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SCREENING AND OPTIMIZATION OF PARAMETERS FOR L-ASPARAGINASE PRODUCTION USING FUNGAL SPECIES

M ANAMIKA[1], Y NEERAJA[1], J SWARNALATHA[1], S VENKATESHWAR[1], L VENKATESWAR RAO[2*]

1: University College of Technology, Osmania University, Hyderabad.

2: Department of Microbiology, Osmania University, Hyderabad.

[*]**Corresponding Author: E Mail: vrlinga@gmail.com, Mobile: +91939111277**

ABSTRACT

The objective of this work was the screening of different fungal species which are potential sources of L-asparaginase with a high specific activity and optimization of fermentation parameters of L-asparaginase production. Thirty six fungal species were screened by rapid plate assay method, *Aspergillus terreus* was selected as a good potential fungal species for L-asparaginase production based on pink colour zone diameter (3.1 cm). Optimization of fermentation parameters such as initial pH (7.5), incubation period (3 days), inoculum level (3 ml), incubation temperature (28°C), aeration rate (5 ml) and agitation rate (12 rpm) were used to produce maximum L-asparaginase activity of 15.6 IU/ml.

Keywords: *Aspergillusterreus*, Modified Czapekdox Medium, Filamentous Fungi, Submerged Fermentation

INTRODUCTION

L-asparagenase (L-asparagine aminohydrolase EC 3.5.1.1) enzyme catalyzes the hydrolysis of L-asparagine to L-asparatic acid and ammonium ion and this catalytic reaction is essentially irreversible under physiological conditions **[1]**. L-asparaginase has been used as a chemotherapeutic agent **[2, 3]**. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma **[4, 5]**. Asparagine is a nutritional requirement of both normal and

cancer cells. Normal cells are able to synthesize L-asparagine and thus are less affected by its rapid depletion produced by treatment with the enzyme L-asparaginase. Cancer cells are not capable of producing L-asparagine, and mainly depend on the L-asparagine from the circulating plasma pools **[6]**. L-asparaginase enzyme inhibits protein synthesis by L-asparagine hydrolysis **[4, 7-9]**.

L-asparaginase is present in a wide range of organisms including animals, microbes, plants, and in the serum of certain rodents but not in human beings **[10]**. Microorganisms such as bacteria, fungi, yeast, actinomycetes and algae have proven to be proficient sources of this enzyme **[11-16]**. A number of undesirable side effects, ascribed to the acute lymphoblastic leukemia presence of contaminating bacterial endotoxins in the enzyme preparations were observed. The search for the other asparaginase sources like eukaryotes, can lead to an enzyme with less adverse effects. It has been observed that eukaryote microorganisms like yeast and filamentous fungi have a potential for asparaginase production. Very little work has been carried out on L-asparaginase from fungal source. Hence, we attempted to study this enzyme from different other microbial source like fungi. The enzyme is produced throughout the world by both submerged and solid-state cultures **[3]**. Mostly submerged fermentation is used to produce L-asparaginase **[17-19]**.

In this study, we report the screening of different fungal species which are potential sources of L-asparaginase with a high specific activity and optimization of fermentation parameters for L-asparaginase production.

MATERIALS AND METHODS

Screening for L-Asparaginase Producing Fungi

Fungal Species

Thirty six fungal species used in the study were obtained from Botany and Microbiology Departments, Osmania University. All the species were maintained in Potato Dextrose Agar slants.

Screening Medium

The screening of the fungal strains for L-asparaginase production was studied by inoculating them on Modified czapekdox medium, pH 6.2 contained (gm/l of Distilled water): glucose 2.; L-asparagine 1.; KH_2PO_4 1.52; KCl .52; $MgSO_4.7H_2O$ .52; $CuNO_3.3H_2O$ tarce; $ZnSO_4.7H_2O$ trace; $FeSO_4.7H_2O$ trace; agar 2.. L-asparaginase and glucose were purchased from Hi-media laboratories (India). All other chemicals of analytical (or) equivalent grade were purchased from local market.

It is generally observed that L-asparaginase production is accompanied by an increase in pH of the culture filtrates. So pH indicator

phenol red was incorporated in the modified czapekdox medium containing L-asparagine as sole nitrogen source. Modified czapekdox medium with 0.9% phenol red was autoclaved and plates were prepared.

