

L-Asparaginase and antioxidant activity of endophytic bacteria associated with ethnomedicinal plants

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The present investigation was aimed at assessing production of L-asparaginase by endophytic bacteria, associated with ethnomedicinal plants of North-east India, keeping in view their relevance as anticancer agents. In recent years, microbial asparaginases have drawn particular interest because of their potential antineoplastic properties and significant application in food industries. Among the endophytes, *Serratia marcescens* cenA, *Bacillus subtilis* cenB, *B. methylotrophicus* PotA and *B. siamensis* C53 produced significant level of L-asparaginase. Their enzyme activity was determined at different incubation periods and the maximum L-asparaginase activity was noted in the culture filtrate of endophytic *S. marcescens* cenA (0.8579 IU.mL⁻¹), followed by *B. methylotrophicus* PotA (0.8379 IU.mL⁻¹) at 96 h. The study also revealed that endophytic bacteria had good antioxidant properties, with *S. marcescens* cenA showing the highest activity (IC₅₀=17±0.013 mg.mL⁻¹).

Keywords: Antioxidant, L-asparaginase, *Bacillus*, ethnomedicinal plants, endophytic bacteria, *Serratia*

Introduction

Compounds derived from natural sources play a dominant role in the discovery and development of conventional drugs for the treatment of diseases like cancer. The tribal people of North-eastern region of India are known to use plants as folk remedies for treating a variety of ailments¹. An alternative approach in investigating plant-microbe interaction and screening the associated bacteria for bioactivity potential similar to their host plants may not only reduce the unwanted destruction of ethnomedicinal plants but also provide an alternative source for the extraction of potential metabolites.

Endophytes are specific group of microorganisms that can be found residing in internal, healthy plant tissues without causing visible damage to their hosts². They produce compounds having pharmacological significance. For example, Taxol, an effective antitumor drug produced by endophytic fungi *Taxomyces andreanae* in the bark of the yew tree, *Taxus brevifolia*³. At present, there are many reports demonstrating fungal endophytes as sources of novel bioactive natural products as they occupy millions of unique biological niches growing in unusual environments⁴.

L-Asparaginase (EC 3.5.1.1) is widely used as anticancer agent⁵. It is preferred over the chemical drugs for its biodegradability and non-toxicity, and it can be administered at the local site quite easily⁶⁻⁸. Current clinical studies indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell diseases in human⁹. Administration of L-asparaginase depletes L-asparagine level at circulating plasma pool in the body, resulting in starvation of the amino acid and impaired protein synthesis^{10,11}. Apart from its therapeutic application, L-asparaginase is also being used in food industry as it is known to reduce the formation of carcinogenic acryl amides in deep fried potato recipes¹². Extracellular L-asparaginase is more advantageous than intracellular ones since they can be abundantly produced and purified economically under normal culture conditions. L-Asparaginase from bacterial source has known to be used as a therapeutic agent for the treatment of acute lymphoblastic leukaemia^{8,13}.

Recently, some studies have been conducted on the antioxidant and antitumor activity of the crude extract of ethnomedicinal plants of North-east India^{14,15}. The present study aims to investigate L-asparaginase production and antioxidant potential of the bacterial endophytes associated with the selected ethnomedicinal plants of North-east India. This is done because the isolation of microorganisms in culture is an important

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process for the study and discovery of novel biological activity from microorganisms. Hence, the endophytic bacteria were evaluated using their culture extracts for their bioproperties.

Materials and Methods

Plant Sample Collection

Healthy plants (*Centella asiatica*, *Litsea cubeba*, *Potentilla fulgens*, *Houttuynia cordata*, *Spilanthes acmella* & *Rubia cordifolia*) used by different Traditional Medicinal Practitioners (TMPs) were collected from different parts of North-east India (N-25°26.737', E-091°44.737'; N-25°36.904', E-091°54.121') based on their ethnomedicinal usages. The taxonomic identity of the plants was confirmed with the help of Herbarium Curator of the parent university. All the samples were collected in previously unused polythene bags and brought to the laboratory and used for the isolation of endophytes within 24 h of collection.

