Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Development of new medium composition for enhanced production of L-asparaginase by *Aspergillus flavus*

Krishna Raju Patro, Uday Chand Basak, Ajay Kumar Mohapatra and Nibha Gupta*

Regional Plant Resource Centre, Bhubaneswar- 751 015, India

*Corresponding Author E-mail: nguc2003@yahoo.co.in

Publication Info

Paper received:
10 September 2012

Revised received:
01 March 2013

Re-revised received:
10 April 2013

Accepted:
03 June 2013

Abstract

The present study on L-asparaginase from *Aspergillus flavus* was aimed to optimize the culture and nutritional conditions in order to obtain enhanced enzyme production under batch culture. *Aspergillus flavus* was grown in different combination of carbon and nitrogen source and L-asparaginase from its biomass was estimated. Finally, L- asparaginase extracted from cell biomass was purified through gel filtration and ion exchange chromatography and characterized for its purity, molecular weight, K_m value thermal tolerance and pH requirement. The fungal species preferred starch and sorbitol as carbon and L-asparagine and L- glutamine as nitrogen source. Addition of ionic compounds or basal nitrogen sources did not help in enhancing enzyme activity. Gel filtration and ion exchange chromatography produced pure enzyme which was later confirmed through PAGE with molecular weight 100.0 kDa. L – asparaginase from *Aspergillus flavus* preferred L-asparagine as substrate, 7 pH , thermally stable upto 50°C and showed $0.806x10^{-3}$ M K_m. L –asparaginase from bacterial origin sometimes cause severe allergic reactions. Search of new sources especially of fungal origin may be useful in development of potential drug. The media composition given in the present study can be further used in continuous fermentation for large scale production of L – asparaginase.

Key words

Aspergillus flavus, L-asparaginase, Microbial enzymes

Introduction

The enzyme L-asapragine amino hydrolase, which catalyzes hydrolysis of L–asparagines into L- aspartic acid and ammonia (Hosmani and Kalliwal, 2011) . L-asparaginase has proved to be particularly promising for treating acute lymphocytic leukemia (Soniyamby *et al*., 2011; Siddalingeshwara and Lingappa, 2011). Microbes are significant source of enzyme, as they can be easily cultured for extraction and purification of desired substances (Gupta *et al.,* 2009; Savitri and Asthana, 2003; Shah *et al.,* 2010) .Asparaginase from two bacterial source (*E. coli* and *Erwinina carotovora*) is currently in clinical use for the treatment of acute lymphoblastic leukemia (Mohana *et al.*, 2011). But long term use of L- asparaginase from bacterial origin can cause hypersensitivity, leading to allergic reactions and anaphylaxis (Reynolds and Taylor, 1993). Under this condition, enzymes from eukaryotic microbes like yeast and filamentous fungi could be abetter option (Theantana *et al*., 2007; Lapmark *et al*., 2010; Jayramu and Hemalatha, 2010; Siddalingeshwara and Lingappa, 2010; Sarquis *et al.,* 2004). Evaluation of nutritional requirements and cultural conditions is an important step for bioprocess development (Venil *et al*., 2009; Baskar and Ranganathan, 2009 and 2011). Carbon and nitrogen ratio and their sources also play an important role in the production of this enzyme (Sreenivasulu *et al*., 2009; Chandra *et al*., 2010; Warangkar and Khobragade, 2009). Nitrogen sources like proline, glutamic acid and sodium nitrate is reported to affect production of extracellular L-asparaginases (Baskar and Ranganathan, 2009). Glucose for instance also play a vital role in gene regulation for L- asparaginase production as in *E. coli* (Ghasemi and Ebrahiminezhad, 2008). The objective of this study was to enhance the L-asparaginase production from *Aspergillus flavus* by optimizing different cultural and nutritional conditions. Further the objective was to characterize the enzyme for its substrate specificity, thermal tolerance and K_m after its purification through chromatography techniques.

