Ann Microbiol (2012) 62:1609–1614
DOI 10.1007/s13213-011-0417-0

ORIGINAL ARTICLE

Production and amplification of an L-asparaginase gene from actinomycete isolate *Streptomyces* ABR2

Ankit P. Sudhir · Bhaumik R. Dave · Kalkal A. Trivedi · Ramalingam B. Subramanian

Received: 6 October 2011 / Accepted: 22 December 2011 / Published online: 19 January 2012

Abstract L-Asparaginase is used as a therapeutic molecule for the treatment of acute lymphoblastic leukaemia (ALL). It is also used in the food industry to remove acrylamide from fried starch-based foods. In an attempt to identify novel organisms as sources of this enzyme, 32 different actinomycetes isolates were obtained from soil samples near Vallabh Vidyanagar, India. From these 32 isolates, 11 L-asparaginase-positive isolates were obtained with a rapid plate assay method. The maximum L-asparaginase producing strain was identified as *Streptomyces* sp. by 16S r-RNA sequencing. The effect of inoculum size, pH and L-asparagine concentration on L-asparaginase production was also checked. An increase in L-asparaginase activity from 12.5 IU/mL to 18.8 IU/mL was found. The gene coding for L-asparaginase (~800 bp) was isolated from *Streptomyces* sp. ABR2.

Keywords Actinomycetes · L-Asparaginase activity · 16S rRNA analysis · *Streptomyces* sp. · Gene amplification

Introduction

L-Asparaginase (EC 3.5.1.1) is an effective antineoplastic agent used in the chemotherapy of acute lymphoblastic leukemia (ALL) (Azmi et al. 2007) for eliminating it from the extraneous cell environment. The enzyme catalyses the hydrolytic conversion of L-asparagine to L-aspartic acid and ammonia. Many investigators have reported that L-asparaginase inhibits tumour growth in mouse, rat, dog and human through the inhibition of tumour-specific cells (Broome 1961, 1965; Mashburn and Wriston 1964; Berenbaum 1970). Bacterial L-asparaginase has been the subject of considerable medical interest and is being employed in the therapy of ALL (Peterson and Ciegler 1969). *Escherichia coli* and *Erwinia chrysanthemi* asparaginases have been used successfully in the treatment of leukemia for the last 40 years (Fu and Sakamoto 2007), and L-asparaginase from *E. coli* and *E. carotovora* is currently in clinical use for the treatment of ALL (Savitri and Azmi 2003). However, L-asparaginase from bacterial sources often causes hypersensitivity in long-term use, leading to allergic reactions and anaphylaxis. Like bacteria, actinomycetes are also a good source for the production of L-asparaginase (Dhevendaran and Annie 1999; Narayana et al. 2008). Among the actinomycetes, several *Streptomyces* species such as *S. karnatakensis*, *S. venezualae*, *S. longisporusflavus* and a marine *Streptomyces* sp. PDK2 have been explored for L-asparaginase production (Narayana et al. 2008). There are also reports of L-asparaginase production from some *Streptomyces* sp. isolated from the gut of the fish *Therapon jarbua* and *Villorita cyprinoides*, *Streptomyces gulbargensis* and *Streptomyces noursei* MTCC 10469 (Dhevendaran and Anithakumari 2002; Amena et al. 2010; Dharmaraj 2011). Among the various sources of L-asparaginase, actinomycetes are the least studied organisms. Much research is being done on these organisms for they have gained attention as rich sources of antibiotics, anti-tumour drugs and other bioactive molecules.

As a result, the search for novel sources of L-asparaginase, with low toxicity is of great current scientific interest. Asparaginases were introduced in food technology after Swedish studies revealed the ubiquitous occurrence of acrylamide in commonly consumed starch-based foods that were baked, roasted or fried (Tareke et al. 2002). Asparaginase promises to be a potential way to reduce the amount of free asparagine

A. P. Sudhir · B. R. Dave · K. A. Trivedi · R. B. Subramanian (✉)
BRD School of Biosciences, Sardar Patel University,
Satellite Campus, Sardar Patel Maidan, Vadtal Road, PO Box 39,
Vallabh Vidyanagar 388 120, Gujarat, India
e-mail: asp.fus@gmail.com

in the starting materials of food production, thus reducing the imminent risk of generating acrylamide, a potentially carcinogenic and neurotoxic compound (Friedman 2003).

