

- XRD patterns of metallic iron surfaces after arsenic incorporation showed presence of iron arsenide (FeAs and FeAs₂) phases when no sulphur was present, and presence of arsenopyrite (FeAsS) and oripment (As₂S₃) when sulphur was present.
- Based on the similarity of XRD patterns irrespective of whether arsenic (III) or arsenic (V) was contacted with the metallic iron surface, it was concluded that arsenic incorporation on the metallic iron surface involved arsenic reduction near the iron surface, followed by deposition of reduced arsenic-bearing phases on the iron surface.
- Theoretical chemical speciation studies of a system containing arsenic, iron and sulphur showed precipitation of arsenic-bearing sulphide phases, i.e. FeAsS (arsenopyrite) and As₂S₃ (oripment) under strongly reducing conditions expected in the vicinity of anaerobically corroding metallic iron surface.

In conclusion, these results indicate that adsorption columns and *in situ* reactive barriers containing metallic iron may potentially be efficient in removing dissolved arsenic from water under strongly reducing conditions.

1. Lackovic, J., Nikolaidis, N. P. and Dobbs, G. M., Inorganic arsenic removal by zero-valent iron. *Environ. Eng. Sci.*, 2000, **17**, 29–39.
2. Farrell, J., Wang, J., O'Day, P. and Conklin, M., Electrochemical and spectroscopic study of arsenate removal from water using zero-valent iron media. *Environ. Sci. Technol.*, 2001, **35**, 2026–2032.
3. Su, C. and Puls, R. W., Arsenate removal by zerovalent iron: kinetics, redox transformation, and implications for in situ ground water remediation. *Environ. Sci. Technol.*, 2001, **35**, 1487–1492.
4. Melitas, N., Wang, J., Conklin, M., O'Day, P. and Farrell, J., Understanding soluble arsenate removal kinetics by zerovalent iron media. *Environ. Sci. Technol.*, 2002, **36**, 2074–2081.
5. Goldberg, S. and Johnston, C. T., Mechanism of arsenic adsorption on amorphous oxides evaluated using macroscopic measurements, vibrational spectroscopy, and surface complexation modeling. *J. Colloid Interface Sci.*, 2001, **234**, 204–216.
6. Utsunomiya, S., Peters, S. C., Blum, J. D. and Ewing, R. C., Nanoscale mineralogy of arsenic in a region of New Hampshire with elevated As-concentrations in the groundwater. *Am. Mineral.*, 2003, **88**, 1844–1852.
7. Loeppert, R. H., Arsenate and arsenite retention and release in oxide and sulfide dominated systems. Technical Report No. 176, Texas Water Resources Institute, College Station, Texas, 1997.
8. Bhowmik, A., Mineralogical study of arsenic contamination of an aquifer system in West Bengal. M.Tech. thesis, Department of Civil Engineering, IIT, Kanpur, 1999.
9. Moore, J. N., Ficklin, W. H. and Johns, C., Partitioning of arsenic and metals in reducing sulfidic sediments. *Environ. Sci. Technol.*, 1988, **22**, 432–437.
10. Battey, M. H., *Mineralogy for Students*, Longman Scientific and Technical, 1990, 2nd edn.
11. Ramaswami, A., Tawachsupa, S. and Isleyen, M., Batch-mixed iron treatment of high arsenic waters. *Water Res.*, 2001, **35**, 4474–4479.
12. Bardwell, J. A., Fraser, J. W., MacDougall, B. and Graham, M. J. Influence of anodic oxide film on pitting of iron. *J. Electrochem. Soc.*, 1992, **139**, 366–370.