The plates were inoculated with 3-4 days cultures of fungal strains. The zone and colony diameters were measured after incubated at 28°C for 72h. Broth studies were also carried out in order to compare the results obtained with those of plate assay. Erlenmeyer flask 25 ml containing 5 ml of modified czapekdox's liquid culture medium was inoculated with each of fungal species. The culture was incubated at 28°C with shaking at 12rpm for 96h. The enzyme activity was examined by using culture filtrated through whatman No1 filter paper as the enzyme preparation by Nesslerization and expressed as IU/ml.

Production Medium

The screened fungal strain was used for further studies. The screened fungal strain had collected from NCIM, Pune. This culture was maintained on modified czapekdox agar medium containing (gm/l of distilled water): glucose 2.; L-asparagine 1.; KH_2PO_4 1.52; KCl 0.52; $MgSO_4.7H_2O$ 0.52; $CuNO_3.3H_2O$ tarce; $ZnSO_4.7H_2O$ trace; $FeSO_4.7H_2O$ trace; at pH 6.2.

Inoculum Preparation

Spore suspension was prepared from 5 days old culture grown on modified czapekdox agar medium slants by adding 1 ml of sterile distilled water containing .1% of Tween-8. Suspend the spores with a sterile loop. 1 ml of spore suspension containing about 1×1^7 spores/ml and this prepare spore suspension was used to inoculate the experimental flasks.

Optimization of Fermentation Parameters

Effect of Initial pH

Initial pH of the culture broth is one of the most critical environmental parameters affecting both mycelial growth and L-asparaginase production. The effect of pH on L-asparaginase production from screened fungal strain was determined by growing the strain in fermentation media of different pH of aqueous solution ranging from 2., 2.5, 3., 3.5, 4., 4.5, 5.,5.5, 6., 6.5, 7., 7.5, 8., 8.5, 9., 9.5 and 1. with 1N NaOH (or) 1N HCl. A47 ml of production medium adjusted with different pH ranges was taken in 25 ml Erlenmeyer flask. After autoclaving 3 ml of spore suspension was added. The inoculated flasks were incubated at 28°C, for 5 days on orbital shaking incubator at 12 rpm. After fermentation, broth was collected and assayed for L-asparaginase production. Change in pH of the fermentation medium affects the essential sites of amino acid residues which involve in catalysis and substrate binding. The optimized pH was fixed for further experiments.

Effect of Inoculum Level

The inoculums level is very much important parameter in fermentation process. The inoculum levels were added to the fermentation medium in the range of .5, 1., 1.5, 2., 2.5, 3., 3.5, 4., 4.5, 5. ml (1×1^7 spores/ml) and optimized pH was maintained. After autoclaving the inoculated flasks were incubated at 28°C, for 5 days on orbital shaking incubator at 12 rpm.

Effect of Incubation Period

Various incubation periods such as 2,3,4,5,6,7,8,9,1 days were employed to study their effect on production of L-asparaginase enzyme. Fermentation was carried out at optimized pH and inoculum level at 28C on orbital shaking incubator at 12 rpm.

Effect of Temperature

The temperature mostly affects the production of L-asparaginase. Temperature is most important factor for L-asparaginase production. Optimal temperature of L-asparaginase enzyme was determined in the range of 26, 28, 3, 32, 34, 36ºC and other fermentation conditions of optimized inoculum level, incubation period and pH of fermentation media were adjusted.

Effect of Aeration and Agitation

Aeration and agitation are one of the fermentation factors which affect the production of L-asparaginase. The effects of aeration and agitation rates on L-asparaginase production from screened fungal strain was determined by growing the strain in fermentation media of different aeration and agitation rates. The different aeration rate such as 25, 5, 75, 1, 125, 15, 175 ml of production medium adjusted with optimized pH was taken in 25 ml Erlenmeyer flask and agitation rates such as 3, 6, 9, 12, 15, 18 rpm was adjusted on orbital shaking incubator.