Materials and methods

Sample collection

Various soil samples were collected from different places in and around Vallabh Vidyanagar with the help of a sterile scalpel at 10 cm depth from the soil surface. These soil samples were collected in sterile polythene bags.

Isolation and screening

Different soil samples were serially diluted in sterile distilled water under aseptic conditions. These were then plated on actinomycete isolation agar (AIA) (Himedia, Mumbai, India) plates and incubated at 30°C for 7 days. After 7 days, suspected actinomycete colonies were selected based on their morphological characteristics. The actinomycetes thus obtained were grown and preserved on starch casein agar (SCA) and yeast malt extract agar (YMA) slants. Screening of potential L-asparaginase producing actinomycetes was carried out with the use of asparagine dextrose salts Agar (ADS Agar) (asparagine 1.0%, dextrose 0.2%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, agar 1.5%) containing 0.009% phenol red as a pH indicator (Gulati et al. 1997). Isolates exhibiting L-asparaginase activity were selected for further study on the basis of a change in the colour of the medium.

Submerged fermentation method

Isolates found to be positive for asparaginase were inoculated separately in 100 mL tryptone glucose yeast extract (TGY) broth (pH 7) (Basha et al. 2009) comprising of glucose, 0.1 g; K_2HPO_4, 0.1 g; yeast extract, 0.5 g; tryptone, 0.5 g; contained in a 250 mL Erlenmeyer flask and incubated at 30°C in a shaker-incubator oscillating at 120 rpm. Daily assays were carried out in a submerged culture for 5-days to determine the change in the enzyme activity every 24 h. The isolate showing maximum L-asparaginase activity was subjected to further studies using the submerged culture technique.

Determination of enzyme activity

Crude enzyme preparation was used for dertermination of enzyme activity. The crude enzyme was prepared by centrifuging the broth at 10,000 rpm for 20 min (Davidson et al. 1977). The cell-free supernatant was taken as the crude enzyme. The enzyme activity was determined in culture broth by asparaginase assay (Imada et al. 1973) using Nessler's reagent. One international unit (IU) of L-asparaginase is the amount of enzyme that liberates one micromole of ammonia in 1 min at 37°C.

Strain identification of selected organism

The selected strain was identified by sequencing the 16S r-RNA gene.

Isolation of total genomic DNA

Total genomic DNA was extracted using a modified cetryltrimethylammonium bromide (CTAB)-NaCl protocol (Kieser et al. 2000).

16S r-RNA amplification by polymerase chain reaction

The genomic DNA was subjected to PCR using universal primers 8 F (5′-AGAGTTTGATCCTGGCTCAG- 3′) and reverse primer 1492 R (5′-GGTTACCTTGTTACGACTT-3′) specific for the 16S r-RNA gene sequence. The final volume of the reaction was 12.5 μL, which comprised 1.3 μL 10x buffer (2.5 mM), 1.0 μL dNTPs, 0.3 μL *Taq* polymerase (3U/μL), 1.0 μL template DNA, 1.0 μL forward primer (10 pM), 1.0 μL reverse primer (10 pM), 6.9 μL Milli Q water. All the reagents used were procured from (Merck, Mumbai, India).

Template denaturation was carried out at 94°C for 1 min; primer annealing was carried out for 1 min at 55°C and extension was carried out at 72°C for 2 min for 35 cycles. The initial template denaturation was carried out at 94°C for 2 min while the final extension cycle was carried out at 72°C for 10 min. The annealing temperature was selected according to the primer pair.

The amplified product was separated in a 1.2% agarose gel by electrophoresis. The amplicon so obtained was eluted with the help of Gel Extraction Kit, (Merck). The amplified 16S r-RNA product was sequenced. The sequences obtained were used as query for Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/) in order to identify the strain. Sequences were aligned to the representative actinomycete 16S r-RNA and neighbour joining tree was constructed using Molecular Evolution Genetic Analysis (MEGA, version 4).