Isolation of Endophytic Bacteria

Endophytic bacteria were isolated from the healthy plant samples, devoid of any apparent pathogens as per the method previously reported^{4,16} with minor modifications. Plant parts (leaves, stems, roots, flowers & fruits) were washed thoroughly with tap water, followed by sterile double distilled water and drained before immersion in 70% ethyl alcohol for 30 sec. They were further washed with sodium hypochlorite solution (2%) for 2 min. The plant parts were drained with blotting paper and immersed in 70% ethyl alcohol again for 30 sec¹⁷. The samples were then rinsed with sterile double distilled water. Each plant sample was cut aseptically into 1 cm long segments using a sterile blade under the laminar flow hood and allowed to dry. The cut surfaces of the segments were placed on Petri plates containing Nutrient Agar (NA) and Tryptic Soya Agar (TSA) (Himedia, India). Plates were then incubated at 32°C for 48 h. Colonies with different morphology and pigmentation were randomly selected from each plate and streaked on fresh NA plates. Isolates were checked for purity by re-streaking on plates of the same medium and then transferred to nutrient agar slants and stored at 4°C. Pure isolates were preserved in 20% glycerol at -20°C for further use.

Molecular Characterization of Isolates

Total genomic DNA was extracted using HiPurA™ bacterial and yeast genomic DNA Isolation Kits

(Himedia, India). PCR amplification and sequencing of 16S rRNA gene was carried out in a 25 µL reaction mixture using general primers 27F 5'-AGAGTTTGATCCTGGCTGAG-3' and 1541R 5'-AAGGAGGTGATCCAGCCGCA-3' with the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and final step was carried out at 72°C for 5 min and 4°C till infinity. The V6-V8 hypervariable region of bacteria-specific 16S rRNA gene was amplified with the primer pair 984F 5'-AACGCGAAGAACCTTAC-3' and 1378R 5'-CGGTGTGTACAAGGCCCGGGAACG-3'¹⁸ with the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, final step was carried out at 72°C for 5 min and then 4°C till infinity using PCR Gene Amp 9700 (Applied Biosystems, USA). DNA template replaced with sterile water was used as negative control. The amplified 16S rRNA gene (approx 1400 base pairs) and V6-V8 region (approx 400 base pairs) was then purified using QIAquick Gel Extraction Spin Kit (QIAGEN, Germany). The purified PCR products were bi-directionally sequenced using both forward and reverse primers in a sequencer Genetic Analyzer (ABI 3130 Applied Biosystems, USA) with Big Dye (3.1) terminator protocol. Sequencing was performed using 20 µL reaction mixture containing about 50 ng DNA template (PCR product), 1 pmol of sequencing primer, 3.5 µL (5X) Big Dye buffer and 1 µL Big Dye. This was carried out by denaturation step at 96°C for 5 min, followed by 30 cycles at 96°C for 10 sec, 55°C (60°C for V6-V8 region) for 10 sec and final step at 96°C for 5 min. Post reaction cleanup was carried out to remove unwanted matters including unincorporated dye terminators by using 125 mM EDTA, 3 M sodium acetate and 70% ethanol and then air dried at room temperature for 45 min before keeping at -20°C. 10 µL formamide was added and then denaturation step was carried out at 96°C for 2 min and immediately kept in ice before loading into the sequencer plate. Sequencing was performed using Genetic Analyzer ABI 3130XL (Applied Biosystems, California, USA).

Phylogenetic Analysis

Sequence similarities were determined by the BLAST program against the database of type strains with validly published prokaryotic names at the EzTaxon 2.1 server¹⁹. Molecular Evolutionary

Genetics Analysis software (MEGA version 4.0)²⁰ was used for phylogenetic analysis. The sequences of identified phylogenetic neighbours were aligned with the sequences of representative strains using Clustal W inbuilt with MEGA 4. Neighbour-Joining method was employed to construct the phylogenetic tree with 1000 bootstrap replications to assess nodal support in the tree. Bootstrap values of 80% or greater were used to define well-supported clusters of the nucleotide sequences in the tree.

