NO_3^- was removed. The OD_{600} value increased with ε-caprolactam removal and reached a maximum value of 0.964 at 45 h. The pH increased to 9.03 at 45 h with ε-caprolactam and NO_3^- removal. The DO was maintained at an extremely low value to facilitate denitrification. The amount of N_2 increased to 190 μmol at 45 h because of NO_3^- and NO_2^- were removed. Since the amount of N_2 accumulated was less than the theoretical amount produced, other gas (NO or N_2O) may be present in the head space of the glass bottle.

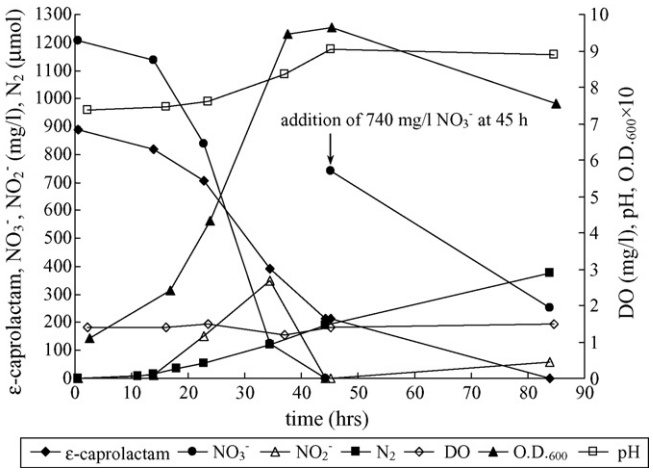

Fig. 1. Time course of the denitrification of the ABS mixed strains with 880 mg/l ε-caprolactam and 1201 mg/l nitrate.

From above study, the initial ε-caprolactam concentration was not completely removed by the ABS mixed strains. The possible reasons could be: (1) the intermediate product (may be ε-aminocaproic acid [11]) of ε-caprolactam inhibited the activity of ABS mixed strains; or (2) the amount of electron acceptor (NO_3^-) was insufficient. In order to distinguish this question, 740 mg/l of NO_3^- was added to the reactor bottle at 45 h to ensure that enough NO_3^- for ε-caprolactam removal was present. After the NO_3^- was added, both NO or N_2O were continuously removed as the reaction proceeded. All ε-caprolactam was removed after 84 h and the NO_3^- concentration remained at 252 mg/l. At 85 h, the OD_{600} value, pH, NO_2^- and N_2 were 0.756, 8.91, 57.6 mg/l and 374.8 μmol, respectively.

In order to understand that ABS mixed strains could utilize higher ε-caprolactam concentration for denitrification, 1631 mg/l of ε-caprolactam and 1177 mg/l of NO_3^- were used to investigate the denitrification of the ABS mixed strains. The ABS mixed strains removed 138 and 603 mg/l of ε-caprolactam after 23 and 35 h, respectively. The residual concentration of ε-caprolactam was 959 mg/l at 45 h. The ABS mixed strains removed 311 and 1175 mg/l of NO_3^- at 23 and 35 h. The residual NO_3^- concentration and the amount of NO_2^- accumulated were 0 and 25 mg/l at 45 h, respectively. Variations of all other monitoring parameters were similar to those presented above and the OD_{600} value, pH and N_2 were 1.017, 9.06 and 195 μmol at 45 h, respectively. ε-Caprolactam concentration was not completely removed by the ABS mixed strains at the end of experiment for a reason similar to that stated above.

