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Isolation, screening, optimization and production of Anti-tumor L-Asparaginase by fungi from karwar coastal region

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Abstract

L-asparaginase is an extracellular enzyme that converts L-asparagine to L-Aspartic acid and has gained considerable attention in the recent years. In the present study, soil samples were gathered from different areas in and around the Karwar regions, Karnataka, India. Several fungal species were isolated and identified using standard manuals, screened primarily for the production of L-Asparaginase on Czapek's Dox medium containing L- Asparagine as main source of carbon. A total of 50 fungal cultures were isolated from soil. The fungal isolates obtained were selected by plate assay method for screening of potential L-Asparaginase production on Modified Czapek Dox's (mCD) medium. Enzyme production was carried out by submerged fermentation process and was performed by using mCD liquid media. Quantitative enzyme assay was performed to determine the rate of hydrolysis of L-Asparagine by measuring the liberated ammonia by nesslerization. From the 50 total isolates 10 fungal cultures showed L-Asparaginase activities. The cultures were identified as Penicillum species, Basidiomycetes species. Aspergillus species, Mucor species. Fusarium species. Among the above species Aspergillus sp showed potential L-Asparaginase production (155U/ml). An attempt is made to optimize the cultural conditions for the production of potential L-Asparaginase by using submerged fermentation. Different temperature (15^0C, 25^0C, 35^0C, 45^0C) different pH (3.5, 5.5, 7.5, 9.5, and 11.5) different carbon and nitrogen source were used. The highest amount of enzyme production was observed at pH 7.5(155 U/ml) and temperature at 35°C (160 U/ml), among the various carbon sources dextrose promoted maximum enzyme activity (176 U/ml) and highest activity was obtained when nitrogen source ammonium sulphate was used (185 U/ml).

Keywords: L-Asparaginase, Anti-tumor activity, Aspergillus, Czapek's Dox medium, submerged fermentation.

Introduction

Cancer has emerged as one of the major cause for human suffering with unprecedented morbidity and mortality. Cancer incidence rate is generally expressed as age standardized incidence rate (ASR) per 100,000 persons. As per 2011 statistic, the ASR was reported to be 300/225 (male/female) and 160/138 (male/female) in more developed and less developed areas, respectively[1]. Cancer is the most precarious disease characterized by uncontrolled proliferation of cells without any physiological demands of the organism. Cancer may be defined as unnecessary tissue growth that occur due to an imbalance between cell division and programmed cell death; caused by various genetic and epigenetic alterations. The specific cause of cancer is elusive, which may be possibly attributed to viral, genetic, chemical, radiation, environmental or immunological factors. The disease remains challenging despite of mammoth research efforts across the world[2]. L-asparaginase is highly suitable for treatment of blood cancer as cancer cells are distributed throughout the body along with the blood. L-asparaginase is known to act by hydrolyzing the asparagine and causing deficiency of the amino acid for cancer cells, whereby it limits the growth of cancerous cell. L-Asparaginase is anticancer agent used with other chemotherapeutic agent[3].

L-asparagine is fundamental amino acid for the growth of tumor cells whereas the growth of normal cell is independent of its requirement[4]. This aminoacid can be produced within the cell by an enzyme called Asparagine synthetase. Most of the normal cells synthesizes L-Asparagine in sufficient amounts for their metabolic needs but the tumour cells (especially Malignant and Carcinoma Cell) require external source of L-Asparagine for their growth and multiplication[5]. In the presence of L-Asparaginase, the tumor cells deprived of an important growth factor and they may failure to survive. Thus this enzyme can be used as a chemotherapeutic agent.

The chemical reactions are catalyzed by enzymes which increase the rate of reactions. In enzymatic reactions, the molecules called substrates are converted into products. In a cell almost all biological processes need enzymes to catalyse reactions at eloquent rates. L-Asparaginase catalyses the conversion of L-Asparagine into L-Aspartate and ammonium[6-9]. Guinea pig contained a high activity of L-Asparaginase[10]. Microbial systems has attracted significant attention for producing potential L-Asparaginase and it is ecofriendly nature. A wide range of microbes such as bacteria, yeast and fungi showed potential source of L-Asparaginase[11].