Screening for L-Asparaginase Production

The endophytes isolated were subjected to rapid screening of L-asparaginase production by plate assay²¹. Modified Czapek Dox's (MCD) medium was used [0.2% (w/v) glucose, 1% (w/v) L-asparagine, 0.152% (w/v) K₂HPO₄, 0.052% (w/v) KCl, 0.052% (w/v) MgSO₄.7H₂O, 0.003% (w/v) CuNO₃. 3H₂O, 0.005% (w/v) ZnSO₄.7H₂O, 0.003% (w/v) FeSO₄.7H₂O, 1.8% (w/v) agar, initial pH 6.2 was supplemented with 0.009% (v/v) phenol red as indicator]. Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase. Control plates were having modified Czapek Dox's medium without asparagine. The plates were incubated at 37°C for 72 h. The isolates that showed L-asparaginase production were selected for determination of enzyme activity.

Extraction of Crude Enzyme

Submerged fermentation for L-asparaginase production was carried out using modified Czapek Dox's liquid medium. Erlenmeyer flask containing 100 mL of medium were inoculated with screened organism. The flasks were incubated at 37°C at different incubation period (48, 72 & 96 h). Uninoculated media served as control. The cultures were harvested by centrifuging at 6,000 rpm for 15 min and the cell free supernatant was used for estimating the extracellular enzyme activity. Enzyme activity was determined at 48, 72 and 96 h.

Spectrophotometric Assay of L-Asparaginase Enzyme

L-Asparaginase activity was determined by measuring the amount of ammonia released by Nesslerization⁵. 0.1 mL of crude enzyme, 0.9 mL of 0.1 M sodium borate buffer (pH 8.5) and 1 mL L-asparagine (0.04 M) solution were combined and incubated for 10 min at 37°C. Control was maintained without addition of crude enzyme. The reaction was stopped by the addition of 0.5 mL of 15% w/v

trichloro acetic acid. After termination of the reaction, 3.7 mL volume of distilled water and 0.2 mL of Nessler's reagent were added to 0.1 mL of the above reaction mixture and incubated for 20 min. The amount of ammonia released during the reaction was determined by measuring the absorbance at 500 nm (Stuart RE300P, UK). Enzyme activity is expressed as one international unit (IU) of L-asparaginase is the amount of enzyme needed to liberate 1 μmol of ammonia in 1 min at 37°C²². Ammonium chloride was used as standard (5.349 mg.mL⁻¹).

Determination of Antioxidant Activity by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method

Each isolate was grown in 100 mL Nutrient Broth incubated at 32°C for 3 d at 120 rpm. The bacterial culture broth was centrifuged at 10000 rpm for 15 min and the supernatant was filtered using autoclaved 0.22 μm membrane filter paper. Methanol was added to the filtrate (2:1) and stirred for 24 h for extraction. The methanolic extract was filtered and concentrated using rotary evaporator (Stuart RE300P, UK) under reduced pressure at 45°C. The concentrated methanolic extract of each isolate was then diluted for antioxidant assay²³. Methanolic extract (0.1 mL) was added to 3.9 mL of a 6 × 10⁻⁵ mol.L⁻¹ methanol DPPH solution. Control consisted of 0.1 mL of distilled water added to 3.9 mL DPPH solution. The reaction mixture was vortexed thoroughly and left in the dark at ambient temperature for 30 min. Fresh DPPH solution was prepared daily and kept in the dark at 4°C between measurements. All determinations were performed in triplicates. Methanol was used to set zero blank and the absorbance was read at 517 nm (Cecil CE7200, UK). A solution of methanol DPPH without extract was used as control. The percentage radical scavenging activity was calculated using the formula:

$$\text{Scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A₀ is the absorbance of control and A₁ is the absorbance of sample extract or standard. Ascorbic acid was taken as known antioxidant for comparative analysis. Then percentage inhibitions were plotted against respective concentrations used and the extract concentration providing 50% inhibition (IC₅₀) was calculated from the standard graph. Ascorbic acid was used as standard (1 mg.mL⁻¹). The experimental results of biological activity tests were expressed as mean ± standard deviation (SD) of three replicates. The results were processed using Microsoft Excel 2007 and Origin 6.0.