Received 5 November 2005; revised accepted 8 March 2006

Determination and quantification of camptothecin in an endophytic fungus by liquid chromatography – positive mode electrospray ionization tandem mass spectrometry

Touseef Amna, Ravi K. Khajuria*, Satish C. Puri, Vijeshwar Verma and Ghulam N. Qazi

Regional Research Laboratory, Canal Road, Jammu 180 001, India

This communication describes the detection of Camptothecin (CPT) in an endophytic fungus and application of gradient reverse phase HPLC method with diode array and MS/MS detection for quantification of the said compound. The extract of fungus *Enterophospora infrequens* isolated from the inner bark of *Nothapodytes foetida* plant was chromatographed on a Merck (250 × 4.6 mm, 5 μm) column, maintained at 30°C, and analysed by positive mode electrospray ionization tandem mass spectrometry on a mass spectrometer in a single reaction monitoring system. The mobile phase consisted of a linear gradient of water, acetonitrile from 10 to 98% in 35 min. The quantity of CPT in the extract was estimated on the basis of linear calibration curves obtained in the concentration range of 5 to 50 ng with standard CPT. Fungus grown under surface culture method accumulated 40 mg CPT/kg dry cell mass, which is far lower than that present in the plant source from where the organism was isolated. This report on the accumulation of CPT in a fungus may be a starting point for improving the productivity of CPT in this isolate.

Keywords: *Enterophospora infrequens*, liquid chromatography, *Nothapodytes foetida*, single reaction monitoring, tandem mass spectrometry.

CAMPTOTHECIN, often abbreviated as CPT (Figure 1), and its analogues are naturally occurring group of quinoline alkaloids depicting profound cytotoxic activity¹. Various plant species such as *Camptotheca acuminata*, *Ophiorrhiza mungo*, *Ervatomia hynaeana* and *Nothapodytes foetida* are known sources of this phytochemical². The supply of CPT depends primarily on the abundant availability of plants such as *C. acuminata*. Many parts of this plant can be used to extract CPTs³. The overexploitation of this source rendered the plant as an endangered species all over the globe, especially in China. The gene pool of this plant is very small in countries like USA⁴. Literature survey on camptothecin revealed that the molecule occupies an important position among the plant-based anti-cancer drugs^{5–21}. In order to conserve the germplasm, a need was felt to look for alternate sources for this class of natural products. Sustained search in this direction led to the isolation of an

*For correspondence. (e-mail: khajuriark@yahoo.com)

endophyte from *N. foetida*. The organism, on extensive qualitative screening, was found to accumulate CPT.

In order to support such studies, there was a need for the development of a sensitive and effective assay technique to quantify the production of CPT in mycelium extracts and fermentation broth. This communication reports the quantification of camptothecin using liquid chromatography – positive mode electrospray ionization tandem mass spectrometry (LC-MS/MS). This method has the advantage over LC-UV (DAD) and LC-MS methods because of its selectivity and specificity. Binary gradient reverse phase HPLC with diode array absorbance and MS/MS detection in the positive mode of ionization under electrospray API interface in a single reaction monitoring (SRM) system was used for detection and quantification of camptothecin.

The HPLC and laboratory-grade methanol, chloroform and acetonitrile were of Rankem make and purchased from Ranbaxy Chemicals Ltd, Mohali (India). Water purified by milli-Q purification system was used in this study. Standard camptothecin was purchased from Sigma-Aldrich, Bangalore (India).

Camptothecin stock solution (1 mg/ml) was prepared in HPLC-grade chloroform:methanol (9 : 1) solvent mixture. The stock solution was stored in the refrigerator at 4°C. From the stock solution, the working solutions were prepared in the concentration range of 0.5 to 5 ng/μl for LC-MS/MS analysis.

Stem explants from fully mature *N. foetida* tree were treated with 95% ethanol to disinfect the outer surface. Pieces of the inner bark of the stem were then placed on aqueous agar and incubated at 28 ± 2°C until fungal growth appeared on the plate. The tips of the fungal hyphae were then removed from the aqueous agar and placed on a mycological solid medium. The pure culture thus obtained was transferred to a number of solid and liquid media,

which supported fungal growth. Incubation of the fungus grown on solid medium was carried out at 28 ± 2°C for 7–8 days. The fungus grows as a white cottony mycelium. The well-developed mycelium is branched, fast-growing and spreads on the solid medium. Aerial hyphae are produced after 5–7 days of growth and turn black due to sporulation.

Total DNA was isolated from the mycelial mass using a slightly modified method described by Vainio *et al.*²².