The bacterial L-Asparaginase (E.coli and Erwinia species) has been considered as effective drugs for the treatment of leukemia[12]. L-Asparaginase isolated from bacteria can cause allergic reactions and side effects like diabetes, leukopenia and coagglutation abnormalities in the long term use[13]. This advances that it is essential to discover novel L-Asparaginase that are serologically different but similar therapeutic effects from eukaryotic microorganisms like yeast, fungi and the enzyme may have less adverse effect[14-17]. The objective of this study is to obtain potential fungi from in and around the Karwar regions. Because there is a continual need to find out newer organisms to obtain potential strains.

Materials and methods

Isolation of microorganism: The fungal species used in the present work were obtained from soil samples by serial dilution method, gathered from different areas in and around Karwar Coastal regions Karnataka India. The isolated microbes were preserved on Czapek Dox medium[18].

Screening of L-Asparaginase producing fungi: A total of 50 fungal cultures were isolated from soil. The fungal isolates obtained were selected by plate assay method for screening of potential L-Asparaginase production. MCD medium used for fungal isolates is composed of, 1% (w/v) L-asparagine, 0.152% (w/v) K_2PO_4, 0.052% (w/v) $MgSO_4.7H_2O$, 0.003% (w/v) $CuNO_3.3H_2O$, 0.005% (w/v) $ZnSO_4.7H_2O$, 0.2% (w/v) glucose, 0.003% (w/v) $FeSO_4.7H_2O$, 0.052% (w/v) KCl, 1.8% (w/v) agar, and was supplemented with phenol red (0.009% (v/v) as an indicator. The fungal isolates were inoculated and incubated at temperature 32°C for 48 h. L-Asparaginase production was indicated by observing pink zone around the colonies, and were selected for determination of enzyme activity. Control plates were maintained with $NaNO_3$ instead of asparagines as nitrogen source on MCD medium. The cultures were identified as *Penicillum species, Basidiomycetes species. Aspergillus species, Fusarium species.* By using plate assay method, the potential fungal strains were identified on the basis of pink zone around the colony. From the total isolates 10 fungal cultures showed L-Asparaginase activity. Among the above species *Aspergillus sp* showed potential L-Asparaginase production.

L-Asparaginase production by submerged fermentation: Enzyme production was performed by submerged fermentation and was carried out by using MCD broth media. The potential organism was inoculated in 100 ml of suitable medium in culture flasks. The culture flasks were incubated at 30°C for 48 and 72 hrs. Uninoculated flasks were treated as controls. The fungal cultures were harvested by filtering through Whatman No. 2 filter paper. The estimation of enzyme activity was performed by crude enzyme obtained from culture filtrate.

Quantitative enzyme assay: The method used was fundamentally that of Imada *et al*[19]. In this assay the rate of hydrolysis of L-Asparagine was determined by measuring the liberated ammonia by nesslerization. The cultures were centrifuged at 10000rpm for 10 min, the reaction was commenced by taking 0.5ml supernatant, 0.5ml 0.04M L-Asparagine and 0.5ml 0.05M tris HCl buffer (pH7.2) make up the volume to 2.0 ml distilled water, incubated at 37^0C for 30 min, the reaction was stopped by addition of 0.5ml of 1.5M trichloroacetic acid (TCA). After centrifugation at 8000rpm, to the 0.1 ml of supernatant 3.75ml of distilled water and 0.2 ml of Nessler's reagent maintained at room temperature for 10 min. Absorbance was read at wavelength of 480 nm by using UV-visual Spectrophotometer.

Enzyme unit: one international unit (IU) is defined as that amount of enzyme capable of producing 1micromole of ammonia per min at 37^0C.

Optimization of fermentation parameters for L-Asparaginase production: The production of L-Asparaginase from fungi mainly depends on factors like temperature, pH, carbon and nitrogen source. Hencethese parameters must be optimized in order to achieve higher yields of L-Asparaginase.

Effect of temperature: Effect of temperature on L-Asparaginase production was examined at 15ºC, 25ºC, 35ºC and 45^0C.The flasks were incubated for 4 days and the supernatant was used as crude enzyme to calculate the L-Aasparaginase activity.