Quantitative Assay for L-Asparaginase Activity

L-asparaginase activity assay was carried out as per Imada **[2]**. In this assay, the rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler's reaction. After fermentation, broth was collected and centrifuged at 8 rpm for 15 min to get clear supernatant. Reaction was started by adding .5 ml supernatant into .5 ml .4M L-asparagine and .5 ml .5M tris-HCl buffer (pH 7.2) and incubated at 37°C for 3 min. The reaction was stopped by adding .5 ml 1.5M trichloroacetic acid (TCA). The reaction mixture was allowed to centrifugation at 1 rpm for 15 min. After centrifugation clear supernatant was collected. A .1 ml supernatant was diluted to 3.75 ml with distilled water and treated with .2 ml Nessler's reagent and incubated at room temperature for 1 min. The absorbance of the supernatant was read at

45 nm using a UV spectrophotometer. The blank was run by adding enzyme preparation after the addition of TCA. A standard curve was prepared with ammonium sulphate. The enzyme activity was expressed in International unit. One unit of L-asparaginase is the amount of enzyme which catalyzed the formation of 1µmol of ammonia per min. All the experiments were carried out in triplicate and mean values were taken.

Determination of Protein Content

Protein was determined by Lowry Method **[21]** using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Among thirty six fungal species tested, fifteen species gave the pink zone diameter from .3-3.1 cm after 72h incubation, while remaining species produced yellow coloured zone which indicate as negative result **(Figure 1b)**. *Aspergillus terreus* is the most active that can give zone diameter of 3.1 cm **(Figure 1a)**. *Aspergillusniger* gave zone diameter of 2.8 cm followed by *Aspergillus oryzae* and *Fusarium* species, *Saccharomyces cerviceae* **(Table1)**. When they were screened on agar plates these strains gave zone diameter more than 1.5 cm. So they were selected as potential good producers of L-asparaginase. A .9% phenol red in the medium keeps the medium yellow colour for better identification of colour change to pink for trace amount of L-asparaginase. High concentrations of phenol red giving the medium colour dark red and it is difficult to identify the pink zone. This plate assay can be directly visualized from the plates without performing time consuming assays **[22, 23]**.

Later secondary screening was done in liquid culture with the eight species which have zone diameter of more than 1.5 cm. The *Aspergillus terreus* gave the highest L-asparaginase activity of 12.8 IU/ml. The screened fungal strain *Aspergillus terreus* was used for further studies. While Soniyambyambi rani **[24]** found *Aspergillus* sp KUFS2, isolated from garden soil, was produced 27 mm diameter pink zone surrounding the colony.

The effect of pH on L-asparaginase activity was studied with pH range of 2. to 1. Maximum of L-asparaginase activity of 13.2 IU/ml was obtained at pH 7.5. Further increase in pH from 7.5 to 1. resulted in reduced L-asparaginase activity. The L-asparaginase enzyme activity is maximum in alkaline pH probably due to the balance between aspatatic acid and L-asparatate. The enzyme activity decreases at low pH and high pH. The optimum pH of L-asparaginase production was 7.5 **(Figure 2)** while Koshy **[25]** found maximum L-asparaginase activity of *streptomyces plicatus*at pH 7. De Angeli **[26]** has reported

pH 7 was optimum for the maximum production of L-asparaginase.

Effect of inoculum levels from .5-5. ml were studied on L-asparaginase production. It results the enzyme activity gradually increased with inoculum level and attained its maximum production at 3 ml. Beyond 3. ml there was gradual decrease of enzyme activity. The optimum inoculum level of L-asparaginase production was 3. ml and the enzyme activity is 13.6 IU/ml **(Figure 3)**. Whereas M. Jayaramu **[27]** found maximum L-asparaginase production by *Emericellanidulans* was 1.11 IU at 1 ml of inoculum size.

The influence of incubation period on L-asparaginase activity was studied the results indicated that the enzyme activity was gradually increased from 2nd to 3rd day and attained its maximum production at 3rd day; after 3rd day it was observed that there is gradual decrease in L-asparaginase production. The optimum incubation period for L-asparaginase production was 3rd day with14.1 IU/ml **(Figure 4)**. Whereas M. Jayaramu **[27]** found maximum L-asparaginase production by *Emericellanidulans* was 1.11 IU at 48 hrs of incubation period.