Materials and Methods

Aspergillus flavus was obtained from the culture collections of Microbiology Laboratory of Regional Plant Resources Centre, Bhubaneswar, Odisha. The fungal isolate was identified through sequence analysis with NCBI sequence accession HQ240106 by using 500 bp of r DNA (amplified by universal primer) with the help of Agharkar Research Institute (ARI), Pune.

Determination of enzyme activity under different carbon and nitrogen sources : A single 5mm disc of inoculum derived from culture plates (4 days old) were inoculated into 150ml volumetric flask containing 50 ml of glucose – asparagine broth (Glucose – 1%, L asparagine-0.5%, KH_2PO_4 -0.1% and $MgSO_4$-0.05%, pH 4.5) and incubated at 30ºC for 10days in static condition as control. Similarly, the fungus was grown with different carbon and nitrogen sources by keeping basic constituents constant. Effect of salt and ionic compounds was studied by adding them individually in medium containing selected carbon and nitrogen sources along with KH_2PO_4 and $MgSO_4$ (0.1%). Finally, a combination of carbon and nitrogen sources were selected and a modified medium was obtained. The fungus was then cultivated in the modified broth medium at different pH level, ranging between 4-9 and incubated at 37º C for 10 days in order to determine the pH requirement. Similarly, cultures were grown in the modified media and were harvested at an interval of 4 days' up to 16 days to know the suitable incubation period in both shake flask and static condition at 37º C. Statistical analysis for standard deviation of three replications was performed according to Sokal and Rohlf (1973).

Production and isolation of crude enzyme : Fungal strain was grown in modified media at 30 °C for 10 days in order to obtain substantial quantity of enzyme. The cells were harvested, homogenized with 0.5 M Tris-HCl buffer (pH-8.5) and centrifuged at 3000 rpm for 20 min. The supernatant was used for ammonium sulphate precipitation with 80% saturation (Thirunavukkarasu *et al.,* 2011) and the fractionated samples were dialyzed overnight at 4ºC (Dialysis bag –Himedia).

Assay of L-asparaginase activity : The enzyme was assayed by measuring the amount of ammonia liberated from L-asparagines (Baskar and Renganathan, 2009). The enzyme activity was expressed in terms of enzyme units (IU ml^{-1}) or international units. One international unit of L-asparaginase is that amount of enzyme, which liberates 1 µmole of ammonia per minute. The protein estimation was done by Bradford's method (Bradford, 1976).

Purification through gel filtration and ion exchange chromatography : Ammonium sulphate precipitated protein was subjected to gel filtration using sephadex G-100-120 (Sigma), with the bead size 40-120 µ, glass column (60cmx2.2 cm) with 0.05M Tris-HCl buffer, pH 8.5 as eluent. The active pooled fractions of the sephadex gel filtration were subjected for ion exchange chromatography through the Biologic LP system- BIO-RAD. The samples were passed through column with Tris-HCl buffer (0.05 M, 8.5 pH) at a flow rate of 0.5 ml min^{-1} for 1hr. Gel electrophoresis was performed (at 40V for 1hr and then 55V for 3hrs.) to estimate the molecular weight. Finally, purified enzyme was characterized for the substrate specificity, pH optima, thermal tolerance and km value.

Results and Discussion

The fungal strain showed 99% sequence similarity (with reference to 13 accession compared) with genus *Aspergillus Micheli* ex Link 1809 species *A. flavus* Link 1809. The L-asparaginase enzyme produced by *Aspergillus flavus* was comprehensively studied and found to possess good enzyme activity with unique substrate specificity, important characteristic of therapeutically important enzyme asparaginase.

Different carbon sources were screened at static flask level to find out the appropriate carbon sources for optimum growth of the organism and L-asparaginase production (Fig. 1). The maximum activity was observed in glucose-asparagine medium supplemented with 1% starch (14.09 IU mg^{-1} protein) and sorbitol (13.14 IU mg^{-1} protein) among nine carbon sources tested.