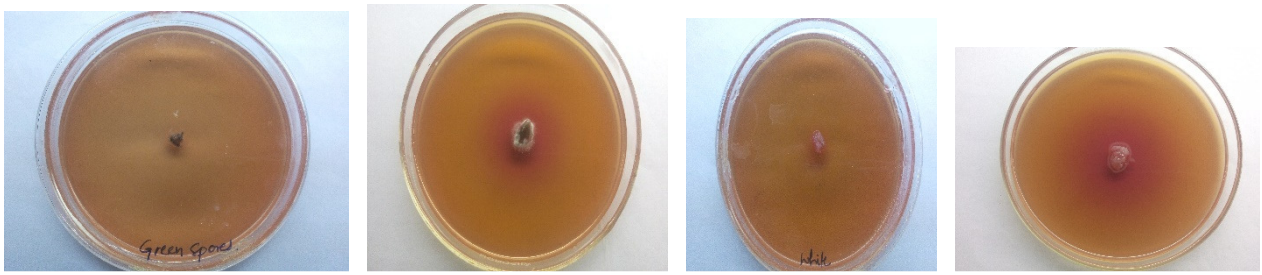
Effect of pH: To examine the effect of pH on maximum enzyme production the medium was adjusted to different pH ranging from pH (3.5, 5.5, 7.5, 9.5.11.5) prior to autoclaving and the organism was inoculated. The flasks were incubated for 4 days and supernatant was used for determining the L-Asparaginase activity**.**

Effect of carbon source: In present study, different carbon sources were added to modified Czapek Dox's liquid media at equivalent weight. Various sources of carbon such as soluble starch, fructose, maltose, dextrose, lactose and sucrose were supplemented with L-Asparagine (0.3%) as nitrogen source in growth media. Thereafter, L-Asparaginase production was investigated. The inoculums was added in the medium and incubated at 35 °C for 4 days under static conditions.

Effect of nitrogen source: As a fact, the nitrogen sources considering the secondary energy source after carbon sources and they play a vital role in the growth of organisms and enzyme production. In microorganisms, amino acids, nucleic acids, proteins and cell wall components are metabolized by nitrogen (both organic and inorganic forms). In the present experiment, the supplementation of additional nitrogen sources (either organic or inorganic) such as urea, peptone, ammonium nitrate, yeast extract, beef extract, ammonium sulphate, sodium nitrate, malt extract, were used to determine the maximum enzyme activity.

Results and discussion

The present study mainly focused on the production of L-Asparaginase enzyme by filamentous fungi, isolated from soil sample from different regions in and around Karwar, Karnataka India. There are total 50 fungal isolates were obtained by using MCD media. The fungal isolates were inoculated and incubated at temperature 32°C for 48 h. L-Asparaginase production was indicated by observing pink zone around the colonies, and were selected for determination of enzyme activity. The fungal strains were stained by using lacto phenol wet mount stain. They were identified on the basis of morphological, cultural and characteristic reproductive structures by using standard reference manuals and were identified as *Penicillum species, Basidiomycetes species. Aspergillus species, Mucor species. Fusarium species*[20,21]. The plate assay method was used to screen the fungus for the production of L-Asparaginase enzyzme. The potential strains were choosed on the basis of pink zone around the colony. From the 50 total isolates 10 fungal cultures showed L-Asparaginase activities among the above species *Aspergillus sp* showed potential L-asparaginase production (Table-1). Growth conditions regarding the temperature, pH of the medium, carbon and nitrogen sources were optimized for the maximum enzyme production. The results for the above parameters are illustrated in Figure-2,3,4,5. The optimum temperature 30 or 37°C was reported in most of the L-Asparaginase producing fungal species[22-24].



Control Organism showing pink zone Control Organism showing pink zone

Figure-1: Isolation and screening of L-Asparaginase producing Bacteria on modified Czapekdox media using L-Asparagine and phenol red indicator.

Table-1: Screening of fungi for L-asparaginase production using plate assay method.