The fungus was identified by amplification of LSU ribosomal gene (~300 bp) and sequenced on ABI Prism310 Genetic Analyzer (ABI, USA). The DNA sequences thus obtained were aligned to the ribosomal gene database (<http://rdp.cme.msu.edu> and <http://ncbi.nlm.nih.gov>), which revealed almost 100% homology with *Entrophospora infrequens*, hitherto reported as ectomycorrhizal fungi²³.

Mycelia and broth were separated by filtration. Mycelia were thoroughly washed with sterile distilled water and homogenized in a cell disintegrator. Both cell homogenate and cell-free broths were extracted four times with equal volume of chloroform:methanol (4 : 1 v/v). After stripping-off the solvent, a small quantity of the residue was applied on silica gel TLC plates (Merck K GaA, 64271 Darmstadt, Germany). The chloroform:methanol (9 : 1 v/v) solvent system was used for TLC. TLC analysis exhibited spots which were superimposable with the standard CPT. The spots were visualized under UV. The rest of the residue was used for quantification of CPT by LC/MS/MS.

Samples were analysed on an Agilent 1100 series HPLC system comprising of binary HPLC pump, diode array absorbance detector, an autosampler, an on-line degasser and a thermostatic column oven. Separations were carried out using 250 × 4.0 mm id, 5 μm, RP-18e column (Merck, Germany). A gradient of water and acetonitrile at a flow rate of 0.5 ml/min was employed as the mobile phase. The gradient used started with 10% acetonitrile (5 min isocratic) and over a period of 35 min, the percentage of acetonitrile was increased to 98 (10 min isocratic) and subsequently decreased again to 10%. The total analysis run time was 50 min. The LC column temperature was maintained at 30°C. After passing through the flow cell of the DAD detector, the column eluate was directly transferred to MS detector without any split. The mass spectrometer was fitted with an electrospray interface. All the interface parameters of LC-MS/MS studies were optimized by infusing the standard solution of CPT. The other parameters for LC-MS/MS analysis were set at dry gas flow of 11 l/min, nebulizer pressure 35 psi and drying gas temperature 320°C. The isolated peak width was taken as 0.8 m/z and fragmentation amplitude value was 2.40.

A mass spectrum of CPT in methanol:chloroform [1 : 3] was recorded under ESI on a Bruker Ion Trap (Esquire 3000) mass spectrometer in the positive ion mode, with a mass range from 50 to 800 amu. Bruker Daltonics Esquire 5.0 software was used to obtain the mass spectra and Chemstation Rev. 06.03 (509) software was used to acquire the LC-MS/MS spectra. The most intense peak in the mass

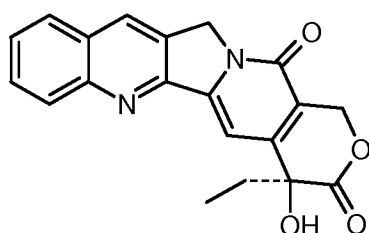
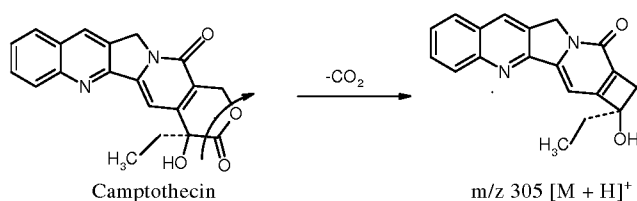


Figure 1. Camptothecin.



Scheme 1. Proposed fragmentation (MS²) of camptothecin.