Remarkable L-asparginase activity (12.69 IU mg^{-1} protein) was observed in the medium containing nitrogen source glutamic acid (0.5% w/v) as compared to other nitrogen sources (Fig. 2) . Addition of xanthin as a nitrogen source in the above medium also exhibited good (11.32 IU mg^{-1} protein) enzyme production (Fig. 3). Among different combination of carbon and amino acid sources tested, the medium supplemented with 1% starch, 0.5% L-asparagine and L-glutamic acid showed higher L-asparagine production (Table 1). Likewise, effect of combined addition of L-asparagine and L-glutamic acid on enzyme production inferred that medium amended with 0.6% L-asparagine and 0.5% L-glutamic acid had maximum hike in enzyme production (Table 2).

A. flavus was grown in modified medium (starch (1%), L-asparagine (0.6%), L-glutamic acid (0.5%), KH_2PO_4 (0.1%), and $MgSO_4$ (0.05%) at 30 °C under static condition for 10 days for different pH ranging between 4.5-8.5. The maximum enzyme activity was observed when *Aspergillus flavus* was grown at 4.5 pH (Fig. 4). The enzyme activity also declined in the presence of $CaCl_2$, KCl, $CoCl_2$, NaCl, $ZnSO_4$, $NaCO_3$ (0.05%). However, addition of $MgCl_2$ did not have much effect on enzyme activity. The organism preferred 10-12 days incubation under static condition to yield maximum enzyme. The enzyme activity declined under shake culture condition (50 rpm).

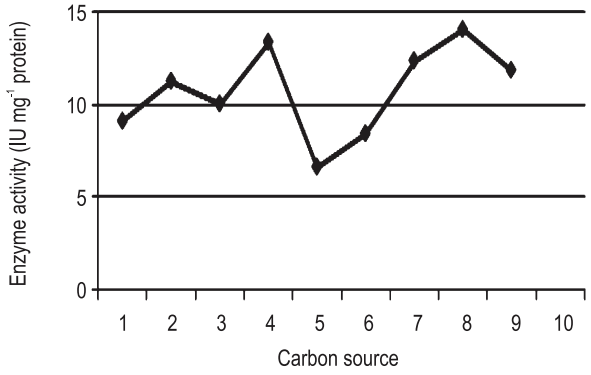

Fig. 1 : L. asparaginase activity of *Aspergillus* sp. With different carbon sources (1%)1, Raffinose, 2- Inositol, 3-lactose, 4- Sorbitol, 5- Arabinose, 6-Galactose, 7-Maltose, 8- Starch, 9- Glucose

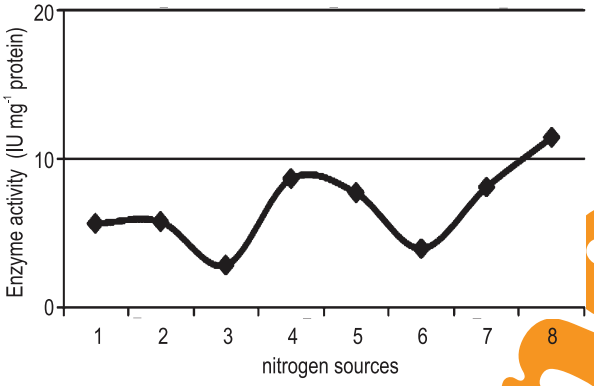

Fig. 3 : L-asparaginase activity of *Aspergillus* sp. with other nitrogen sources 1-citric acid, 2-oxalic acid, 3-pyruvic acid, 4-sorbic acid, 5-Azelic acid, 6-yeast extract7-Guanine, 8-Xanthine

The total enzyme activity increased from 9.04 to 153.38 Iu mg^{-1} protein in ammonium sulfate precipitation step. The gel filtration profile of L-asparaginase done on sephadex G100-120 column is shown in Fig. 5. A sharp peak at fraction no. 27 confirmed the purity of enzymatic protein. The purified protein was obtained by applying highest protein containing fractions from sephadex column to anion exchange chromatography (Fig. 5 and 6).

