N-methyl-2-pyrrolidone-degrading bacteria from activated sludge

Karel Křížek, Jan Růžička, Markéta Julinová, Lucie Husárová, Josef Houser, Marie Dvořáčková and Petra Jančová

ABSTRACT

N-methyl-2-pyrrolidone (NMP) is a widely used solvent for many organic compounds and a component found in a vast array of chemical preparations. For this research paper, NMP degrading bacteria were isolated from two samples of activated sludge. They pertained to both Gram-negative and Gram-positive members, and belong to the *Pseudomonas*, *Paracoccus*, *Acinetobacter* and *Rhodococcus* genera. All the strains utilized 300 mg/L of NMP as the only source of carbon, energy and nitrogen over several days, and they were shown to additionally be able to degrade *N*-acetylphenylalanine (NAP). The growth of all the isolated strains was recorded at different NMP concentrations, to a maximum of 20 g/L.

Key words | activated sludge, bacteria, degradation, isolation, nitrogen, *N*-methyl-2-pyrrolidone

Karel Křížek
Jan Růžička (corresponding author)
Markéta Julinová
Lucie Husárová
Josef Houser
Marie Dvořáčková
Petra Jančová
Department of Environmental Engineering,
Faculty of Technology,
Tomas Bata University in Zlin,
TGM Square 275,
762 72 Zlin,
Czech Republic
E-mail: *ruzickaj@ft.utb.cz*

Jan Růžička
Markéta Julinová
Centre of Polymer Systems,
TGM Square 5555,
76001 Zlin,
Czech Republic

INTRODUCTION

N-methyl-2-pyrrolidone (NMP) is a clear to slightly yellowish liquid with a characteristic smell. It is an inflammable, chemically stable organic solvent easily miscible with water and other organic solvents, alcohols and ketones. Due to its inherent properties, NMP has been widely adopted by a wide range of industries, e.g. as a solvent for processing petroleum, resins, acetylene, olefins, diolefins and for desulfurizing gases. Furthermore, it is applied in a variety of chemical reactions, as well as in paint, surface coatings, paint stripper and cleaning products. In the pharmaceutical industry, NMP is used as a penetration enhancer for more rapid transfer of substances through the skin. NMP is also used as a solvent by the microelectronics industry and in agriculture, as a solvent/co-solvent in a variety of pesticides. In comparison to other, more toxic organic solvents, especially chlorinated hydrocarbons, NMP had never been considered as posing a severe risk to the environment (Ward & Phang 2006). However, a study by Lan *et al.* (2004) revealed toxicity affecting *Daphnia magna* (LC_{50} 1.23 mg/L 48 h^{-1}), and EC_{50} 1500 mg/L affecting *Vibrio fisheri* had been found earlier by Campbell & Striebig (1999).

Despite the broad application of NMP and its potential effect on water ecosystems, only a few papers actually describe NMP biodegradation or bacterial strains capable of utilizing it. Chow & Ng (1983) first investigated NMP degradation by microorganisms using air-dried activated sludge and concluded that NMP is degradable under normal conditions of sewage treatment. The authors discovered the formation of an unknown carbonyl metabolite; however, they did not identify the bacteria responsible for degrading NMP. Similarly, Lee *et al.* (2010) observed the degradation of several xenobiotics, including NMP, by an immobilized mixture of five bacterial strains (*Acinetobacter* sp., *Cupravidus* sp., *Paracoccus* sp. and two strains of *Pseudomonas* sp.) and by activated sludge, discovering that NMP had been completely degraded over 9 days. Nevertheless, they also did not specify the strain(s) responsible for NMP degradation. Hence the only work describing a bacterial NMP degrader is by Oceguera-Cervantes *et al.* (2007), which focused on polyurethane (PU) degradation. The authors isolated two strains of *Alicycliphilus* sp. capable of degrading both PU and NMP. The strains proved able to utilize NMP as the sole source of carbon and nitrogen.

doi: 10.2166/wst.2015.031

On the basis of such poor knowledge on bacteria capable of degrading NMP in a wastewater treatment process we decided to obtain these from two samples of activated sludge and to describe and identify the most effective isolates.

MATERIALS AND METHODS

Chemicals and biological material

NMP and *N*-acetylphenylalanine (NAP) were purchased from Sigma-Aldrich and ordinary chemicals were obtained from local suppliers.