No	Place of isolation	Fungi code	Fungi	L asparaginase Activity U/ml
1	Asnoti	ASN4	*Penicillum sp*	121
2		ASN3	*Fusarium sp*	130
3	Hanakon	HNK1	*Aspergillus sp,*	135
4	Belur	BEL6	*Penicillum sp*	98
5	Devalamkki	DVMK1	*Basidiomycetes sp*	85
6	Majali	MJL2	*Mucor sp.*	116
7		MJL7	*Fusarium sp*	107
8	Devbag	DEV3	*Penicillum sp*	106
9	Sunkeri	SNK2	*Mucor sp.*	116
10		SNK5	*Basidiomycetes sp*	93
11	Kajubag	KJB6	*Aspergillus sp,*	149
12	Ulga	ULG4	*Aspergillus sp,*	155
13		ULG2	*Fusarium sp*	130
14	Sadashivagad	SDGD3	*Aspergillus sp,*	121

Effect of temperature: The microbial growth and enzyme secretion is highly influenced by incubation temperature as it is a critical environmental factor for L-Asparaginase production by microbes. Temperature influences the rate of the chemical reaction thus affecting rate of enzymatic activity. In present study, the maximum activity obtained was 160U/ml at 35^0.The significance of the incubation temperature could determine the effects of inhibition, cell viability and death. However, the enzyme production reduced gradually with further increase in incubation temperature. This may be due to heat that accumulates in the medium during process. The results obtained was compared with other reports, Sarqius et al have reported 30°C is suitable for L-Asparaginase production through submerged fermentation by using *A. terreus* and *A. tamarii*[25]. Siddalingeshwara et al observed a temperature of 37^0C was found to be optimum for asparaginase production by *Aspergillus species*[26]. Monica Tet et al noticed the maximum activity at 30^0C *by Mucor hiemalis*[27]. Kotra et al have reported optimized temperature as 30^0C by *Penicillium sp*[28].

Effect of pH: The enzyme activity can be either enhanced or inhibited depending on the change in the pH, and hence can influence the growth of microorganisms[29]. The surface charges present on the amino acids influence the microbial enzyme activity. Different organisms have different pH optima and any modification in their pH optima could result in a decrease in their enzyme activity. In present study experiments were carried out in order to maintain the favorable conditions and to find the optimum pH to obtain increased L-Asparaginase production. This was performed by carrying out the fermentation process by using various pH from 3.5-11.5 (adjusted with 1N HCl or 1N NaOH).

In this study the maximum enzyme activity was observed at pH 7.5 with an activity of 155 U/ml. A decline in the enzyme activity noted after the optimum pH may be due to the fact that both acidic and alkaline pH has an inhibitory effect on the growth and enzyme production. A change in pH prevents the binding of a substrate to the enzyme owing to change of shape and properties of an enzyme and/or the substrate. The outcome of the present work was correlated with other columnists, Chandrasekhar AP observed maximum activity at pH by *Aspergillus* species[30]. Thirunavukkarasu et al observed pH of 6.2 as optimum for asparaginase production by *Fusarium species*[31]. Mohsin et al reported pH of 6.0 was the optimal pH for L-asparaginase production in *Penicillium species*[32]. Niharika Yadav et al reported a pH of 5.0 was found to be optimal for L-asparaginase production by *F.oxysporum*[33]. G. Thirumurugan et al. reported an optimum asparaginase production at pH 8.0 by Aspergillus Terreus[34]. Selvakumar observed peak activity of asparaginase at pH 8.0 by Streptomyces noursei MTCC 10469[13].

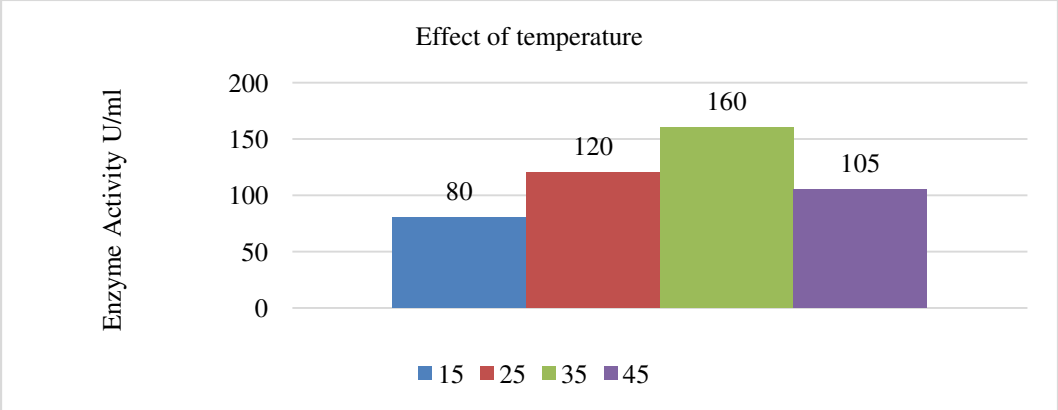


Figure-2: Effect of Temperature on L-Asparaginase production.