The specific activity increased to 153.59 IU mg^{-1} protein in ammonium sulfate precipitation and 97.088 and 176.47 IU mg^{-1} protein after sephadex 100 -120 and anion chromatography, respectively (Table 3). Electrophoretic separation of enzyme preparation from different purification steps revealed one distinguished band of L-asparaginase. Determination of molecular weight was done by using different standard proteins with known molecular weight. It was discovered that the approximate molecular weight of L- asparaginase from this fungi was 100 kDa (Fig.7).

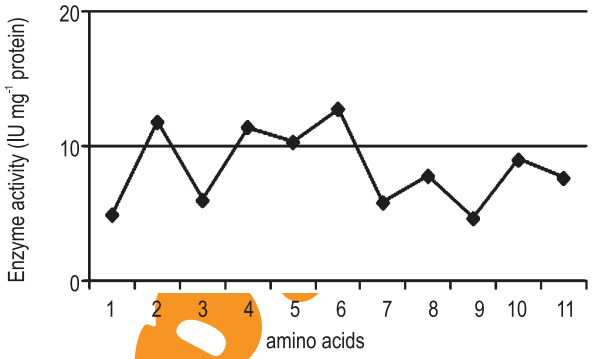

Fig. 2 : L. asparaginase activity of *Aspergillus* sp. with different amino acids 1-Asparagine, 2-Threonine, 3-trptophan, 4-proline, 5-aspartic acid, 6-glutamic acid, 7-phenylalanine, 8-Valine, 9-cysteine, 10-methonine, 11-tyrosine

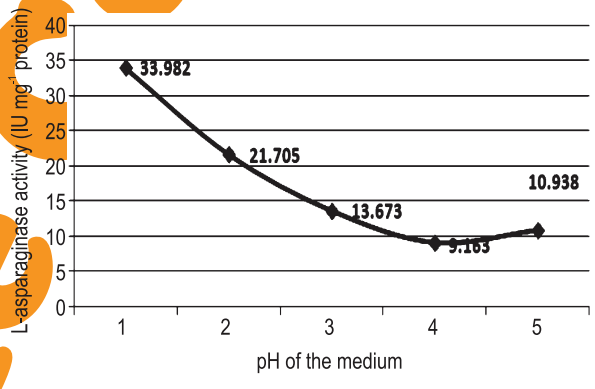

Fig. 4 : Enzyme activity in modified medium of different pH1-4.5, 2-5.5, 3=6.5, 4-7.5, 5-8.5

The effect of pH on enzyme was studied using 0.01 M of phosphate buffer of different pH ranging from 4-10 .The enzyme gradually increased upto pH 7 at which the maximum activity was observed. At higher pH, the activity decreased (Fig.8). The reaction rate of L-asparaginase was also measured at different temperature. The enzyme was found to be stable at 30 ºC- 50 ºC and showed maximum activity with L- asparagine as substrate. A Lineweaver- Burk plot analysis gave K_m value of 0.806×10^{-3} M.

Several fungi are reported as producer of L –asparaginase enzyme (Siddalingeswara and Kingapp, 2011; El-Bessoumy *et al.,* 2004). *Aspergillus flavus* preferred starch and sorbitol for enhanced enzyme production where as glucose showed lowest enzyme production as compared to other. The synthesis of L – asparaginase in *Aspergillus* sp. was almost suppressed in presence of glucose at 0.05% concentration. This observation is supported by the studies on L- asparaginase from *E. coli* W and *E.coli* K 12 and *E. carotovora*. This could be due to glucose caused catbolite repression and catabolic inhibition of the components involved in lactate transport and lactate