One sample of activated sludge originated from a municipal wastewater treatment plant (hereinafter 'WTP') in Zlin, Czech Republic, and the other from an industrial WTP in Slovenska Lupca, Slovak Republic (pharmaceutical production).

Growth media and agars

The mineral medium (MM) used for degradation tests (in g/L, if not otherwise stated) are: K_2HPO_4 0.18, $Na_2HPO_4{\cdot}12H_2O$ 1.92, NH_4Cl 0.3, $MgSO_4{\cdot}7H_2O$ 0.1, $FeSO_4{\cdot}7H_2O$ 0.01, $CaCl_2$ 0.01, NaCl 0.5, yeast extract 0.02, trace element solution 1 mL (Muchova *et al.* 2009).

Nitrogen-free mineral medium is the same composition as MM, excluding NH_4Cl and yeast extract.

Mineral agar (MA) for strain isolation and cultivation are: K_2HPO_4 1.0, NH_4Cl 1.1, $MgSO_4{\cdot}7H_2O$ 0.2, $FeSO_4{\cdot}7H_2O$ 0.01, $CaCl_2$ 0.01, yeast extract 0.03, agar 19.0, trace element solution 1 mL.

NMP biodegradability in activated sludge

The process was monitored using the MicroOxymax respirometric system (Columbus Instruments, USA) in accordance with Muchova *et al.* (2009) and evaluated as the relation ship between measured biological oxygen demand and theoretical oxygen demand. NMP concentration equaled 100 mg/L.

Isolation of NMP degrading bacteria

Mineral agar and mineral agar with NMP (500 mg/L) were prepared so as to isolate the degrading bacteria. Both enriched sludge suspensions from the tests of NMP biodegradation were diluted with sterile saline solution and streaked on both agar plates and grown at 25 °C for 10 days. Any morphologically different colonies that showed more conspicuous growth on MA with NMP than on MA were then isolated, purified and tested for their growth in liquid MM with NMP.

Degradation tests

NMP degradation tests were carried out in 250 mL bottles containing 50 mL of sterile MM, to which NMP was added at a concentration of 300 mg/L. After inoculation, all the tests were incubated in the dark at 25 °C on a rotary shaker for up to 10 days. Samples were taken daily from each test bottle and an automatic analyzer (Shimadzu 5,000A) was used for dissolved organic carbon (DOC) determination, after cell removal by centrifugation (10,000 *g*, 12 minutes, 15 °C). Bacterial growth was monitored by optical density at 600 nm (OD_{600}) measurements. All the tests were carried out in triplicate, if not otherwise stated.

Negative controls were made in parallel using autoclaved cells as inoculums.

In order to determine maximum NMP concentration degraded by the isolates, NMP concentrations of 0.3, 0.5, 1.0, 2.0, 3.0, 5.0, 7.5, 10.0, 20.0 and 50.0 g/L in MM were used and bacterial multiplying was detected by OD_{600} measurements.

NMP utilizations as the nitrogen source were investigated in nitrogen-free MM amended with 300 mg/L of NMP. Inoculated bottles were cultivated at 25 °C for up to 4 days and inoculated MMs with NMP and NH_4Cl were used as positive blank. The growth of the isolates was monitored each day and the highest OD_{600} value was recorded. Initial and final nitrogen concentrations were measured on a Formacs^{HT} TOC/TN analyzer (Skalar Analytical B.V., The Netherlands), after cell removal.

Degradation of NAP was tested in sterile MM containing 1.0 g/L of filter sterilized NAP. Test bottles after inoculation were incubated at 25 °C for 10 days and the results were evaluated by DOC determination, as mentioned above. Tests of NAP degradation were carried out in duplicate.

Identification

All the isolates responsible for degrading NMP were gram-stained and characterized using common microbiological characteristics (cell shape, oxidative and fermentative glucose utilization, catalase and oxidase production, growth at different temperatures) and identified by 16S rDNA sequence analysis.