From the previous studies, ABS mixed strains could utilize ε-caprolactam as a substrate for denitrification, when the initial ε-caprolactam concentration was less than 1631 mg/l. The complete removal of ε-caprolactam depends on the supply of sufficient electron acceptors (NO_3^-). Table 1 presents the specific growth rate and the ε-caprolactam utilization rate of the ABS mixed strains and *Paracoccus versutus* MDC-3 with various initial ε-caprolactam concentrations. Either the

Table 1
Specific growth rate and ε-caprolactam utilization rate of the ABS mixed strains and *Paracoccus versutus* MDC-3 with different initial ε-caprolactam concentration

Strain	ABS mixed strains		*P. versutus* MDC-3	
ε-Caprolactam (mg/l)	880	1631	784	1539
ε-Caprolactam removal rate (mg/[l h])	20.7	20.9	11.8	13.0
Specific growth rate (h^{-1})	0.065	0.077	0.041	0.034
ε-Caprolactam/nitrate (mg/[l mg l])	0.56	0.59	0.43	0.47

ε-caprolactam removal rate or the specific growth rate of the ABS mixed strains was no great difference varied significantly with the initial ε-caprolactam concentration. The ε-caprolactam/nitrate ratio was 0.56–0.59 for the ABS mixed strains, when the ε-caprolactam concentration was under 1631 mg/l.

3.2. Isolation and identification of ε-caprolactam denitrifying bacteria

Three strains were isolated from the YA plate and one utilized 1014 mg/l of ε-caprolactam as a substrate for growth. This was strain MDC-3. The strain was a Gram-negative rod and the oxidase test yielded positive results. Strain MDC-3 was identified as *P. versutus* by approaches based on 16S rDNA gene sequence.

3.3. Denitrification with ε-caprolactam by P. versutus MDC-3

Two ε-caprolactam concentrations were used to understand the denitrification performance of *P. versutus* MDC-3. Fig. 2 presents the results of the denitrification of *P. versutus* MDC-3 with 784 mg/l of ε-caprolactam and 1806 mg/l of NO_3^-. At 67 h, *P. versutus* MDC-3 had removed 651 mg/l of ε-caprolactam; all had been removed at 88 h. The nitrate concentration also declined as the reaction proceeded and the amounts removed were 61, 248 and 1169 mg/l at 37, 47 and 67 h, respectively. All had been removed at 114 h. *P. versutus* MDC-3 still removed

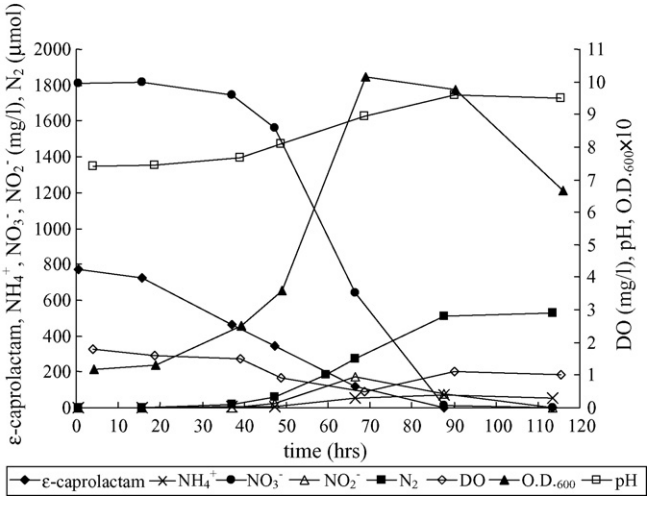

Fig. 2. Time course of the denitrification of *Paracoccus versutus* MDC-3 with 784 mg/l ε-caprolactam and 1806 mg/l nitrate.

both NO_3^- and NO_2^- after the depletion of ε-caprolactam, perhaps because *P. versutus* MDC-3 utilized the intermediate product (may be ε-aminocaproic acid [11]) of ε-caprolactam metabolism for denitrification. The OD_{600} value increased with the ε-caprolactam removal to a maximum value of 1.016 at 69 h. It then decreased as the reaction proceeded further because *P. versutus* MDC-3 was in the endogenous respiration phase. The pH value increased with the denitrification and rose to 9.5 by the end of the study. The NH_4^+ increased with the removal of ε-caprolactam to 55 mg/l at 114 h. If 1 mol of ε-caprolactam [$CH_2(CH_2)_2NHCO$] generates 1 mol of NH_4^+, then the increase in the amount of NH_4^+ should be 125 mg/l after 784 mg/l of ε-caprolactam has been removed. The increase in NH_4^+ concentration was less than the theoretical value perhaps for the following reasons. (1) Some ε-caprolactam was adopted for biomass yield. (2) The ε-caprolactam was not completely mineralized to CO_2 and some may have been transformed to ε-aminocaproic acid. N_2 (525.8 μmol) was accumulated by the end of the experiment.