Results and Discussion

L-asparaginase production was detected on the basis of formation of pink colour around the colony (Fig. 1). Among the plant associated endophytic bacteria (Table 1), *Serratia marcescens* cenA and *Bacillus subtilis* cenB associated with *Centella asiatica*, *B. siamensis* C53 associated with *Litsea cubeba* and *B. methylotrophicus* PotA associated with *Potentilla fulgens* showed positive results for L-asparaginase production. L-asparaginase activity was observed in the range of 0.6643-0.857 IU.mL⁻¹ (Fig. 2). From the experimental results, optimum L-asparaginase activity was observed at 72 to 96 h with the maximum activity noted in culture filtrate of *S. marcescens* cenA with 0.8579 IU.mL⁻¹, followed by *B. methylotrophicus* PotA with 0.8379 IU.mL⁻¹ at 96 h of incubation (Fig. 2). L-asparaginase production was found to be accompanied with the rise in pH as observed by colour change to pink in the plates. The acid-base indicator dye, phenol red converted to pink colour at basic pH. Lack of L-asparaginase in the culture medium

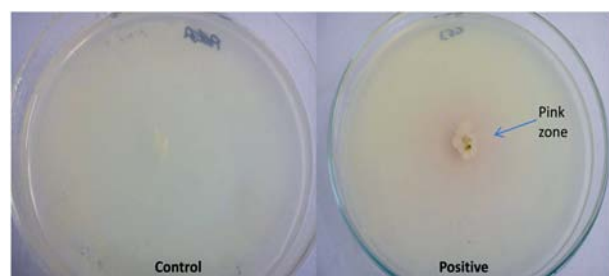


Fig. 1—Plates showing control (left) and positive result for L-asparaginase (right) where pink zone was observed surrounding bacterial growth due to L-asparaginase activity.

did not depict any colour change, which proved that colour transformation was due to L-asparaginase production⁹.

Similar to earlier reports^{24,25}, the present study has also shown that culture incubation for 96 h was the optimum time required for L-asparaginase activity²⁶. L-Asparaginase, which is currently used clinically, is extracted from bacterial sources²⁷. These findings suggest that this ecological group of bacteria isolated from ethnomedicinal plants are an excellent source of L-asparaginase and natural antioxidant that could have great relevance as therapeutic agents. In food industry, pre-treatment of unprocessed food materials with the enzyme L-asparaginase leads to the reduction of free asparagine to a significant level, thus reducing the

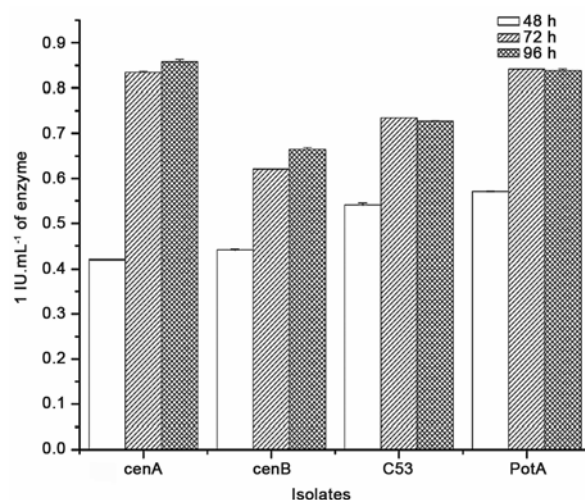


Fig. 2—L-Asparaginase activity (IU) of endophytic isolates at different incubation period (n = 3).