At first, the primers fD1 and rD1 (AGAGT TTGAT CCTGG CTCAG and AAGGA GGTGA TCCAG CC, respectively) were used to amplify nearly a full-length 16S rDNA (Weisburg *et al.* 1991). Each 25 μL PCR reaction contained 12.5 μL of FastStart PCR master mix for hot start PCR (Roche), 1 μL of each primer solution (12.5 pmol), 9.5 μL of water for molecular biology, and 1 μL (5-10 ng) of bacterial DNA. All amplifications were carried out on a Piko Thermal Cycler (Finnzymes); the temperature profile was as follows: initial denaturation at 94 °C for 5 minutes; 30 cycles at 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute; followed by a final extension at 72 °C for 10 minutes. The size and amount of the PCR products (7 μL) was confirmed by agarose gel (2%) electrophoresis.

Purifying the PCR products was carried out using a commercial protocol of the Gel/PCR DNA Fragments Extraction kit (Geneaid). Consequently, these products were sent for sequencing (GATC Biotech). The acquired sequences of 16S rDNA were compared with the public database (GenBank).

NMP determination in water samples

For the clean-up procedure, Isolute ENV+ solid phase extraction (SPE) columns (100 mg, 3 mL, IST, Biotage) were used. Each column was initiated by adding 3 mL of methanol and 3 mL of sterile distilled water (at a flow rate of 0.05 mL/s) and dried by airflow for 10 minutes. 6 mL of the sample was applied to the initiated column using the same flow rate, then the column was dried by air suction for 10 minutes. The column was then eluted by 6 mL of methanol. So as to determine the recovery, standard solutions of NMP were prepared in methanol at the desired concentrations. The resulting solutions were analyzed by a gas chromatograph (GC) device equipped with a flame-ionization detector (7,890A, Agilent Technologies Inc., USA) using an HP-5 capillary column (30 m × 0.32 mm with 0.25 μm film thickness). The initial oven temperature of 80 °C was held for 1 minute and then it was gradually increased by 10 °C min^{-1} up to the temperature of 160 °C. The injection volume was 1 μL and the injector and detector temperatures were 250 °C and 300 °C, respectively. Nitrogen was used as a carrier gas at the flow rate of 1.5 mL/min.

A recovery for NMP by the above mentioned method was found to be 99%.

A calibration curve (1–100 mg/L) was used for the evaluation of NMP concentration used.

RESULTS AND DISCUSSION

The biodegradability of NMP in activated sludge and isolation of bacterial strains

In order to confirm the activities of the activated sludge and to enrich NMP degrading bacteria, tests for NMP biodegradation were conducted.

In compliance with the results of Chow & Ng (1983), NMP proved to be readily degraded over 4 days in both sludge samples, while the levels of biochemical oxygen demand (BOD)/theoretical oxygen demand (ThOD) parameter of around 0.5 suggested that complete degradation had taken place (data not shown).

Both acclimated sludge suspensions were used as inoculums for the isolation procedure. Initially, after cultivation on agar plates with and without NMP, three isolates were obtained from the municipal activated sludge Zlin (designated as MP10, MP11 and MP12), and four isolates originated from the industrial sample Slovenska Lupca (MP1, MP2, MP3 and MP4). Most bacteria obtained formed distinctly larger colonies on the MA amended with NMP than on the MA without it; only in the case of MP3 and MP4 isolates was such phenomenon only slightly evident. This fact led to the necessity to perform the tests in liquid medium accompanied by measurement of NMP consumption.

NMP utilization by isolates

A total of seven bacterial strains were individually tested for their degradation activities in liquid culture. The results of 300 mg/L NMP utilizations are reported in Figures 1 and 2.

Negative control tests showed no NMP removal during 10 days.

It is obvious that MP3 and MP4 strains were not capable of NMP degradation, even though their cultivations were prolonged by up to 10 days (data of prolonged cultivations not shown); five other isolates proved capable of NMP utilization as the only carbon and energy source. In these five strains, DOC removals from 74 to 92% were found in 3 days (see Table 1) indicating a high level of substrate mineralization; these removals were linked to important bacterial growth recorded by increasing OD_{600} levels during degradation. Slightly lower levels of OD_{600} in the isolates MP10 and MP11 were recorded due to relatively strong cell flocculation occurring during the incubation of the cultures, in comparison with fine cell suspensions formed by the other bacteria. In order to check that all the NMP was