PCR amplification of L-asparaginase gene

The gene for L-asparaginase was amplified using different primer pairs generated on the basis of the sequences of L-asparaginase gene of the same genus. All these primer pairs were supplied by Integrated DNA Technologies (http://eu.idtdna.com).

Good quality genomic DNA was used as the template for PCR. The final volume of the reaction was 12.5 μL, which comprised of 1.3 μL 10x buffer (2.5 mM), 1.0 μL of 2.5 mM dNTPs, 0.3 μL *Taq* polymerase (3U/μL), 1.0 μL template DNA, 1.0 μL forward primer (10 pM), 1.0 μL reverse primer (10 pM), 6.9 μl Milli Q water. All reagents used were from Merck Genei, Bangalore, India. The PCR conditions were same as used for amplification of 16S r-RNA.

The amplified product was separated using 1.2% agarose gel electrophoresis.

Gel elution of PCR product

A Merck Gel extraction kit was used for the elution of PCR product from the agarose gel. The product so obtained was rechecked on an agarose gel by loading a minimum amount of the eluted product. The eluted PCR product was sent for sequencing.

Optimisation of inoculum size

Autoclaved detergent suspension of Triton X-100 was used to scrape off the colonies grown on AIA slants. The spore suspension obtained was diluted in sterile distilled water to a manageable level and spores were counted using a Neubauer's chamber under 40X magnification with a light microscope. 10^6, 10^7, 10^8 and 10^9 spores were then inoculated into the TGY broth for submerged culture.

Optimisation of pH

TGY broths of pH 6.0, 7.0, 8.0 and 9.0 were prepared to check the effect of pH on asparaginase production. They were inoculated with the optimum inoculum size by the spore count method.

Effect of L-asparagine concentration on L-asparaginase production

TGY broths incorporating different concentrations of L-asparagine (0.5, 1.0, 1.5 and 2.0 %) as an N source were used to check for stimulation of L-asparaginase production.

Results and discussion

Isolation

A total of 32 different actinomycete isolates were obtained from the various soil samples collected from in and around Vallabh Vidyanagar. The isolates were selected on the basis of their peculiar morphology on solid agar media and corroboration was provided by performing Gram's staining of the suspected actinomycetes. These were then screened for L-asparaginase production.

Screening

Each isolate was subjected to screening on ADS agar containing 0.009% phenol red dye as an indicator. Phenol red at acidic pH has a yellow colour and at alkaline pH turns pink (Gulati et al. 1997). The colour of phenol red exhibits a gradual transition from yellow to red over the pH range 6.8 to 8.2. As a result of the ammonia produced due to the breakdown of L-asparagine, medium containing phenol red turns pink in the presence of L-asparaginase-producing organisms. Isolates exhibiting L-asparaginase activity were selected for further study on the basis of a change in the colour of the medium. They were subjected to further studies using a submerged culture technique.

Determination of enzyme activity

Of the 32 isolates obtained, 11 were found to produce L-asparaginase by the primary screening method. These actinomycete isolates were inoculated in TGY broth by the agar plug technique. The enzyme activity was determined in culture broth using Nessler's reagent (Imada et al. 1973). One international unit (IU) of L-asparaginase is the amount of enzyme that liberates 1 μmol ammonia min^{-1} at 37°C. Isolate 5 showed maximum L-asparginase activity (Fig. 1). The annotated L-asparaginases of *S. coelicolor, S. avermitilis* and *S. griseus* are distributed within cells and possess no signal sequences for secretion. Hatanaka et al. (2008) reported that amino peptidases were secreted at high levels into the medium even though they lacked signal peptides for secretion. It was observed in the present study also that L-asparaginase activity was higher in the medium, which supports the observations of Hatanaka et al. (2008).

Identification of selected organism

The quality and quantity of DNA was checked by agarose gel electrophoresis and UV absorption method, respectively.