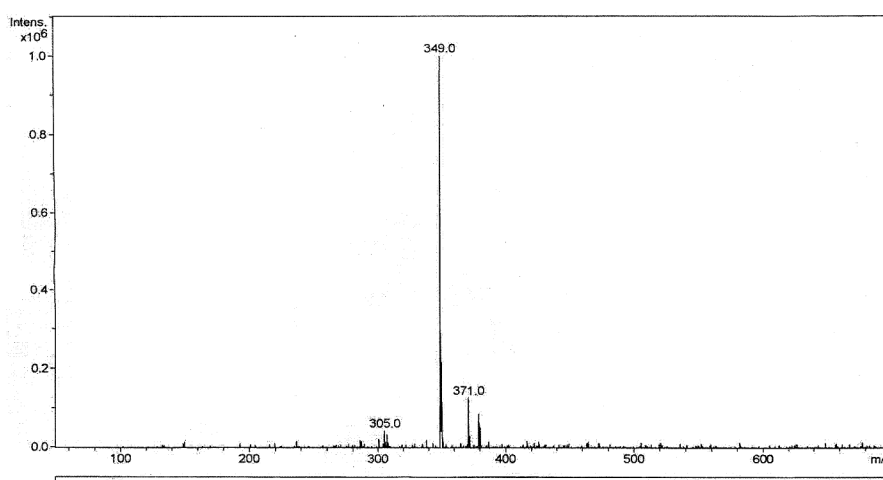


Figure 2. ES-MS spectra of CPT.

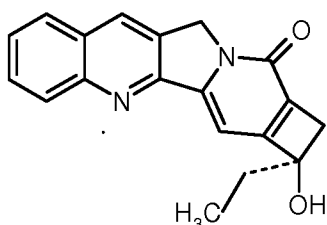


Figure 3. m/z 305 $[M + H]^+$.

spectrum corresponded to the $[M + H]^+$ ions of CPT at m/z 349 (Figure 2). The sodium adduct of CPT was also formed and was visible in the mass spectrum at m/z 371 $[M + Na]^+$. The molecular ion peak at m/z 349 $[M + H]^+$ was taken for MS/MS studies²⁴. This molecular ion peak on isolation and fragmentation, exhibited daughter ion peak (Scheme 1) at m/z 305 $[M^+ + H - 44]^+$, which was due to the loss of carbon dioxide (Figures 3 and 4) moiety. This daughter ion peak at m/z 305 was taken up for quantification in a SRM system.

Using the above conditions, satisfactory results were obtained in the positive mode ESI-MS/MS. Figure 5 shows the total ion current (TIC) trace from SRM [a], LC-UV (DAD) chromatogram (256 nm) [b] and LC-ES-MS/MS spectra [c] of CPT under the positive mode of electrospray ionisation. Figure 6 shows the TIC chromatogram from SRM (a), LC-UV-(DAD) chromatogram at 256 nm (b) and LC-ES-MS/MS (c) of the sample of endophytic fungal extract, wherein presence of CPT has been observed.

Quantification of CPT in extracts prepared from mycelia and broth was done on the basis of calibration curves established by injecting six concentrations of the CPT standard in the range of 5 to 50 ng each time before sample analysis. Quantification of CPT was carried out using SRM detection of the molecule at the above-mentioned concen-

trations. Linear calibration curves for the CPT within the concentration range of 5 to 50 ng ($R^2 =$ curve coefficient 0.9998695) were obtained. Validation of the method was carried out by spiking 10 μ g of standard camptothecin to 100 mg of the endophytic fungal extract and the recovery was within the range of 98.5 to 101.5%.

A fungal endophyte identified on the basis of LSU rDNA sequence homology as *E. infrequens* was isolated from the inner bark of *N. foetida*, a known producer of CPT. The organism was difficult to grow under stirred aerobic conditions in shaken flask as well as in jar fermentors. However, the organism formed a fungal mat on the liquid-medium surface. The organic solvent extracts of the fungal mats thus obtained were screened for CPT.

A compound having chromatographic properties comparable to CPT was identified by comparative TLC, HPLC, LC-MS and LC-MS/MS in the extracts. CPT accumulation in the fungal mat was followed as a function of time of incubation. Organic solvent extracts of mycelia and broth of surface culture were found to contain CPT after 14 days. The maximum yield of CPT per 100 g dry weight of mycelia was found to be 4.28 ± 0.05 (the results are mean of six experiments) mg/100 g mycelium (dry weight) and 250 ± 20 μ g/l of broth. No CPT was detected in uninoculated as well as in inoculated culture broth at the beginning of incubation (zero-day). The experiment was carried out six times in replicates of three to confirm the results. Biological activity of fungal extract was assayed *in vitro*²⁵ against human cancer cell lines (A-549) for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer and compared with standard CPT (10^{-5} M). A comparable cytotoxic activity profile was observed (data communicated elsewhere).