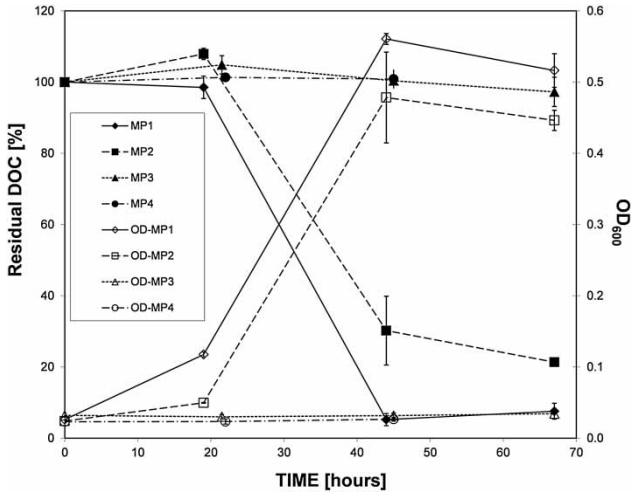

Figure 1 | NMP biodegradation by individual isolates obtained from industrial WTP; % of residual DOC concentrations: black symbols; the growth (expressed as OD_{600}): white symbols.

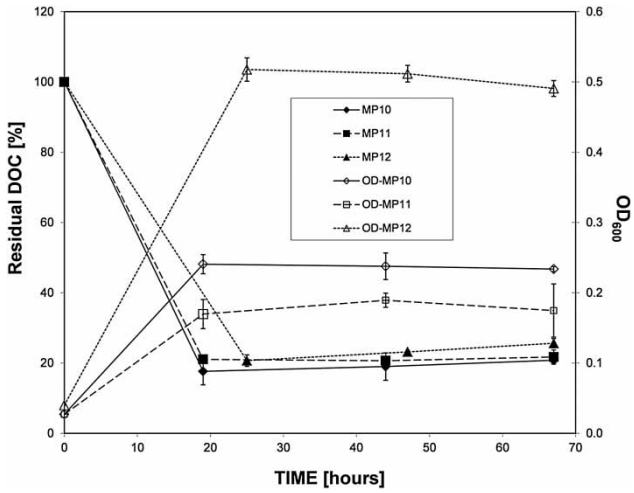

Figure 2 | NMP biodegradation by individual isolates obtained from municipal WTP; % of residual DOC concentrations: black symbols, the growth (expressed as OD_{600}): white symbols.

consumed by the individual isolates, further analyses were conducted of the final supernatant liquors taken from the degradation tests using gas chromatography after the SPE procedure, so as to observe any potentially remaining NMP concentrations. No NMP peak was found in the chromatograms obtained in all cases, which confirmed complete NMP removal (data not shown). Nevertheless, several other peaks, in shorter retention times, were found on all the chromatograms obtained by analyzing the final supernatant liquors. To find whether the peaks were metabolites originating from NMP degradation or the natural products from the bacteria under investigation, degradation trials of lactate sodium as common substrate were performed in all degrading bacteria, adhering to the same procedure as for NMP degradation, in addition to which the final supernatant liquors were analyzed. Comparing the groups of peaks originating from NMP and lactate degradation, respectively, showed that all the compounds produced by single strains during MNP degradation and detectable by the SPE procedure followed as well as with the GC procedure and were the same metabolites as those recorded after lactate utilization. One example of such a chromatogram pair including chromatogram with standard NMP solution is given in Figure 3 (strain MP2).

These results proved that the compounds recorded at the end of NMP degradation by GC after SPE were common metabolic products of the tested isolates not associated with NMP degradation.

In addition to the tests mentioned above, the maximum NMP concentrations utilized by individual isolates were investigated. Slightly surprisingly, despite exhibiting the solvent character of NMP, all the degrading strains were able to grow at relatively high concentrations (up to 20 g/L), while none of the strains were extremely sensitive to the compound.

Table 1 | NMP degradation and its use as the only nitrogen source in active isolates

		Maximum growth on nitrogen source (OD_{600})[a]		
Isolate	**NMP degradation (% DOC removed)**	**NMP**	**NMP + NH_4Cl**	**Nitrogen removal[b] (%)**
MP1	92.45 ± 2.24	0.450 ± 0.013	0.451 ± 0.020	41.81 ± 2.57
MP2	78.65 ± 1.06	0.539 ± 0.034	0.480 ± 0.008	34.91 ± 1.47
MP10	79.21 ± 0.91	0.356 ± 0.007	0.364 ± 0.011	47.64 ± 3.02
MP11	78.29 ± 1.99	0.354 ± 0.008	0.209 ± 0.013	59.48 ± 4.27
MP12	74.38 ± 1.36	0.377 ± 0.011	0.470 ± 0.012	36.41 ± 0.99

Mean ± SD, for $n = 3$.
[a]Initial OD_{600} values < 0.015.
[b]NMP as the only nitrogen source.