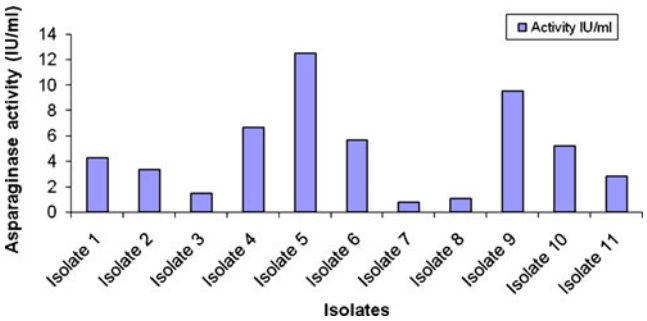

Fig. 1 L-Asparaginase activity of the screened isolates

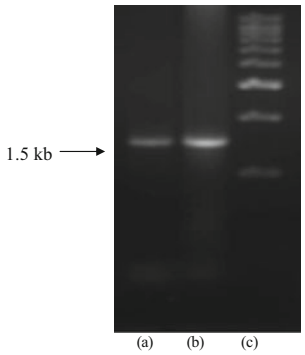

Fig. 2 PCR amplification of 16S r-RNA from genomic DNA of selected organism. Lanes: *a*, *b* 1.5 kb gene amplified band; *c* molecular weight markers (1 kb step up marker)

Genomic DNA thus obtained was used for the 16S r-RNA amplification by PCR using universal primers.

The amplified product was found to be 1.5 kb in size (Fig. 2). Stackebrandt et al. (1991) reported the molecular identification of several *Streptomyces* species by 16S r-RNA gene amplification. The amplified product was eluted from the agarose gel successfully and sequenced at Xcelris Labs (Ahmedabad, India). The sequences thus obtained were used as a query for Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/). This was used to compare the homology between the 16S r-RNA sequences of various already characterised organisms and our query sequences (Fig. 3). Isolate 5 was found to have maximum sequence homology with *Streptomyces* A310 Ydz-XM, showing 99% identity. The sequence thus obtained was submitted to GenBank as *Streptomyces* ABR2 with accession number JN256028.1 and GI: 343126488.

Optimisation parameters

Effect of inoculum size on L-asparaginase production

Amena et al. (2010) reported maximum L-asparaginase production with an inoculum size of $1x10^8$ spores of *Streptomyces gulbargensis*. The inoculum size in the current study was varied by the use of the spore count method. The broths were inoculated with 10^6, 10^7, 10^8 and 10^9 spores, respectively.

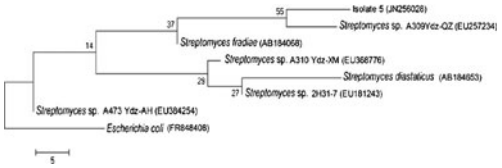

Fig. 3 Phylogenetic tree produced using the 16S r-RNA gene sequences corresponding to isolate 5 and other members of the genus *Streptomyces* showing that isolate 5 falls within the *Streptomyces* genus. *Escherichia coli* was used as outgroup in the neighbour-joining method of MEGA 4

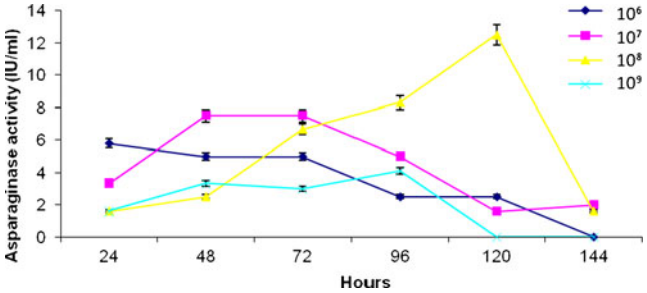

Fig. 4 Effect of inoculum size on L-asparaginase production

The optimum inoculum size was found to be 10^8 spores (Fig. 4), yielding maximum L-asparaginase activity of 12.5 IU/mL.

Effect of pH on L-asparaginase production

The *Streptomyces* strain under study was subjected to a wide pH range of culture media (pH 6.0, 7.0, 8.0 and 9.0). It was found that the strain grows well at a neutral pH of 7.0 and any sharp increase or decrease in the pH leads to an inhibition of the growth of the organism and thus of production of L-asparaginase (Fig. 5). Khamna et al. (2009) showed maximum L-asparaginase production by actinomycete strain CMU H002 at pH 7.0. Cedar and Schwartz (1968) reported *E. coli* giving maximum L-asparaginase production between pH 7 and pH 8.