Table 1—L-Asparaginase production by endophytic bacteria characterized from ethnomedicinal plants

Host plant	Endophytes	GenBank acc. no.	% similarity	Nucleotide length (base pairs)	L- Asparaginase production
<i>Centella asiatica</i>	<i>Serratia marcescens</i> cenA	JN613282	99.9	1368	+
	<i>Bacillus subtilis</i> cenB	JN613283	99.8	1411	+
<i>Litsea cubeba</i>	<i>B. siamensis</i> C53	JN585960	100	401	+
	<i>Paenibacillus uliginis</i> C22	JN585959	100	373	-
<i>Rubia cordifolia</i>	<i>B. mycoides</i> M31	JX298809	99.9	1456	-
<i>Potentilla fulgens</i>	<i>B. methylotrophicus</i> PotA	JQ236632	100	1418	+
<i>Spilanthes acmella</i>	<i>Pseudomonas palleroniana</i> Y1	JQ770187	99.7	1406	-
<i>Houttuynia cordata</i>	<i>Bacillus</i> sp. F21	JX298807	100	1420	-
<i>Aloe vera</i>	<i>Herminiimonas saxobsidens</i> AA	JQ770186	99.8	1399	-

+ = Presence ; - = Absence

imminent risk of the synthesis of acrylamide²⁸. These endophytic organisms indicate a possibility of mimicking mechanisms to produce similar products as their host plants.

Methanolic extracts of endophytic isolates with L-asparaginase production were able to reduce the stable radical DPPH to a yellow-coloured diphenylpicrylhydrazine. Ascorbic acid showed the highest radical scavenging activity ($IC_{50}=0.062\pm 0.006$ mg.mL⁻¹), followed by the culture extract of the endophytic *S. marcescens* cenA ($IC_{50}=17.0\pm 0.013$ mg.mL⁻¹) (Fig. 3). In the present study, bacterial endophytes producing L-asparaginase showed better antioxidant activity than the isolates lacking L-asparaginase. Based on their phylogenetic analysis (Fig. 4), endophytes belonging to *Bacillus* spp. showed significant biological activities.

The IC_{50} value is a widely used parameter to measure the free radical scavenging activity. Lower

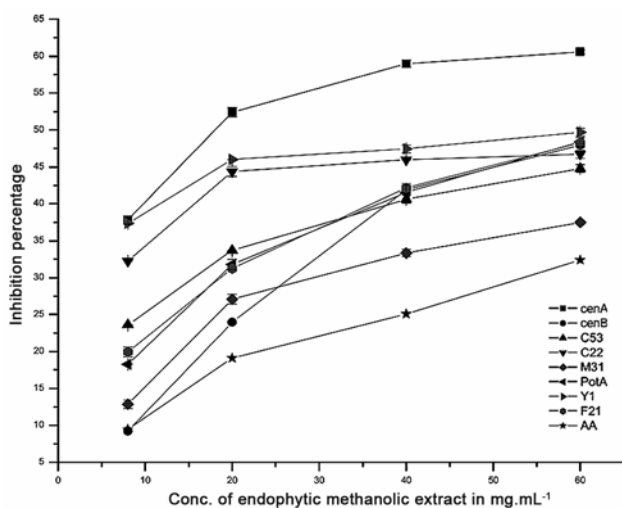


Fig. 3—DPPH radical scavenging activity of endophytic methanolic extract at different concentration (n = 3).

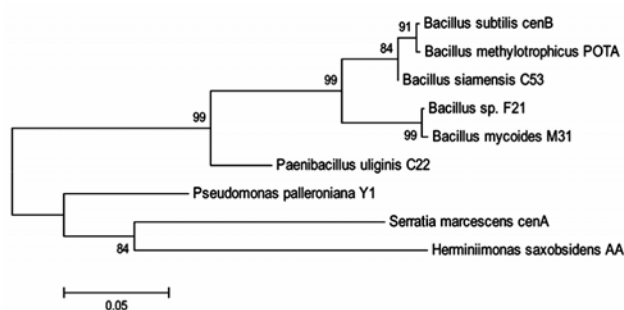


Fig. 4—Rooted neighbour-joining tree for endophytic isolates based on 16S rRNA gene sequences (the numbers at the nodes are bootstrap values based on 100 replications).