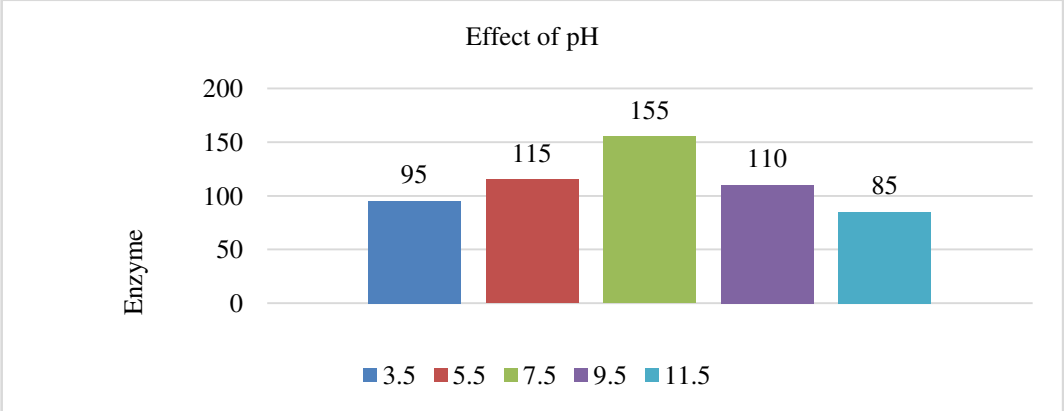


Figure-3: Effect of pH on L-Asparaginase production.

Effect of carbon sources on L-Asparaginase production: Generally carbohydrates are used as carbon sources in the fermentation processes. During industrial fermentation process the energy for the growth of microorganism is achieved either from the oxidation of medium components or from light. The growth and maximum enzyme production are derived from Carbon sources which is normally observed in the synthesis of primary metabolites, such as enzymes. The carbon concentration had a positive effect on L-Asparaginase production and high titres can be obtained in a medium rich of carbon source. The maximum enzyme activity was promoted when dextrose used as a carbon source and enzyme activity obtained was 176 U/ml. while the lowest L-Asparaginase production was recorded when using soluble starch 97 U/ml.The results of the present investigation are also in agreement with other authors concerning the production of L-Asparaginase enzyme[35,36]. Baskar and Renganathan reported that glucose was found to be best carbon source for maximum L-Asparaginase production Aspergillus terrus MTCC 1782[37].

Effect of nitrogen source on L-Asparaginase production: Nitrogen sources have been preferred for enhancing the production of L-Asparaginase. The organic form or inorganic form sometimes both, nitrogen source is utilized by most of the industrial enzymes. In most of the industrial fermentation process growth will be faster with supply of organic and inorganic nitrogen source. Among the various nitrogen sources tested, ammonium sulphate in the medium promoted enhanced growth of microorganism and consequently the L-Asparaginase production (185U/ml). These results are in good agreement with those reported for the production of L-Asparaginase by other microorganisms[38]. Kalyanasundaram et al. used ammonium sulfate as a nitrogen source for the maximum production of l-Asparaginase from *A. terreus*[39]. Gaffar and Shethna observed the positive effect of supplementation of ammonium sulphate in the production of L-asparaginase[40]. Sreenivasulu et al. have reported ammonium sulphate exhibited maximum enzyme production by the isolated fungus VS-26[41].