Effect of L-asparagine on L-asparaginase production

This study was carried out based on the earlier reports that synthetic medium with asparagine as an N source stimulated more enzyme production than natural medium by *Streptomyces* (Mostafa and Salama 1979). Thus, media containing different concentrations (0.5–2.0%) of L-asparagine were used in the study. At a concentration of 1.0% of L-asparagine in the culture medium, the isolate *Streptomyces* ABR2 showed maximum L-asparaginase activity of 18.8 IU/mL (Fig. 6).

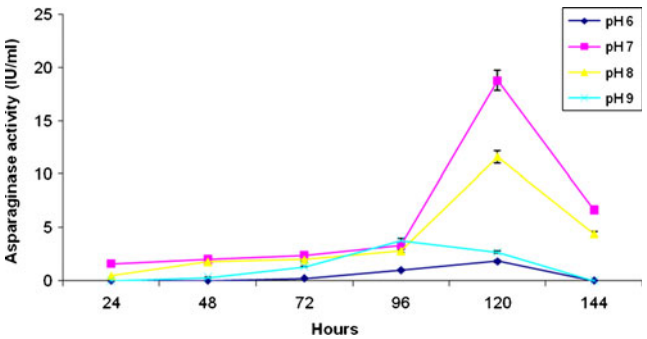

Fig. 5 Effect of pH on L-asparaginase production

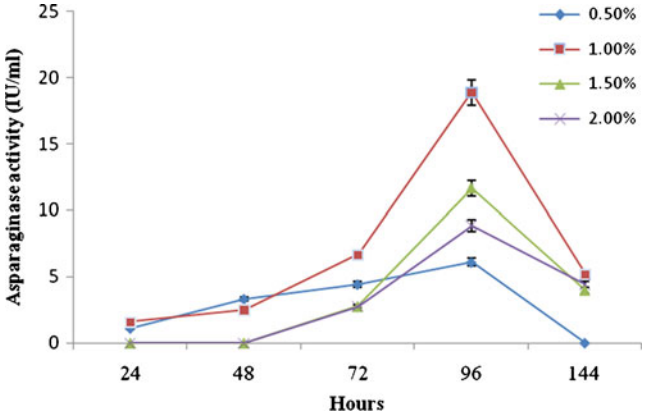

Fig. 6 Effect of L-asparagine concentration on L-asparaginase production

Amplification of L-asparaginase gene

The gene for L-asparaginase in *E. coli* was reported by Wang et al. (2001) to be 1,044 base pairs long. The L-asparaginase genes from *Streptomyces griseus*, *Streptomyces coelicolor* and *Streptomyces avermitilis* have already been sequenced.

The gene for L-asparaginase was amplified using different primer pairs generated on the basis of the sequences of the L-asparaginase gene from *S. griseus*, *S. avermitilis* and *S. coelicolor*. Sequence details of the L-asparaginase genes from the above *Streptomyces* strains were obtained from NCBI GenBank. Using these sequences, different primer pairs were designed manually as well as with the help of the primer generation tool PRIMER3 (Table 1). Manual primers were generated in order to cover the full length of the L-asparaginase gene from the L-asparaginase gene sequences of the respective *Streptomyces* sp., as full gene length primer pairs for L-asparaginase could not be generated with the primer generation tool PRIMER3. Thus, primer pairs designed with the help of PRIMER3 tool that covered the maximum possible gene length were selected for L-asparaginase gene amplification.