An LC/ESI/MS/MS assay for quantification of CPT in mycelia and fermentation broth of a culture isolated from *N. foetida* has been developed and validated on Bruker

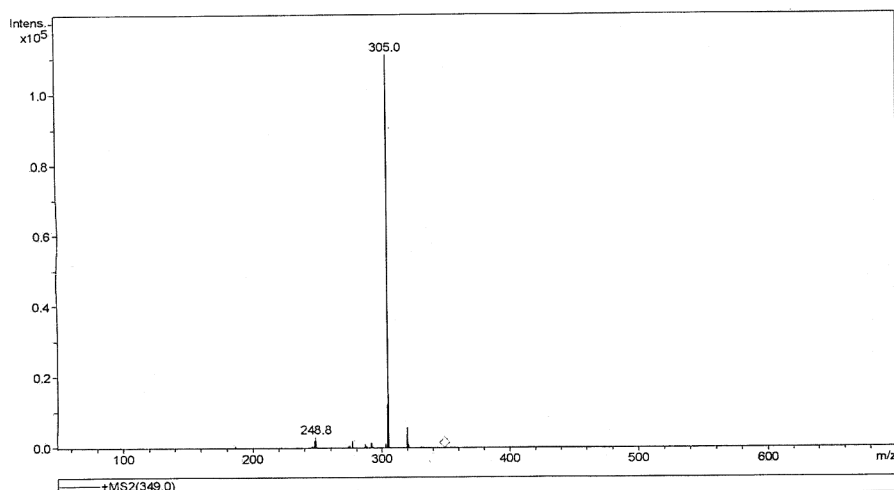


Figure 4. ES-MS/MS spectra of CPT at m/z 349.

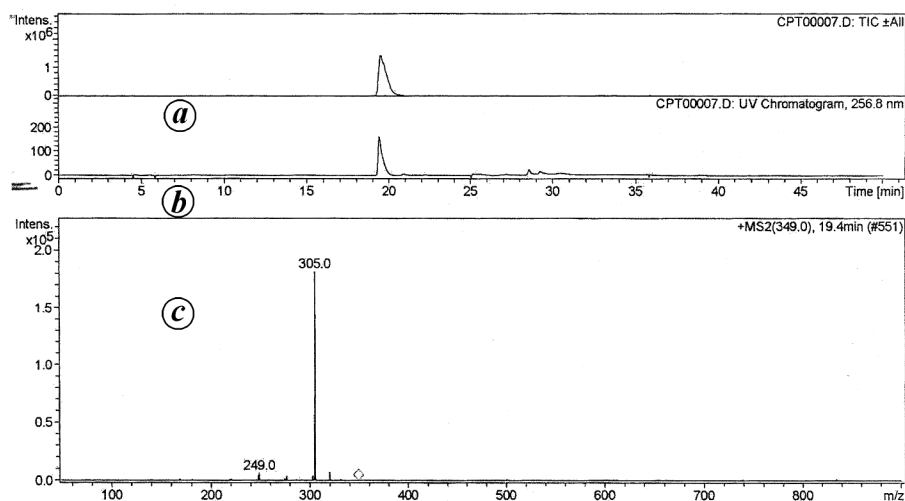


Figure 5. Total ion current trace from SRM (a), UV-(DAD) chromatogram at 256 nm (b) and LC-ES-MS/MS (c) of CPT.