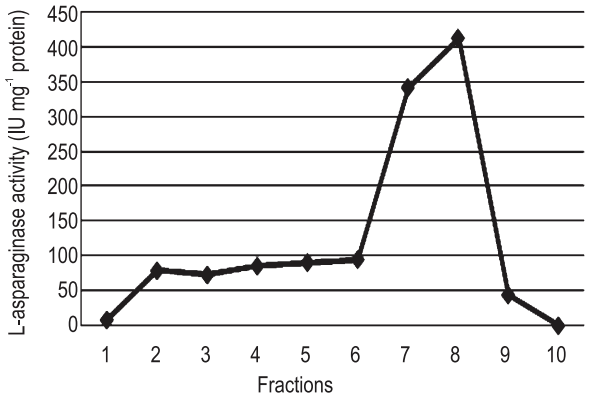

Fig. 5 : Purification of L -asparaginase from *Aspergillus* sp. Gel filtration column chromatography(Sephadex G100-120) of the fractions collected from ammonium sulfate precipitation fractions nos. 1-6, 2=9, 3=12, 4=15, 5=18, 6=21, 7=24, 8=27, 9=30, 10=33

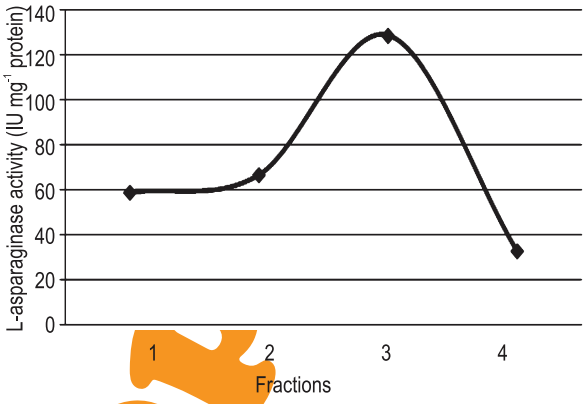

Fig. 6 : Purification of L -asparaginase from the Aspergillus sp. ion exchange column chromatography (Sephadex G100-120) of the fractions collected from ammonium sulfate precipitation fractions nos. 1-4, 2=5, 3=6, 4=7

stimulated L- asparagine synthesis (Warangkar and Khobragade, 2009; Sahu and Sivakumar, 2007).

The high yielding organisms are the prime factor in any biotechnological process (Shah *et al.,* 2010) To achieve this, the component of the medium should be supplied in a specific quantity for growth and energy yielding fermentation process. The environmental conditions, nutrient, temperature and incubation period are important factor that affect the product formation. In the present study, organisms preferred 4.5 acidic pH to give best enzyme production. Evaluation of enzyme production at different incubation period showed 10 days as the most prefered period for maximum organism to produce. Sometimes, microbes performed better in absence of amino acids as naturally available amino acids supported their L– asparaginase production (Mathew *et al*., 1994, Dhevendaran and Anitha , 2002). It was found that nitrogen as limiting factor, inhibited L- asparaginase activity. The strain studied may be producer of L- asparaginase II enzyme, which is an inducible enzyme. For induction it may require amino acid, L-asparagine in the growth medium. Other nitrogen sources like yeast extract, peptone, tryptone caesin hydrolysate were found to be potent for the production of L-asparaginase in some bacteria (Barenes *et al.,* 1997). All these components did not show any promising effect on L–asparaginase production by *Aspergillus* sp.

Nutritional requirement for maximum synthesis of L-asparagine may vary in different organisms. The rate of synthesis also varied in the same organism as function of culture conditions . In the present study, effect of complex interaction in carbon and nitrogen sources, pH of the medium, incubation time and temperature and specific requirement of amino acids were reported. L- asparagine is the natural substrate but the maximal enhancement of enzyme synthesis occurred by addition of glutamine to the medium . Asparaginase synthesis is induced by the presence of a group of amino acid particularly by glutamic and