In order to understand that *P. versutus* MDC-3 could utilize higher ε-caprolactam concentration for denitrification, 1539 mg/l of ε-caprolactam and 1771 mg/l of NO_3^- were used to study the denitrification of *P. versutus* MDC-3. *P. versutus* MDC-3 removed 121 mg/l of ε-caprolactam after 48 h; the concentration of ε-caprolactam at 88 h was 777 mg/l. *P. versutus* MDC-3 removed 80, 191 and 601 mg/l of NO_3^- at 38, 48 and 67 h, respectively. All was completely removed after 88 h. The amount of NO_2^- accumulated at 88 h was 177 mg/l. Variations of all other monitoring parameters were similar to those presented above and the OD_{600} value, pH, NH_4^+ and N_2 were 1.129, 9.31, 76 mg/l and 451 μmol, respectively, at the end of experiment. ε-Caprolactam concentration was not completely removed by *P. versutus* MDC-3 at the end of experiment for a reason similar to that stated above.

From above study, *P. versutus* MDC-3 used ε-caprolactam as a substrate for denitrification, when the initial ε-caprolactam concentration was below 1539 mg/l. From Table 1 results, neither the ε-caprolactam removal rate nor the specific growth rate of *P. versutus* MDC-3 was any great difference varied significantly with the initial amount of ε-caprolactam. The ratio of ε-caprolactam/nitrate was 0.43–0.47 for *P. versutus* MDC-3, when the ε-caprolactam concentration was below 1539 mg/l.

From Figs. 1 and 2, Table 1 and the above studies, it showed that ε-caprolactam removal efficiency and strain specific growth rate of the ABS mixed strains was better than *P. versutus* MDC-3. *Paracoccus thiophilus* TDC-2 was isolated from the wastewater treatment system manufactured with polyacrylonitrile fiber and it could utilize ε-caprolactam for growth [12].

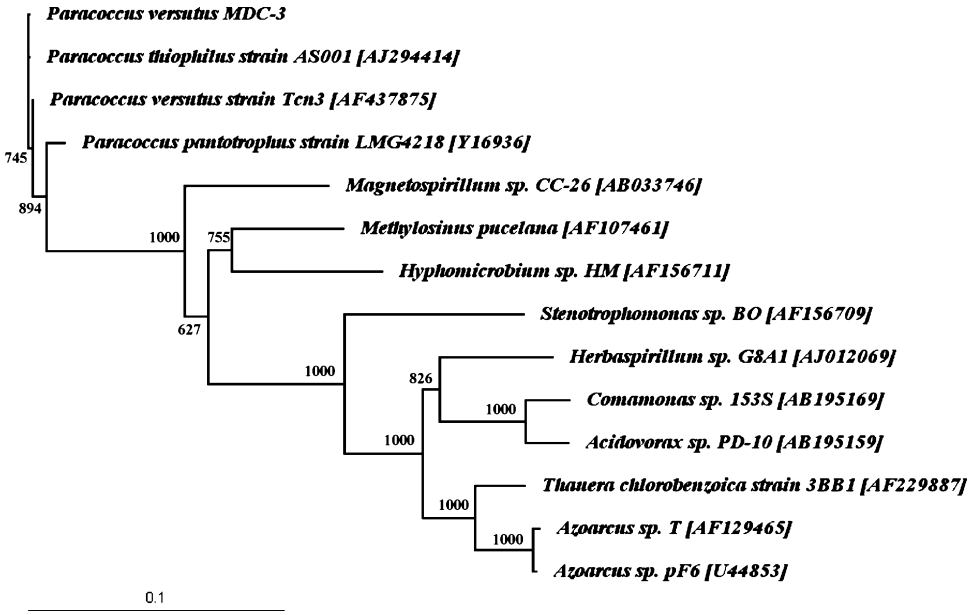

Fig. 3. Phylogenetic analyses of 16S rDNA sequences of the denitrifying bacteria.