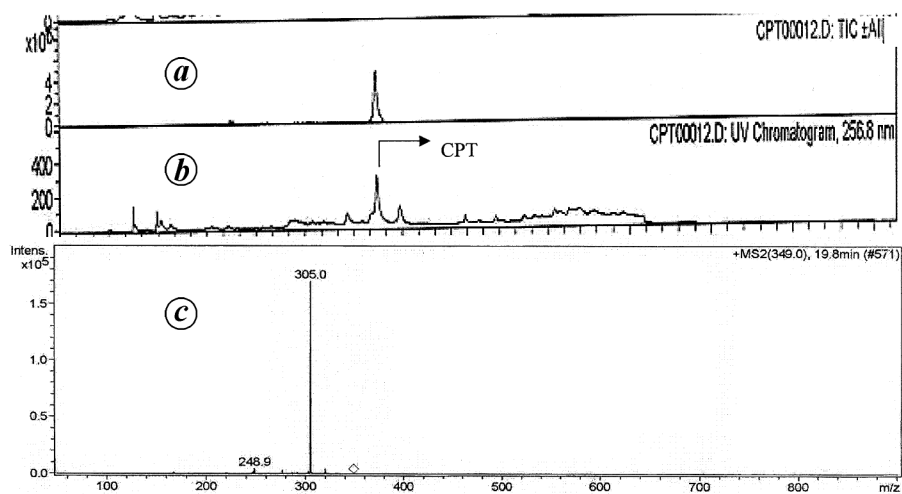


Figure 6. Total ion current trace from SRM (a), LC-UV-(DAD) chromatogram (b), and LC-ESI-MS/MS of the endophytic fungal extract sample showing the presence of CPT (c).

Ion Trap (Esquire 3000) mass spectrometer, which gives monoisotopic resolution throughout the mass range of 3000 amu. The method is linear over a wide concentration range with a correlation coefficient greater than 0.996. The analytical protocol based on MS/MS has successfully been employed by us for analysis of camptothecin in crude extracts obtained from plant sources, fermentation broths and mycelia cultured in the laboratory. The assay method is sensitive and selective, allows minimal matrix interferences and requires a simple sample clean-up procedure. The experimental results obtained by us during the fermentation experiments for the production of camptothecin demonstrate the usefulness of the assay in monitoring the metabolic flux of CPT in endophytes residing in the plants.