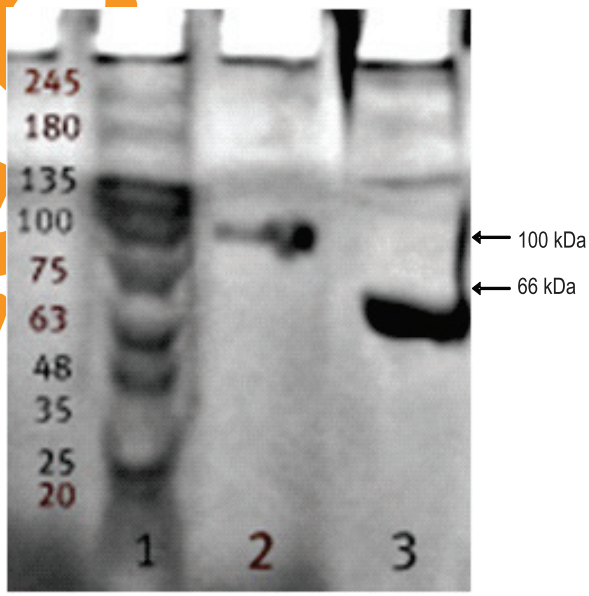

Fig. 7 : Gel electrophoretic separation of purified L-asparaginase from *Aspergillus flavus*. Lane 1- Molecular weight markers (kDa), 2- purified L-asparaginase (approx.100 kDa), 3- standard albumin (66 kDa)

or glutamine (Baskar and Eenganathan, 2011). In the present study, presence of L- asparagine and L-glutamine under certain ratio acted as inducer and produced more enzymes.

The purification steps followed for L-asparaginase from *Aspergillus* sp. achieved a protein of single peptide chain with molecular weight 92 kDa. In this report, the enzyme was more or less higher than that of bacterial L-asparaginase. The temperature tolerance of the enzyme showed that it had maximum activity at 37°C and was quite stable at high temperature too. Enzymatic activity was optimum at pH 7. This

Table 1 : L-asparaginase activity (IU mg^{-1} protein) of *Aspergillus flavus* grown under different concentrations of carbon and amino acid

Concentration %	L-asparagine (0.5%)	L-asparagine (0.5%)+ L-glutamic acid (0.5%)	L-asparagine (0.5%)+ L-threonine (0.5%)
Starch			
1	20.884 ± 2.8	40.317±0.65	13.452±0.89
2	10.229 ± 0.11	21.417±0.82	10.690±0.79
3	9.110 ± 0.73	5.869±0.413	11.491±0.23
Sorbitol			
1	11.73±0.79	36.996±9.0	13.635±1.76
2	9.871±0.62	21.415±0.84	27.615±2.39
3	-	-	31.275±0.40

No activity; Values are ± SD of three replications

Table 2 : L-asparaginase activity of *A. flavus* grown under different concentrations of L-asparagine and L – glutamic acid

L-asparagine (%)+ L-glutamic acid (%)	L-asparaginase activity IU mg^{-1} protein
0.5+0.5	40.885±6.91
0.6+0.5	42.359±5.57
0.7+0.5	32.457±1.12
0.8+0.5	34.290±3.17
0.9+0.5	34.719±3.63
1+0.5	31.476±9.29
0.5+0.6	21.959±1.45
0.5+0.7	22.624±4.806
0.5+0.8	25.283±9.24
0.5+0.9	35.463±7.82
0.5+1	39.120±4.89

Values are mean ± SD of three replications

Table 3 : Purification status of L-asparaginase from *Aspergillus flavus*

Steps	Collected volume (ml)	Total activity (IU)	Total Protein (mg)	Specific activity (IU mg^{-1})	Purification (fold)	Yield (%)
Crude extract	600	1058.4	46.8	22.615	0	100
Ammonium sulphate precipitation	70	967.61	6.3	153.588	6.791	91.421
Sephadex gel filtration	45	589.815	6.075	97.088	4.293	55.727
Ion-exchange chromatography	10	264.7	1.5	176.466	7.803	25.009

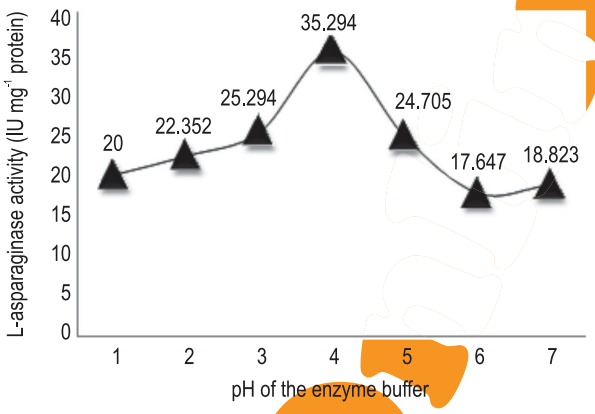

Fig. 8 : Effect of pH on enzyme purified through gel filtration and anion exchange chromatography

clearly indicates that L-asparaginase, from this fungal species was pH dependant (Sahu and Sivakumar, 2007).