Different incubation temperatures such as 26-36°C were studied for their effect on L-asparaginase production and the maximum enzyme activity was observed at 28°C (14.5 IU/ml). It was observed that there was decrease in the enzyme activity when the temperature was higher or lower than the optimum incubation temperature **(Figure 5)**. While *Streptomyces albidoflavus* produced maximum amount of enzyme when cultured at 35°C **[18, 28]**. Sarquis **[11]** reported 3°C is the suitable for L-asparaginase production through submerged fermentation by using *A. terreus*and *A. tamari.*

Effect of aeration rates from 25-175 ml were studied on L-asparaginase production. It results the enzyme activity gradually increased and attained its maximum production at 5 ml. Beyond 5 ml there was gradual decrease of enzyme activity. The optimum aeration rate of L-asparaginase production was 5 ml and the enzyme activity was 15.1 IU/ml **[Figure 6]**. Various agitation rates such as 3-18 rpm were studied and the maximum enzyme activity was observed at 12 rpm (15.6 IU/ml) **[Figure 7]**. Gurunathan Baskar observed that the modified Czapek-Dox medium gives the maximum enzyme activity of 24.1 IU/ml at the agitation rate of 16 rpm **[29]**.

Table1: Colony and Zone Size of Fungal Species on Modified Czapekdox Medium

S. No	Fungal species	Colony diameter (cm)	Zone diameter (cm)	L-asparaginase activity (IU/ml)
1	*Aspergillusniger*	1.53	2.8	9.2
2	*Aspergillusterreus*	1.7	3.1	12.8
3	*Fusariumsolani*	1.62	2.3	5.4
4	*P.chrysogenum*	1.71	1.8	2.1
5	*S.cerveciae*	1.58	1.9	4.7
6	*A.oryzae*	1.68	2.6	7.6
7	*A.nidulus*	1.81	1.7	1.8
8	*P.nigricans*	1.54	1.6	1.2