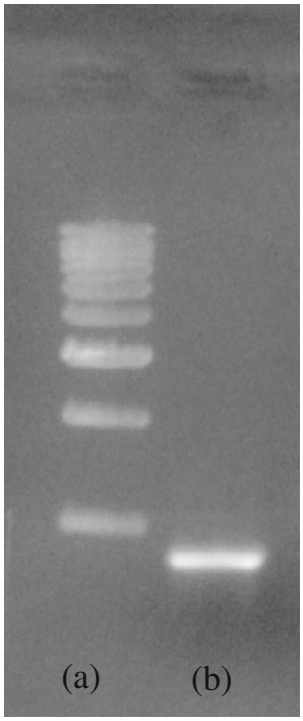

Fig. 7 Amplification of L-asparaginase gene. The expected product size of 800 bp was confirmed by agarose gel electrophoresis. Lanes: *a* 1 kb step up marker, *b* 800 bp amplicon

One of the primer pairs designed with the help of PRIMER3 from *Streptomyces coelicolor* showed amplification of the L-asparaginase gene in *Streptomyces* ABR2. This set of primers gave specific amplification, producing a sharp band on an agarose gel, while the other primer pairs were found to be non-specific as they either amplified the desired product too inefficiently, or they gave non-specific amplification. The expected product size for this primer pair was 808

Table 1 Primer pairs designed using L-asparaginase gene sequences of *Streptomyces coelicor*, *Streptomyces avermitilis* and *Streptomyces griseus*. The primer pairs were designed manually and with the help of the primer designing tool PRIMER3. Sequences of L-asparaginase genes were obtained from NCBI GenBank. All primer pairs were used for L-asparaginase gene amplification; primer pair (2) showed specific amplification

Sequence source	Primer pair no.	Method used for primer design	Forward and reverse sequence	Primer sequence (5′ to 3′)
S. coelicolor	1	Manual	F	ATGCAGCCGTCACACCCGT
			R	TCAGAAGGACGCCCGGATCTC
	2	Primer3	F	CCGCGTTCCTCCAACAAG
			R	AGAAGGACGCCCGGATCT
S. avermitilis	3	Manual	F	ATGTCCGCCCCTGCCACG
			R	TCAGAACGCCGCCCGGATC
	4	Primer3	F	GAGGTCGTGCGTTCCGGCT
			R	TCAGAACGCCGCCCGGATC
S. griseus	5	Manual	F	ATGACGTCCACCGACGCCCC
			R	TTCCCCGACCTCGGCCGCC
	6	Primer3	F	CCCGTCCGCCATATCCTC
			R	GCGAATTTCCCCGACCTC

base pairs, and nearly 800 base pairs of specific amplification was obtained, as confirmed by agarose gel electrophoresis (Fig. 7). The PCR product thus obtained was eluted from the gel using a Merck gel extraction kit and sequenced. The sequence thus obtained was fed as a query sequence in Nucleotide BLAST (http://ncbi.nlm.nih.gov./). The result of the BLAST query revealed 99% sequence similarity with the gene sequence from which the Primer pair was generated. The L-asparaginase gene sequence was submitted to GenBank under the accession no. JN399994.

Conclusions

Among the various organisms screened for L-asparaginase, actinomyces are the least studied. The present study thus focussed on L-asparaginase from actinomycetes. Among the different isolates, *Streptomyces* ABR2 showed highest L-asparaginase activity. Production optimisation parameters like pH, inoculum size and L-asparagine concentration, were also checked to enhance L-asparaginase production. Optimum pH and inoculum size were found to be pH 7 and 10^8 spores, respectively. Incorporation of L-asparagine into the medium also increased L-asparaginase production, and 1.0% L-asparagine showed maximum activity. Isolation of the gene encoding L-asparaginase was attempted using six different primer pairs designed manually as well as with the help of a primer generation tool, PRIMER3. An amplicon of 800 bp was obtained and BLAST results showed 99% sequence homology with the L-asparaginase gene of *Streptomyces coelicolor*. To the best of our knowledge, there are very few reports showing L-asparaginase gene amplification from *Streptomyces* sp.

Acknowledgement The University Grant Commission, New Delhi is highly acknowledged for providing financial support to carry out this work.