L-asparaginase from different microbes has different substrate affinity and species play different ecophysiological roles in enzyme activity (Warangkar and Khobragade ,2009) . In the present study, fungal strain produced enzyme of $0.806x10^{-3}$ M. It was comparatively higher than other organisms like *Vibrio succinogens* (0.0745 mM) and *Pseudomonas aeruginosa* (0.147 mM). K_m value indicates high affinity of the enzyme to substrate (Thirunavukkarasu *et al*., 2011; Willis and Woolfolk , 1974) .

A few nutritional components have been identified for the improved production of L-asparaginase by this organism. Though the study has been done under batch fermentation, better performance of this organism may be observed under continuous fermentation. However, the most suitable medium for the production of L-asparaginase at high scale was found in this study. The excellent property of this enzyme such as high K_m value, thermal stability, substrate specificity and high molecular weight make it more valuable model for the chemotherapeutic use.

Acknowledgment

The financial support received from Ministry of Earth Sciences, Government of India under the research project no. No. MoES/11/MRDF/1/30/P/08 is gratefully acknowledged.

References

Baskar, G. and S. Ranganathan: Statistical screening of process variables for the production of L-asparaginase from cornflour by *Aspergillus terreus* MTCC 1782 in submerged fermentation.

Indian J. Sci. Technol., **2**, 45-48 (2009).

Baskar, G. and S. Ranganathan: Optimization of media components and operating conditions for exogenous production of fungal L asparaginase. *Chiang Mai J. Sci.,* **38**, 270-279 (2011) .

Barnes, W.R., G.L. Dorn and G.R. Vela: Effect of culture conditions on synthesis of L-asparaginase by *Escherichia coli* A-1. *App. Environ. Microbiol.,* **33**, 257-261 (1997).

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochem.*, **72**, 248-254 (1976).

Chandra, R., P. Ghosh and S.P. Bhatnagar: effect of growth factors, amino acids and minerals on L-asparaginase production by wild stain of *Alcaligenes faecalis* isolated from wild mushroom *Scleroderma* sp. *Inter. J. Biosci. Engin.*, **1**, 17-26 (2010) .

Dhevendaran, K. and Anitha Kumari Y.K.: L-asparaginase activity in growing conditions of *Streptomyces* sp. associated with *Therapon jarbua* and *Villorita cyprinoids* of Veli lake, South India. *Indian J. Marine Sci.,* **39**, 155-159 (2002).

El-Bessoumy, A.A., M. Sarhan and J. Mansour: Production, Isolation, and Purification of L-asparaginase from *Pseudomonas aeruginosa* 50071 using solid-state fermentation. *J. Biochem. Molecular Biol.,* **37**, 387-393 (2004).

Ghasemi, Y. and A. Ebrahiminezhad: An optimized medium for screening of L-asparaginase production by *Escherichia coli*. *Ame. J. Biochem. Biotechnol.*, **4**, 422-424 (2008).

Gupta, N., S. Dash and U.C. Basak: L-asparaginases from fungi of Bhitarkanika mangrove ecosystem. *Asia Pacific J. Molecular biol. Biotechnol.,* **17**, 27-30 (2009).

Hosamani, R. and B.B. Kaliwal: Isolation, molecular identification and optimization of fermentation parameters for the production of l-asparaginase, an anticancer agent by *Fusarium equiseti*. *Inter. J. Microbiol. Rese.,* **3**, 108-119 (2011).

Jayaramu, M. and N.B. Hemalatha: A novel approach for detection, confirmation and optimization of L-asparaginase from *Emericella nidulans*. *Curr. Pharma. Rese.,* **1**, 20-24 (2010).