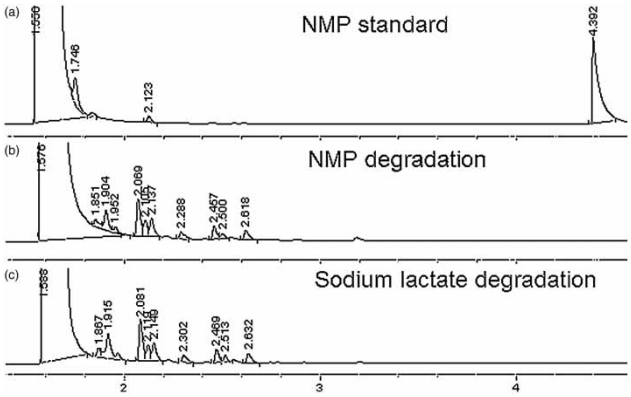

Figure 3 | GC chromatograms: (a) analytical standard of 100 mg/L NMP; (b) and (c) supernatant liquors after 300 mg/L NMP and lactate degradation, respectively, and after the SPE procedure (strain MP2; the greatest peak is methanol).

NMP utilization as the only nitrogen source

As many heterocyclic compounds may serve as a nitrogen source to numerous microorganisms, the tests for such NMP utilization were performed in all degrading strains. Their growth in nitrogen-free mineral media amended with 300 mg/L of NMP was detected during 4 days of cultivation and the maximum values are given in Table 1, in comparison with growth in MM with NMP and NH_4Cl as positive blank. In addition to it, the amounts of nitrogen utilized by each strain when NMP was the only nitrogen source are presented.

In accordance with the findings of Oceguera-Cervantes *et al.* (2007), all the NMP degraders were able to use NMP as the sole nitrogen source, although they did not utilize all the nitrogen available in the medium, since the remaining nitrogen concentrations found in the supernatant liquors reached approx. 40–65% of initial concentrations. This phenomenon can be explained by the relatively low C/N ratio in NMP (5/1), which indicates a certain degree of nitrogen excess in the medium containing NMP where it was the only organic substrate. The different remaining nitrogen concentrations in the individual strains were probably done by some physiologic properties of isolated bacteria, such as diverse levels of both their nitrogen assimilation and an excretion of its unnecessary portion.

Degradation of N-acetyl phenylalanine

Due to NAP being commonly used as a specific substrate for detecting gamma-lactamase-producing bacteria (Brabban *et al.* 1996; Taylor *et al.* 1999), investigation was conducted on NAP degradation by the NMP degrading bacteria. All the isolates were inoculated to MM with the addition of NAP, and after 10 days of incubation the DOC levels were measured. The final data are listed in Table 2, showing that NAP was degraded in all cases. These results indicate that carrying out an enzyme study on at least some of the degrading strains is greatly desired, and suggests that NMP could potentially be used for screening and detecting gamma-lactamase producing bacteria.

Identification

All the degrading bacteria were revealed to be non-fermenting, catalase producing bacteria. The base properties of the strains corresponded well with the common characteristics of the genera (Sedlacek 2007) found by BLAST on the base of 16S rDNA analyses. In three cases (MP1, MP10 and MP11) 99% similarity with some species of known bacteria were recorded, whilst lower similarities were observed in the strains MP2 and MP12. MP1 bacterium turned out to be a member of the genus *Paracoccus*, closely related to 14 species of the genus. The isolates MP10 and MP11 were discovered as being greatly similar to one other and to several *Pseudomonas* species: *Ps. entomophila*, *Ps. mosselii*, *Ps. taiwanensis*, *Ps. monteilii*, *Ps. plecoglossicida*, and *Ps. putida*. The isolates MP2 and MP12 were not identical to any bacterial species recorded in the GenBank. The closest relatives of the MP2 strain were *Rhodococcus opacus* (Acc. No. NR_074632.1), *Rh. wratislaviensis* (Acc. No. NR_026524.1), and *Rh. koreensis*