References

Amena S, Vishalaksh N, Prabhakar M, Dayanand A, Lingappa K (2010) Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. Braz J Microbiol 41:173–178

Azmi W, Narta UK, Kanwar SS (2007) Pharmacological and clinical evaluationof L-asparaginase in the treatment of leukemia. Crit Rev Oncol Hematol 61:208–221

Basha SN, Rekha R, Komala M, Ruby S (2009) Production of extracellular anti-leukemic enzyme L-asparaginase from marine actinomycetes by solid state and submerged fermentation: purification and characterization. Trop J Pharm Res 8(4):353–360

Berenbaum MC (1970) Immunosuppression by L-asparaginase. Nature 225:550–552

Broome JD (1965) Antilymphoma activity of L-asparaginase in vivo. Clearance rates of enzyme preparations from guinea pig serum and yeast in relation to their effect on tumor growth. J Natl Cancer Inst 35:967–974

Broome JD (1961) Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. Nature 191:1114–1115

Cedar H, Schwartz JH (1968) Production of L-asparaginase II by *Escherichia coli*. J Bacteriol 96(6):2043–2048

Davidson L, Brear DR, Wingard P, Hawkins J, Kitto GB (1977) Purification and properties of an L-glutaminase-L-asparaginase from *Pseudomonas acidovorans*. J Bacteriol 129:1379–1386

Dharmaraj S (2011) Study of L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge *Callyspongia diffusa*. Iran J Biotechnol 9(2):102–108

Dhevendaran K, Annie K (1999) Antibiotic and L-asparaginase fo Streptomycetes isolated from fish, shellfish, and sediments of veli estuarine along Kerala Coast. Indian J Mar Sci 28:335–337

Dhevendaran K, Anithakumari YK (2002) L-asparaginase activity in growing conditions of *Streptomyces* sp.associated with *Therapon jarbua* and *Villorita cyprinoides* of Veli Lake, South India. Fishery Technol 39(2):155–159

Friedman M (2003) Chemistry, biochemistry, and safety of acrylamide. A review. J Agric Food Chem 51:4504–4526

Fu CH, Sakamoto KM (2007) PEG-asparaginase. Expert Opin Pharmacother 8(12):1977–1984

Gulati R, Saxena RK, Gupta R (1997) A rapid plate assay for screening L-asparaginase producing micro-organisms. Lett Appl Microbiol 24:23–26

Hatanaka T, Onaka H, Arima J, Uraji M, Uesugi Y, Usuki H, Nishimoto Y, Iwabuchi M (2008) pTONA5: a hyperexpression vector in *Streptomyces*. Prot Expr Purif 62:244–248

Imada A, Igarasi S, Nakahama K, Isono M (1973) Asparaginase and glutaminase activities of micro-organisms. J Gen Microbiol 76:85–99

Khamna S, Yokota A, Lumyong S (2009) L-Asparaginase production by actinomycetes isolated from some Thai medicinal plant rhizosphere soils. Int J Integr Biol 6:1–22

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (eds) (2000) Practical Streptomyces genetics. John Innes Centre, Norwich, England

Mashburn LT, Wriston JC (1964) Tumor inhibitory effect of L-asparaginase from *Escherichia coli*. Arch Biochem Biophys 105:450–452

Mostafa SA, Salama MS (1979) L-Asparaginase producing *Streptomyces* from soil of Kuwait. Zentralbl Bakteriol 134:325–334

Narayana KJP, Kumar KG, Vijayalakshmi M (2008) L-Asparaginase production by *Streptomyces albidoflavus*. Ind J Microbiol 48:331–336

Peterson RE, Ciegler A (1969) L-Asparaginase production by *Erwinia aroideae*. J Appl Microbiol 18:64–67

Savitri NA, Azmi W (2003) Microbial L-asparaginase: a potent antitumour enzyme. Indian J Biotechnol 2:184–194

Stackebrandt E, Liesack W, Webb R, Witt D (1991) Towards a molecular identification of *Streptomyces* species in pure culture and in environmental samples. Actinomycetology 5(1):38–44

Tareke E, Rydberg P, Karlsson P, Eriksson S, Törnqvist M (2002) Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J Agric Food Chem 50:4998–5006

Wang Y, Qian S, Meng G, Zhang S (2001) Cloning and expression of L-asparaginase gene in *Escherichia coli*. Appl Biochem Biotechnol 95:93–103