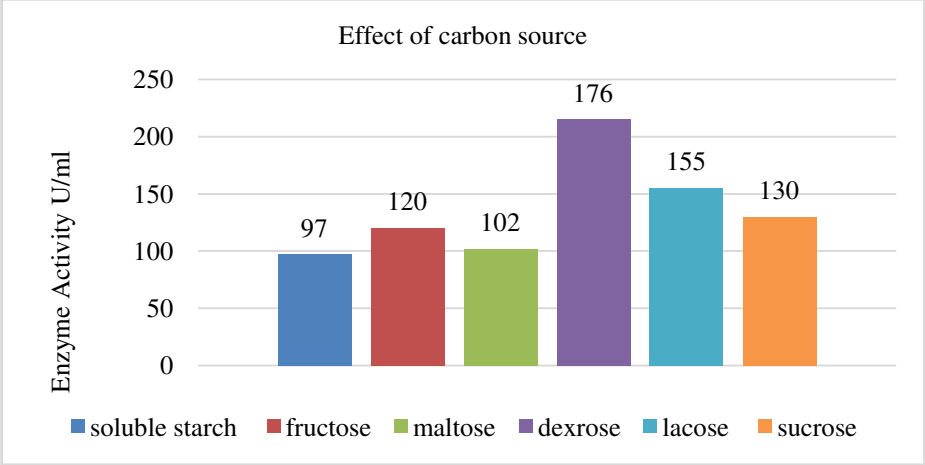


Figure-4: Effect of carbon source on L-Asparaginase production.

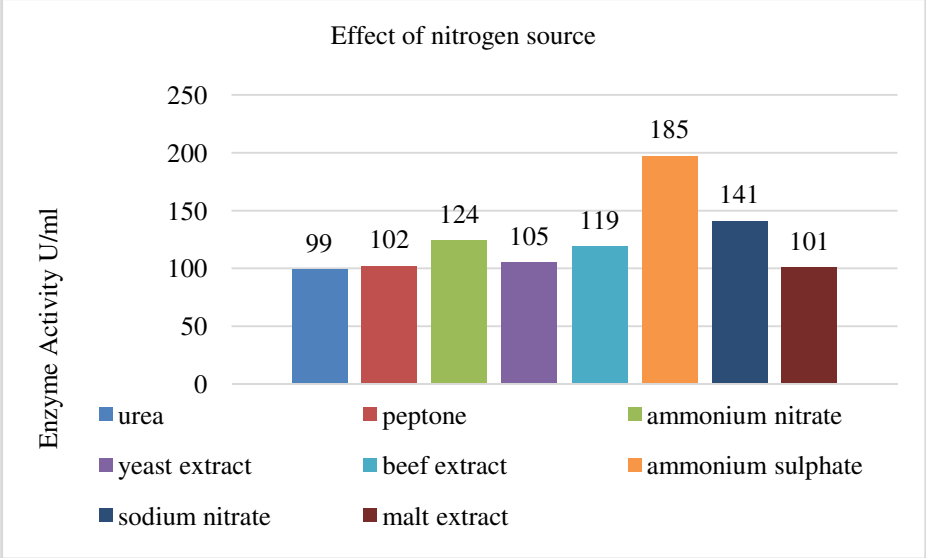


Figure-5: Effect of nitrogen source on L-Asparaginase production.

Conclusion

The present investigation has revealed that *Aspergillus species have* potential for L-Asparaginase production on modified Czapekdox medium under submerged fermentation methodology. It was found that optimum temperature 35^0C and optimum pH 7.5 and dextrose, ammonium sulphate were identified as the best operating conditions for the maximum L-asparaginase activity of 160 U/ml and 155 U/ml,176U/ml and 185U/ml respectively.

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References

1. Jemal A., Bray F., Center M.M., Ferlay J., Ward and E. Forman D. (2011). Global cancer statistics. *CA Cancer J Clin.*, 61(2):69-90. doi: 10.3322/caac.20107. Erratum in: CA Cancer J Clin. 2011 Mar-Apr; 61(2):134.

2. Weinberg R.A. (1996). How cancer arises. *Scientific American*, 275(3), 62-71.

3. Kotzia G.A. and Labrou N.E. (2007). L-Asparaginase from Erwinia chrysanthemi 3937, Cloning, Expression and Characterization. *Journal of Biotechnology*, 127(4), 657-669.

4. Berenbaum M.C., Ginsburg H. and Gilbert D.M. (1970) Effects of L-asparaginaseon lymphocyte target cell reactions in Vitro. *Nature,* 227(5263), 1147-1148.