ε-Caprolactam removal rate of *Paracoccus thiophilus* TDC-2 were 8.8 and 5.0 mg/(l h) for ε-caprolactam concentration of 784 and 1446 mg/l, respectively. Strain specific growth rate of *Paracoccus thiophilus* TDC-2 were 0.022 and 0.012 h^{-1} for ε-caprolactam concentration of 784 and 1446 mg/l, respectively. Comparing *P. versutus* MDC-3 with *Paracoccus thiophilus* TDC-2, it could be found that the ε-caprolactam removal ability was better for *P. versutus* MDC-3 than for *Paracoccus thiophilus* TDC-2.

P. aeruginosa MCM B-407 was isolated from activated sludge used to treat waste from a factory producing nylon-6 [3]. This organism was able to remove 497 mg/l of ε-caprolactam with simultaneous reaction in chemical oxygen demand (COD). *P. putida* MCM B-408 was isolated from cow dung by adaptation to wastewater of the nylon-6 industry [4]. *P. putida* MCM B-408 could use 1000 mg/l ε-caprolactam for growth. The research of Fukumura demonstrated that *A. cycloclastes* W-2 and *C. aurantiacum* B-2 (rough) could split 40 mM ε-caprolactam and produced 3.5 and 3.2 mM ε-aminocaproic acid, respectively. [2]. *Y. lipolytica* DS-1 was isolated from contaminated soil at a local oil refinery and it could decrease COD by as much as 57% at COD load of 73,600 ppm [5]. *P. versutus* MDC-3 could utilize 1539 mg/l ε-caprolactam for denitrification and the removal efficiency of ε-caprolactam by *P. versutus* MDC-3 exceeded that by *P. aeruginosa* MCM B-407 and *P. putida* MCM B-408. Hence, *P. versutus* MDC-3 is expected to play an important role in Taiwan for treating ε-caprolactam contaminated material.

3.4. Close relationship between the ε-caprolactam denitrifying bacteria and other substrate denitrifying bacteria

Fig. 3 shows phylogenetic analyses of 16S rDNA sequences of the denitrifying bacteria. It revealed that *P. versutus* MDC-3, *Hyphomicrobium* sp. HM (methanol denitrifying bacteria) [13], *Methylosinus pucelana* (methane denitrifying bacteria) [14] and *Magnetospirillum* sp. CC-26 (phenol denitrifying bacteria) [15] were strongly related to each other (with relative distances of 0.117–0.145), according to the phylogenetic analyses of 16S rDNA sequences. Aforementioned four strains were weakly related to other substrate denitrifying bacteria according to the phylogenetic analyses of 16S rDNA sequences. The main reason was that above-mentioned four stains all belong to alphaprotebacteria; other strains belong to betaproteobacteria or gammaproteobacteria. Whether the structure of substrate was another reason still need further confirmed in the further study.

4. Conclusions

The ABS mixed strains used ε-caprolactam as a substrate for denitrification, when the initial ε-caprolactam concentration was below 1631 mg/l.

P. versutus MDC-3 was isolated from the ABS resin-manufactured wastewater treatment system and utilized ε-caprolactam as a substrate for denitrification, when the initial ε-caprolactam concentration was below 1539 mg/l.

The use of ABS mixed strains or *P. versutus* MDC-3 for the complete removal of ε-caprolactam depended on the supply of enough electron acceptors (nitrate). The ε-caprolactam/nitrate ratio was 0.56–0.59 for the ABS mixed strains and 0.43–0.47 for *P. versutus* MDC-3.

P. versutus MDC-3, *Hyphomicrobium* sp. HM, *M. pucelana* and *Magnetospirillum* sp. CC-26 were closely related, according to the phylogenetic analyses of 16S rDNA sequences.

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