- Bodley, A. F., Cumming, J. N. and Shapiro, T. A., Effects of camptothecin, a topoisomerase-I inhibitor, on *Plasmodium falciparum*. *Biochem. Pharmacol.*, 1998, **55**, 709.
- Aiyama, R., Nagai, H., Kokata, K., Shinonara, C. and Swada, S., Camptothecin derivative from *Nothapodytes foetida*. *Phytochemistry*, 1988, **27**, 3663–3664.
- Li, S. Y. and Adair, K. T., *Camptotheca acuminata* Decaisne, Xi Shu, a Promising Anti-Cancer and Anti-Viral Tree for the 21st Century. A Henry M. Rockwell Monograph (ed. Stephen, F.), Austin State University, Texas, 1994.
- Li, S. Y., Wang, Y. J., Beasley, R. S. and Northrup, K., Anti-cancer happytrees Decaisne Research Report. Arthur Temple College of Forestry, Stephen en F. Austin State University, Texas, 2000.
- Hsiang, Y-H., Hertzberg, R., Hecht, S. and Liu, L. F., Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase-1. *J. Biol. Chem.*, 1985, **260**, 14873.
- Cai, C. J. and Hutchinson, C. R., Camptothecin. In *The Alkaloids* (eds Brossi, A. and Manske, R. H. F.), Academic Press, New York, 1983, vol. XXI, pp. 101–137.
- Bacon, C. W. and White, J. F., *Microbial Endophytes*, Marcel Dekker, New York, 2000.
- Palumbo, M., Sissi, C., Gatto, B., Moro, S. and Zagotto, G., Quantitation of camptothecin and related compounds. *J. Chromatogr. B*, 2001, **764**, 121–140.
- Yamakazi, Y. et al., Metabolite profiling of alkaloids and strictosidine synthase activity in camptothecin producing plants. *Phytochemistry*, 2003, **62**, 461–470.
- Sheriha, G. M. and Rapoport, H., Biosynthesis of *Camptotheca acuminata* alkaloids. *Phytochemistry*, 1976, **15**, 505–508.
- Pirillo, A., Verotta, L., Garibodi, P., Torrigiani, E. and Bombardelli, E. J., Constituents of *Nothapodytes foetida*. *Chem. Soc. Perkin Trans. I*, 1995, **5**, 583–587.
- Thomas, C. J., Rahierm, N. J. and Hecht, S. M., *Camptothecin: Current Perspectives*, 2004, vol. 12, pp. 1585–1604.
- McCutcheon, T. L., Carrol, G. C. and Schwab, S., Genotypic diversity in populations of a fungal endophyte from Douglas fir. *Mycologia*, 1993, **85**, 180–186.
- Wani, M. C. and Wall, M. E., Plant antitumour agents. II. The structure of two new alkaloids from *Camptotheca acuminata*. *J. Org. Chem.*, 1969, **34**, 1364–1367.
- Wall, M. E., Wani, M. C., Cook, C. E. Palmer, K. H., Mcphail, H. T. and Sim, G. A., Plant antitumour agents I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. *J. Am. Chem. Soc.*, 1966, **88**, 3888–3890.
- Fulzele, D. P., Satdive, R. K. and Pol, B. B., Growth and production of camptothecin by cell suspension cultures of *Nothapodytes foetida*. *Planta Med.*, 2001, **67**, 150–152.
- Li, S., Yi, Y., Wang, Y., Zhang, Z. and Beasley, R. S., Camptothecin accumulation and variation in *Camptotheca acuminata*. *Planta Med.*, 2002, **68**, 1010–1016.
- Strobel, G., Daisy, B., Cstillo, U. and Harper, J., Natural products from endophytic microorganisms. *J. Nat. Prod.*, 2004, **67**, 257–268.
- Wall, M. E. and Wani, M. C., *Econ. Med. Plant Res.*, **5**, 111–127.
- Tsai, T. H., Chen, Y. F., Chou, C. J. and Chen, C. F., Measurement and pharmacokinetics of unbound 20(S)-camptothecin in rat blood and brain by microdialysis coupled to microbore liquid chromatography with fluorescence detection. *J. Chromatogr. A*, 2000, **870**, 221–226.
- Hutchinson, C. R., Heckendorf, A. H., Daddona, P. E., Hagaman, E. and Wenkert, E., Biosynthesis of Camptothecin I. Definition of the over all pathway assisted by carbon-13 nuclear magnetic resonance analysis. *J. Am. Chem. Soc.*, 1974, **96**, 5609–5611.
- Vainio, E. J., Korhonen, K. and Hantula, J., Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. *Mycol. Res.*, 1998, **102**, 187.
- Ames, R. N. and Schneider, R. W., Entrophospora, a new genus in the Endogonaceae. *Mycotaxon*, 1979, **8**, 347.
- Lemoine, J., Fournat, B., Despeyroux, D., Jenneys, K. R., Rosenberg, R. and De Hoffmann, E., Collision gas values. *J. Am. Mass Spectrosc.*, 1993, **4**, 197.
- Skehan, P., New colorimetric cytotoxicity assay for anticancer drug screening. *J. Natl. Cancer Inst.*, 1990, **82**, 1107.

Received 5 July 2005; revised accepted 10 March 2006

Microbial genetic resource mapping of East Calcutta wetlands

S. Ray Chaudhuri* and A. R. Thakur

West Bengal University of Technology, BF 142, Sector I, Salt Lake, Kolkata 700 064, India

East Calcutta Wetland (ECW) is the world's largest natural treatment plant for solid and soluble waste, where bioremediation and biodegradation of complex compounds is mainly based on microbial activity and is recognized as a potential source of bacteria of biotechnological impact. Here we discuss the microbial resource mapping of this important Ramsar site. Culture-independent analysis of the microbial population from soil and water through community DNA isolation, amplification, cloning and partial sequencing (320 clones) of 16Sr RNA gene sequence and finally secondary structure-based phylogenetic analysis of the novel sequences (292 GenBank submissions) using ARB software indicated the presence of microorganisms from 12 different main bacterial phyla, thus revealing the rich natural microbial resource at ECW.

Keywords: ARB software, bioremediation, East Calcutta wetlands, microbial biodiversity, phylogenetic analysis.

*For correspondence. (e-mail: shaon@wbut.ac.in)