Table 2 | NAP degradation in individual isolates and their identification

Isolate	NAP degradation (% DOC removed)	Gram stain	Identification	% similarity
MP1	87.0	G− cocci	*Paracoccus* sp.	99
MP2	81.2	G+ rods	*Rhodococcus* sp.	97
MP10	74.6	G− rods	*Pseudomonas* sp.	99
MP11	73.1	G− rods	*Pseudomonas* sp.	99
MP12	84.6	G− cocci	*Acinetobacter* sp.	95

(Acc. No. NR_024973.1) with 97% similarity. As for the strain MP12 *Acinetobacter johnsonii* (Acc. No. NR_044975.1) and *Acinetobacter beijerinckii* (Acc. No. NR_042234.1), these showed 95% similarity to it.

Owing to their novelty, partial 16S rDNA sequences of the *Rhodococcus* MP2 and *Acinetobacter* MP12 have been deposited in the GenBank under Acc. No. KM081653 and Acc. No. KM099074, respectively.

The results of identification revealed that all the NMP degrading bacteria are members of well-known genera capable of removing various pollutants, including degradation of nitrogen containing heterocyclic compounds. *Pseudomonads* were revealed as capable of degrading nicotine (Li *et al.* 2010), atrazine (Udikovic-Kolic *et al.* 2012) and caffeine (Gummadi *et al.* 2012), while *paracocci* were described in the degradation of pyridine (Qiao & Wang 2010), piperazine (Cai *et al.* 2013), *N,N*-dimethylformamide (Sanjeevkumar *et al.* 2013) and several amide pesticides (Sun *et al.* 2013). Some *Acinetobacter* strains can utilize carbazole (Singh *et al.* 2011) or epsilon-caprolactam (Rajoo *et al.* 2013) and also *rhodococci* can degrade nitrogenous compounds such as pyridine (Follner & Babel 2001) and carbendazim (Zhang *et al.* 2013). Hence, the results of this study are fully in accordance with previous findings and they illustrate well the prominent role of these bacteria in nitrogenous pollutant removal.

CONCLUSION

Bacteria degrading *N*-methyl-2-pyrrolidone are easily isolated from common activated sludge and belong to both gram-negative and gram-positive species. Such bacteria are able to degrade relatively high NMP concentrations; consequently they ensure rapid NMP removal during the wastewater treatment process. If needed, they could be potentially used for NMP removal from environmental spheres with low potential of autochthonous microbial communities to degrade NMP.

ACKNOWLEDGEMENTS

This work was performed with the support of the Operational Program Research and Development for Innovations, co-founded by the European Regional Development Fund (ERDF), and the national budget of the Czech Republic, within the framework of the projects Center of Polymer Systems (reg. number: CZ.1.05/2.1.00/03.0111) and IGA/FT/2014/005.

REFERENCES

Brabban, A. D., Littlechild, J. & Wisdom, R. 1996 Stereospecific gamma-lactamase activity in a *Pseudomonas fluorescens* species. *Journal of Industrial Microbiology & Biotechnology* **16**, 8–14.

Cai, S., Li, X., Cai, T. M. & He, J. 2013 Degradation of piperazine by *Paracoccus* sp. TOH isolated from activated sludge. *Bioresource Technology* **130**, 536–542.

Campbell, H. L. & Striebig, B. A. 1999 Evaluation of *N*-methylpyrrolidone and its oxidative products toxicity utilizing the microtox assay. *Environmental Science & Technology* **33**, 1926–1930.

Chow, S. T. & Ng, T. L. 1983 The biodegradation of *N*-methyl-2-pyrrolidone in water by sewage bacteria. *Water Research* **17**, 117–118.

Follner, C. G. & Babel, W. 2001 Degradation of pyridine by *Rhodococcus opacus* UFZ B408. In: *Innovative Methods In Support Of Bioremediation* 6 (4), (V. Magar, T. M. Vogel, C. M. Aelion & A. Leeson, eds), 6th International In Situ and on-Site Bioremediation Symposium 4–7 June, San Diego, CA, pp. 81–90.

Gummadi, S. N., Bhavya, B. & Ashok, N. 2012 Physiology, biochemistry and possible applications of microbial caffeine degradation. *Applied Microbiology and Biotechnology* **93**, 545–554.