5. Broome J.D. (1963). L-asparaginase EC-II from Escherichia coli.Some substrate specificity characteristics. *Biochemistry* 8, 3766-3772.

6. Wriston J. and Yellin T. (1973). L-Asparaginase: A Review**.** *Adv Enzymol Relat Areas Mol Biol,* 39, 185-248.

7. Yellin T. and Wriston J. (1973). Purification and Properties of Guinea Pig Serum Asparaginase. *Biochemistry***,** 5(5), 1605-1612.

8. Capizzi R.L., Poole M., Cooper M.R., Richards F., Stuart J.J., Jakson D.V., White D.R., Spurr C.L., Hopkins J.O. and Muss H.B. (1984). Treatment of poor risk acute leukaemia with sequential hig-done ARA-C and asparaginase. *Blood* 63(3), 694-700.

9. Lubkowski J., Palm G.J., Gilliland G.L., Derst C., Röhm K.H. and Wlodawer A. (1996). Crystal structure and amino acid sequence of *Wolinella succinogenes* l-asparaginase. *European Journal of Biochemistry,* 241(1), 201-207.

10. Clementi A. (1922). La désamidation enzymatique de l'asparagine chez les différentes espéces animales et la signification physio logique de sa presence dans l'organisme. *Archives Internationales de Physiologie,* 19(4), 369-398.

11. Gulati R., Saxena R.K. and Gupta R. (1997). A rapid plate assay for screening L-asparaginase producing micro-organisms. *Lett Appl Microbiol,* 24(1), 23-26

12. Dhevagi P. and poorani E. (2006). Isolation and characterization of l asparaginase from marine actinomycetes. *international journal of biotechnology*, 5, 514-520

13. Selvakumar N. (1991). vanajakumar and Natarajan R. *Partial purification, characterization and anti tumor properties of l asparaginase from vibrio in bioactive compounds from microorganisms,* 289-300.

14. Tiwari N. and Dua R.D. (1996). purification and preliminary characterization of l asparaginase from Erwinia aroideae NRRL B- 138. *Ind. J Biochem Biophys,* 33(5), 371-376.

15. Wade H.E., Robinson H.K. and Philips B.W. (1971). Asparaginase and glutaminase activities of bacteria. *J.Gen Microbiol*., 69(3), 299-312.

16. Wiame J.M., Grenson M. and Arst N.H. Jr. (1985). Biodiversity of higher yeasts and filamentous fungi. *AdvMicrobial Physiol.,* 26, 1-88.

17. Pinheiro I.O., Araujo J.M., Ximenes E.C.P.A., Pinto J.C.S. and Alves T.L.M. (2001). Production of L-Asparaginase by *zymononas mobiles* strain Cp4. *Biomaterial and Diagnostic*, 6, 243-244.

18. Saxena R.K. and Sinha U. (1981). L-asparaginase and glutaminase activities in the culture filtrates of Aspergillus nidulans. *Cur Sci.,* 50, 218-219.

19. Imada A., Igarasi S., Nakahama K. and Isono M. (1973). Asparaginase and glutaminase activities of micro-organisms. *J Gen Microbiol,* 76(1), 85-99.

20. Ellis M.B. (1976). More "dematiaceous hyphomycetes". Commonwealth Mycological Institute, Kew, Survey. England 507, ISBN 0-79235957-7.

21. Raper K.B. and Fennell D.I. (1965). The *Aspergillus*, Baltimore: Williams and Wilkins. 1-686.

22. Lapmak Kodchakorn, Lumyong Saisamorn, Thongkuntha Sutheera, Wongputtisin Pairote and Sardsud Uraporn (2010). L-ASPARAGINASE PRODUCTION BY BIPOLARIS SP. BR438 ISOLATED FROM BROWN RICE IN THAILAND. *Chiang Mai Journal of Science*., 37, 160-164.

23. Hosamani R. and Kaliwal B.B. (2011). Isolation, molecular identification and optimization of fermentation parameters for the production of L-asparaginase, an anticancer agent by Fusarium equiseti. *Int J Microbiol Res*, 3(2), 108-119.