Figure 1a: *Aspergillus terreus*on Modified Czapekdox Agar Medium

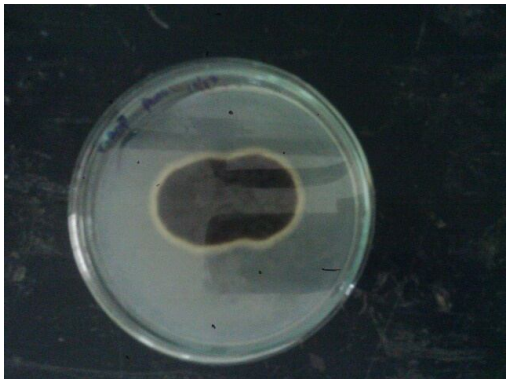

Figure 1b: *Alternaria*species on Modified Czapekdox Agar Medium

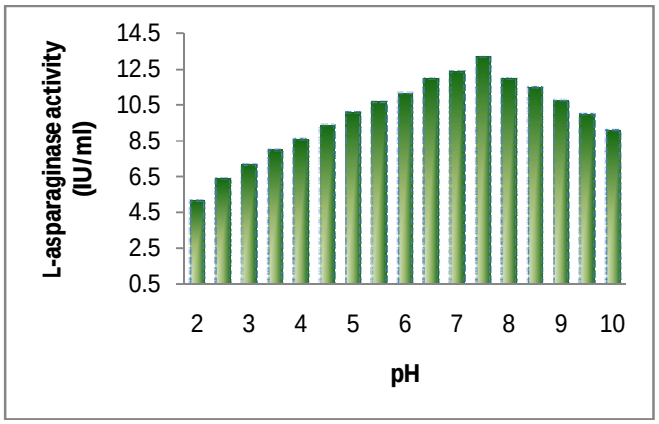

Figure 2: Effect of pH on L-Asparaginase Activity

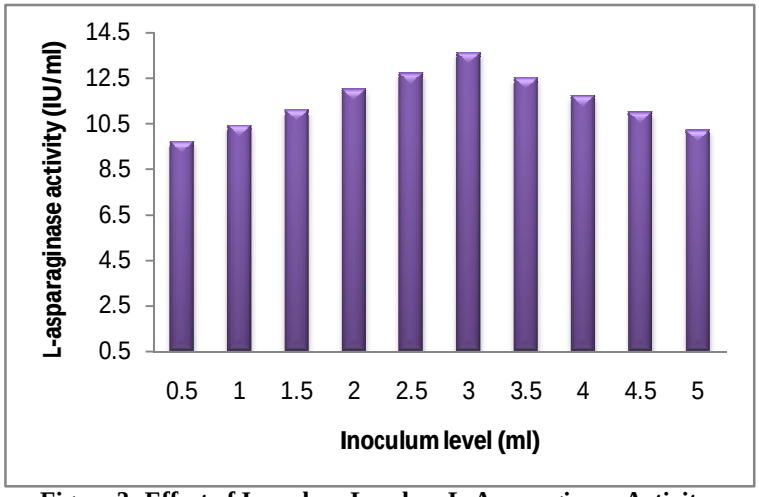

Figure 3: Effect of Inoculum Level on L-Asparaginase Activity

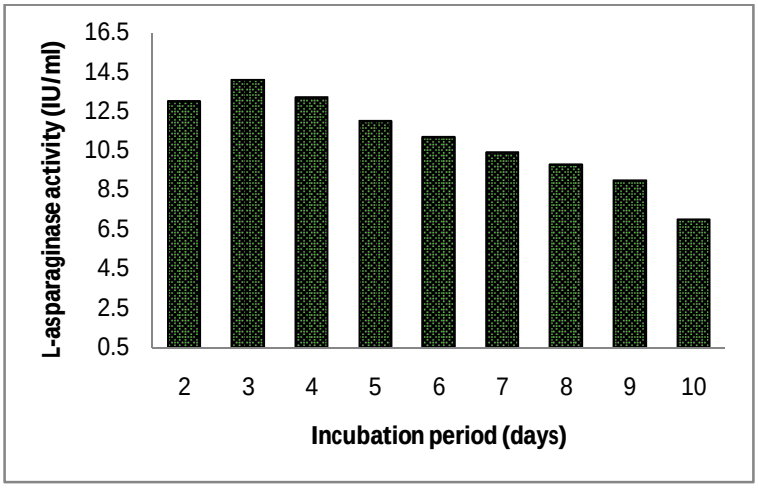

Figure 4: Effect of Incubation Time on L-Asparaginase Activity

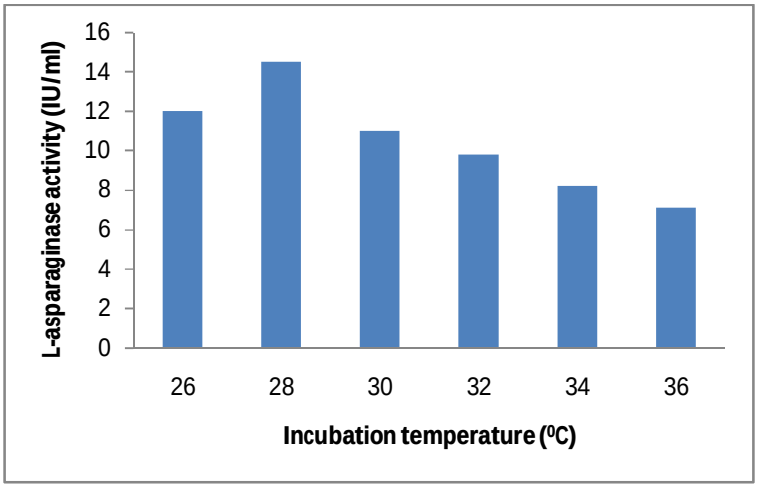

Figure 5: Effect of Temperature on L-Asparaginase Activity

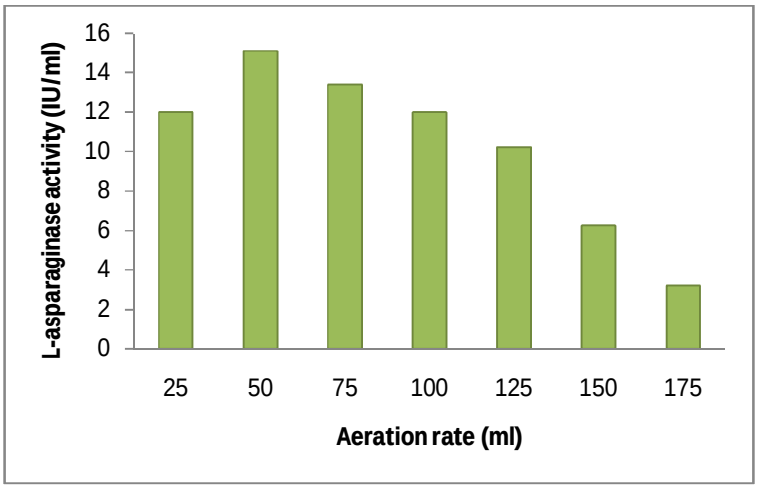

Figure 6: Effect of Aeration Rate on L-Asparaginase Activity

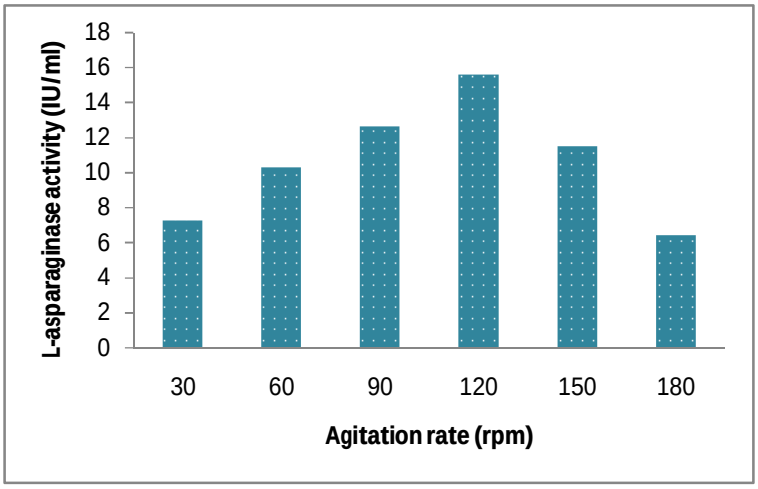

Figure 7: Effect of Agitation Rate on L-Asparaginase Activity

CONCLUSION

From this work we conclude that *Aspergillus terreus* is the most active and a good potential strain among all fungal species for L-asparaginase production on modified czapekdox medium under submerged fermentation. We achieved optimum production of L-asparaginase15.6 IU/ml with optimized conditions.

REFERENCES

[1] Lubkowski J, Palm GJ, Gilliland GL, Derst C, Rohm KH and Wlodawer A, Crystal structure and amino acid sequence of Wolinella succinogenes L-asparaginase, Eur. J. Biochem., 241, 1996, 21-27.