Lan, C. H., Peng, C. Y. & Lin, T. S. 2004 Acute aquatic toxicity of N-methyl-2-pyrrolidinone to *Daphnia magna*. *Bulletin of Environmental Contamination and Toxicology* **73**, 392–397.

Lee, J. K., Lee, W. J., Cho, Y. J., Park, D. H., Lee, Y. W. & Chung, J. W. 2010 Variation of bacterial community immobilized in polyethylene glycol carrier during mineralization of xenobiotics analyzed by TGGE technique. *Korean Journal of Chemical Engineering* **27**, 1816–1821.

Li, H. J., Li, X. M., Duan, Y. Q., Zhang, K. Q. & Yang, J. K. 2010 Biotransformation of nicotine by microorganism, the case of *Pseudomonas* spp. *Applied Microbiology and Biotechnology* **86**, 11–17.

Muchova, M., Ruzicka, J., Julinova, M., Dolezalova, M., Houser, J., Koutny, M. & Bunkova, L. 2009 Xanthan and gellan degradation by bacteria of activated sludge. *Water Science and Technology* **60**, 965–973.

Oceguera-Cervantes, A., Carrillo-Garcia, A., Lopez, N., Bolanos-Nunez, S., Cruz-Gomez, J. M., Wacher, C. & Loza-Tavera, H. 2007 Characterization of the polyurethanolytic activity of two *Alicycliphilus* sp. strains able to degrade polyurethane and *N*-methylpyrrolidone. *Applied Microbiology and Biotechnology* **73**, 6214–6223.

Qiao, L. & Wang, J. L. 2010 Microbial degradation of pyridine by *Paracoccus* sp. isolated from contaminated soil. *Journal of Hazardous Materials* **176**, 220–225.

Rajoo, S., Ahn, J. O., Lee, H. W. & Jung, J. K. 2013 Isolation and characterization of a novel epsilon-caprolactam-degrading microbe, *Acinetobacter calcoaceticus*, from industrial

wastewater by chemostat-enrichment. *Biotechnology Letters* **35**, 2069–2072.

Sanjeevkumar, S., Nayak, A., Santoshkumar, M., Siddavattam, D. & Karegoudar, T. B. 2013 *Paracoccus denitrificans* SD1 mediated augmentation with indigenous mixed cultures for enhanced removal of *N,N*-dimethylformamide from industrial effluents. *Biochemical Engineering Journal* **79**, 1–6.

Sedlacek, I. 2007 Taxonomie prokaryot (*Taxonomy of prokaryotes*) Masaryk University, Brno.

Singh, G. B., Gupta, S., Srivastava, S. & Gupta, N. 2011 Biodegradation of carbazole by newly isolated *Acinetobacter* spp. *Bulletin of Environmental Contamination and Toxicology* **87**, 522–526.

Sun, L. N., Zhang, J., Kwon, S. W., He, J., Zhou, S. G. & Li, S. P. 2013 *Paracoccus huijuniae* sp nova, an amide pesticide-degrading bacterium isolated from activated sludge of a wastewater biotreatment system. *International Journal of Systematic and Evolutionary Microbiology* **63**, 1132–1137.

Taylor, S. J. C., Brown, R. C., Keene, P. A. & Taylor, I. N. 1999 Novel screening methods – the key to cloning commercially successful biocatalysts. *Bioorganic & Medicinal Chemistry* **7**, 2163–2168.

Udikovic-Kolic, N., Scott, C. & Martin-Laurent, F. 2012 Evolution of atrazine-degrading capabilities in the environment. *Applied Microbiology and Biotechnology* **96**, 1175–1189.

Ward, R. T. & Phang, W. 2006 *Reassessment of One Exemption from the Requirements of a Tolerance for N-Methylpyrrolidone*. US EPA, Washington, DC. http//www.epa.gov/opprd001/inerts/methyl.pdf (accessed 29 August 2014).

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 1991 16S Ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697–703.

Zhang, X. J., Huang, Y. J., Harvey, P. R., Li, H. M., Ren, Y., Li, J. S., Wang, J. N. & Yang, H. T. 2013 Isolation and characterization of carbendazim-degrading *Rhodococcus erythropolis* djl-11. *PLoS One* **8**, e74810.

First received 19 September 2014; accepted in revised form 12 January 2015. Available online 23 January 2015