Lapmark, K., S. Lumyong and S. Thongkuntha: L-Asparaginase production by *Bipolaris* sp. BR438 isolated from brown rice in Thailand. *Chian Mai J. Sci.,* **37**, 160-164 (2010).

Mathew, A., K. Dhevendraran, M.I. Geogekutty and P. Natarajan: L-asparaginase activity in antagonistic *Stroptomycetes* associated with clam *Villorita cyprinoides* (Hanley). *Indian J. Mar. Sci.,* **23**, 204-208 (1994) .

Mohana Priya, P., M. Radhakrishanan and R. Balaguranathan: Production and optimization of L asparaginase from *Streptomyces* sp. (TA 22) isolated from Western Ghats, India. *J. Chem. Pharma. Sci.,* **3**, 618-624 (2011).

Reynolds, D.R. and J.W. Taylor: The fungal holomorph: A consideration of mitotic meiotic and pleomorphic speciation.CAB Int. Wallingford, UK (1993).

Sahu, M.K. and K. Sivakumar: Studies on L-asparaginase enzyme of actinomycetes isolated from estuarine fishes. *J. Environ. Biol.,* **28**, 465-474 (2007).

Sarquis, M.I.D.M., E.M.M. Oliveira, A.S. Santos and G.L.D. Costa. Production of L-asparaginase by filamentous fungi. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, **99**, 489-492 (2004).

Savitri, N. and A.W. Asthana. Microbial L-asparaginase: A potent antitumour enzyme. *Indian J. Biotechnol.,* **2**, 184-194 (2003).

Shah, A. J. , R.V. Karadi and P.P. Parekh: Isolation, optimization and production of L-asparaginase from coliform bacteria. *Asian J. Biotechnol.*, **2**, 169-177 (2010).

Siddalingeshwara, K.G. and K. Lingappa: Screening and optimization of L-asparaginase- A tumour inhibitor from *Aspergillus terreus* through solid state fermentation. *J. Advance Sci. Rese.,* **1**, 55-60 (2010).

Siddalingeshwara, K.G. and K. Kingapp: Production and characterization of L asparaginase– A tumour inhibitor. *Inter. J. Pharmace. Rese.,* **3**, 314-319 (2011).

Sokal, R.R. and J.F. Rhohlf: Introduction to Biostatistics. Freeman, San Fransisco (1973).

Soniyamby, A.R., S. Lalitha, B.V. Pravessh and V. Priyadarshini: Isolation, production and anti-tumor activity of l-asparaginase of *Penicillium* sp. *Inter. J. Microbiol. Res.,* **2**, 38-42 (2011).

Sreenivasulu, V., K.N. Jayaveera and P.M. Rao: Optimization of process parameters for the production of L-asparaginase from an isolated fungus. *Res. J. Pharmacol. Phtyochem.,* **1**, 975-2331 (2009).

Theantana, T. , K.D. Hyde and S. Lumyong: Asparaginase production by endophytic fungi isolated from some Thai medicinal plants. *KMITL Sci. Technol. J.,* **7**, 13-18 (2007).

Thirunavukkarasu, N., T.S. Suryanarayanan, T.S. Murali, J.P. Ravishankar and S.N. Gummadi: L-asparaginase from amrine derived fungal endophytes of sea weeds. *Mycosphere,* **2**, 147-155 (2011).

Venil, C.K., K. Nanthakumar, K. Karthikeyan and P. Lakshmanaperum alsamy: Production of L-asparaginase by *Serratia marcescens* SB08: Optimization by response surface methodology. *Iranian J. Biotechnol.,* **7**, 10-18 (2009).

Warangkar, S. and C.N. Khobragade: Screening, enrichment and media optimization for L-asparaginase production. *J. Cell Tissue Rese.,* **9**, 1963-1968 (2009).

Willis, R.C. and R. Woolfolk: Aspraginase utilization in *E. coli*. *J. Bacteriol.,* **118**, 231-235 (1974).