IC_{50} value indicates a higher activity²⁹. The present study revealed that isolates producing L-asparaginase also showed good antioxidant activity. However, all isolates with good DPPH radical scavenging activity does not produce L-asparaginase. *S. marcescens* and *B. methylotrophicus* showed good L-asparaginase and antioxidant activity, indicating a potential source for the production of L-asparaginase enzyme on a large scale. Agar plate assay and spectrophotometric method were compared for L-asparaginase activity and it was found that some isolates, such as, *B. methylotrophicus*, showed better enzyme activity in agar plate assay but not by Nesslerization assay, which corroborates with the earlier reports³⁰.

The major problem facing the future of endophyte biology and natural product discovery is the rapid depleting of rainforests together with the disappearance of traditionally used ethnomedicinal plants, which hold the greatest potential resource for acquiring novel microorganisms and their products. Thus, screening and optimization of bioactive compounds of industrial relevance, such as, L-asparaginase, from bacterial endophytes associated with ethnomedicinal plants will not only assist in conservation of plants but will also help in larger recovery of the microbial metabolites as products of pharmacological importance.

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References

- 1 Syiem D, Sharma R & Saio V, *In vitro* study of the antioxidant potential of some traditionally used medicinal plants of North-east India and assessment of their total phenolic content, *Pharmacologyonline*, 3 (2009) 952-965.
- 2 Hallmann J, Quadt-Hallmann A, Mahaffee W F & Kloepper J W, Bacterial endophytes in agricultural crops, *Can J Microbiol*, 43 (1997) 895-914.
- 3 Pezzuto J, Taxol production in plant cell culture comes of age, *Nat Biotechnol*, 14 (1996) 1083.
- 4 Strobel G, Daisy B, Castillo U & Harper J, Natural products from endophytic microorganisms, *J Nat Prod*, 67 (2004) 257-268.