24. Rani S.A., Lalitha S. and Praveesh B.V. (2011). In vitro antioxidant and anticancer activity of L-asparaginase from

Aspergillus flavus (KUFS20). *Asian J. Pharm. Clin. Res*., 4, 174-177.

25. Sarquis M.I., Oliveira E.M., Santos A.S. and Costa G.L. (2004). Mem Inst Oswaldo Cruz. 99(5), 489-92.

26. Siddalingeshwara K.G. and Lingappa K. (2011). Production and characterization of L-asparaginase-a tumour inhibitor. *Int J Pharm Tech Res*, 3(1), 314-319.

27. Monica T., Lynette L., Niyonzima F.N. and Sunil S.M. (2013). Isolation, Purification and Characterization of Fungal Extracellular L- Asparaginase from Mucor Hiemalis. J Biocatal Biotransformation 2: 2. 9, 12-14.

28. Kotra S.R., Prudvi N., Sada Sai K.R.A., Mannava K.K., Peravali J.B., Kumar Anmol, Sambasiva Rao K.R.S. and Pulicherla K.K. (2013). Cost effective process for the production of fungal L-asparaginases from Penicillium sps isolated from local soil sample. *Mintage Journal of Pharmaceutical and Medical Sciences,* 2(1), 45-50.

29. Jalgaonwala R.E. and Mahajan R.T. (2014). A review: bacterial endophytes and their bioprospecting. *J Pharm Res*., 4, 795-799.

30. Chandrasekhar A.P. (2012). Isolation, purification and characterization of asparaginase from aspergillus species. *Int J Res Chem Environ*, 2, 38-43.

31. Thirunavukkarasu N., Suryanarayanan T.S., Murali T.S., Ravishankar I.P. and Gummadi S.N. (2011). *Mycosphere,* 2(2), 147-15.

32. Mohsin S.M., Dutt Sunil, Siddalingeshwara PLNSN, Karthik K.G., Jayaramu J., Naveen M., Vishwanatha M. and Prathiba T.K.S. (2012). *J Acad* , *Ind Res,*1(4), 180-182.

33. Yadav Niharika and Sarkar Supriya (2014). *Intl J Pharmaceutical Science Invention*, 3(6), 32-40.

34. Thirumurugan G., Moses Jeyakumar Rajesh, Leelavathy Rajesh, Vanapalli VSV and Rajarammohan Sivasubramanian (2011). Effect of Inducers and Physical Parameterson the Production of L-Asparaginase Using Aspergillus Terreus. *J Bioprocess Biotechniq,* 1, 1-6. doi:10.4172/2155-9821.1000110.

35. Dange V. and Peshwe S. (2015). Purification and biochemical characterization of L-asparaginase from *Aspergillus niger* and evaluation of its antineoplastic activity. *Int. J. Sci. Res.,* 4(2), 564-569.

36. Kalyanasundaram J., Nagamuthu B., Srinivasan A., Pachayappan S. and Muthukumarasamy (2015). Production, purification and characterization of extracellular L-asparaginase from salt marsh fungi endophytes. *World J. Pharma. Sci.,* 4(3), 663-667.

37. Baskar G. and Renganathan S. (2011). Design of experiments andartificial neural network linked genetic algorithm formodelling and optimization of L-asparaginase production by Aspergillus terreus MTCC 1782. *Biotechnology and Bioprocess Engineering*, 16(1), 50-58.

38. Murali T.S. (2011). L-Asparaginase from marine derived fungal endophytes. *Mycosphere*, 2(2), 147-155.

39. Kalyanasundaram I., Nagamuthu J., Srinivasan B., Pachayappan A. and Muthukumarasamy S. (2015). Production, purification and characterization of extracellular L-asparaginase from salt marsh fungi endophytes. *World J. Pharma. Sci.,* 4(3), 663-667.

40. Gaffar S.A. and Shethna Y.I. (1977). Purification and Some Biological Properties of Asparaginase from Azotobactervinelandii. *Appl Environ Microbiol.,* 33(3), 508-514.

41. Sreenivasulu V., Jayaveera K.N. and Mallikarjuna Rao (2009). Optimization of process parameters for the production of Lasparaginasefrom an isolated fungus. *Research J.Pharmacognosy and Phytochemistry*, 1(1), 30-34.