- 5 Wriston J C Jr & Yellin T O, L-Asparaginase: A review, *Adv Enzymol Relat Areas Mol Biol*, 39 (1973) 185-248.
- 6 Ferrara M A, Mattoso J M V, Bon E P S & Pereira N, Kinetics of asparaginase II fermentation in *Saccharomyces cerevisiae* ure2dal80 mutant: Effect of nitrogen nutrition and pH, *Appl Biochem Biotechnol*, 113-1156 (2004) 299-305.
- 7 Stecher A L, de Deus P M, Polikarpov I & Abrahao-Neto J, Stability of L-asparaginase—An enzyme used in leukemia treatment, *Pharm Acta Helv*, 74 (1999) 1-9.
- 8 Verma N, Kumar K, Kaur G & Anand S, L-Asparaginase: A promising chemotherapeutic agent, *Crit Rev Biotechnol*, 27 (2007) 45-62.
- 9 Singh Y & Srivastava S K, Screening and characterization of microorganisms capable of producing antineoplastic drug, L-asparaginase, *Int J Biol Med Res*, 3 (2012) 2548-2554.
- 10 Keating M J, Holmes R, Lerner S & Ho D H, L-Asparaginase and PEG asparaginase—Past, present and future, *Leuk Lymphoma*, 10 (1993) 153-157.
- 11 Kotzia G A & Labrou N E, L-Asparaginase from *Erwinia chrysanthemi* 3937: Cloning, expression and characterization, *J Biotechnol*, 127 (2007) 657-669.
- 12 Kornbrust B A, Stringer M A, Lange N E K & Hendriksen H V, Asparaginase—An enzyme for acrylamide reduction in food products, in *Enzymes in food technology*, 2nd edn, edited by R J Whitehurst and M V Oort (Wiley-Blackwell, Oxford) 2010, 59-87.
- 13 Gallagher M P, Marshall R D & Wilson R, Asparaginase as a drug for treatment of acute lymphoblastic leukaemia, *Essays Biochem*, 24 (1999) 1-40.
- 14 Radhika M, Ghoshal N & Chatterjee A, Comparison of effectiveness in antitumor activity between flavonoids and polyphenols of the methanolic extract of roots of *Potentilla fulgens* in breast cancer cells, *J Complement Integr Med*, 9 (2012) 24. [doi: 10.1515/1553-3840.1644]
- 15 Syiem D, Syngai C, Kharbuli B, Kayang H & Khongwir B S, Anti-tumor activity of crude root extract of *Potentilla fulgens*, *Indian Drugs*, 40 (2003) 124-125.
- 16 Long H H, Furuya N, Kurose D, Takeshita M & Takanami Y, Isolation of endophytic bacteria from *Solanum* sp. and their antibacterial activity against plant pathogenic bacteria, *J Fac Agric Kyushu Univ*, 48 (2003) 21-28.
- 17 Barzanti R, Ozino F, Bazzicalupo M, Gabbrielli R, Galardi F *et al*, Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant *Alyssum bertolonii*, *Microb Ecol*, 53 (2007) 306-316.
- 18 Das M, Royer T V & Leff L G, Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream, *Appl Environ Microbiol*, 73 (2007) 756-767.
- 19 Chun J, Lee J H, Jung Y, Kim M, Kim S *et al*, EzTaxon: A web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences, *Int J Syst Evol Microbiol*, 57 (2007) 2259-2261.
- 20 Tamura K, Dudley J, Nei M & Kumar S, MEGA4: Molecular evolutionary genetics analysis (MEGA) software, version 4.0, *Mol Biol Evol*, 24 (2007) 1596-1599.
- 21 Gulati R, Saxena R K & Gupta R, A rapid plate assay for screening L-asparaginase producing micro-organisms, *Lett Appl Microbiol*, 24 (1997) 23-26.
- 22 Imada A, Igarasi S, Nakahama K & Isono M, Asparaginase and glutaminase activities of micro-organisms, *J Gen Microbiol*, 76 (1973) 85-99.
- 23 Brand-Williams W, Cuvelier M E & Berset C, Use of a free radical method to evaluate antioxidant activity, *LWT-Food Sci Technol*, 28 (1995) 25-30.
- 24 Theantana T, Hyde K D & Lumyong S, Asparaginase production by endophytic fungi from Thai medicinal plants: Cytotoxicity properties, *Int J Integr Biol*, 7 (2009) 1-8.
- 25 Upadhyay R, Saxena A & Kango N, Screening and production of tumour inhibitory L-asparaginase by bacteria isolated from soil, *Asian J Pharm Clin Res*, 5 Suppl (2012) 135-137.
- 26 Soniyamby A R, Lalitha S & Praveesh B V, Isolation and screening of L-asparaginase producing fungi from soil samples, *Int J Pharm Pharm Sci*, 4 (2012) 279-282.
- 27 Schrey D, Borghorst S, Lanvers-Kaminsky C, Hempel G, Gerb J *et al*, Therapeutic drug monitoring of asparaginase in the ALLBFM 2000 protocol between 2000 and 2007, *Pediatr Blood Cancer*, 54 (2010) 952-958.
- 28 Lindsay R C & Jang S, Chemical intervention strategies for substantial suppression of acrylamide formation in fried potato products, *Adv Exptl Med Biol*, 561 (2005) 393-404.
- 29 Maisuthisakul P, Suttajit M & Pongsawatmanit R, Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants, *Food Chem*, 100 (2007) 1409-1418.
- 30 Holker U, Hofer M & Lenz J, Biotechnological advantages of laboratory-scale solidstate fermentation with fungi, *Appl Microbiol Biotechnol*, 64 (2004) 175-186.