[2] Moorthy V, Ramalingam A, Sumantha A and Shankaranaya RT, Production, purification and characterisation of extracellular L-asparaginase from a soil isolate of *Bacillus* sp, Afr. J. Microbiol. Res., Vol. 4 (18), 21, 1862-1867.

[3] Amena S, Vishalakshi N, Prabhakar M, Dayanand A and Lingappa K, Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*, Br. J. Microbiol., 41, 21, 173-178.

[4] Stecher AL, Morgantetti P, De Deus, Polikarpov I and Abrahao-Neto J, Stability of L-asparaginase-an enzyme used in Leukemia treatment. Pharmaceut. ActaHelv., 74, 1999, 1-9.

[5] Verma NK, Kumar G, Kaur and Anand S, L-asparaginase: A promising chemotherapeutic agent. Crit. Rev. Biotechnol., 27 (27), 45-62.

[6] Swain AL, JaskÓlski M, Housset D, Rao JKM, and Wlodawer A, Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy, Proc. Natl. Acad. Sci., 9, 1993, 1474-1478.

[7] Marlborough DI, Miller DS and Cammack KA, Comparative study on conformational stability and subunit interactions of two bacterial asparaginases, Biochim. Biophys. Acta, 386, 1975, 576-589.

[8] Ravindranath Y, Abella E, Krischer JP, Wiley J, Inoue S, Harris M, Chauvenet A, Alvarado CS, Dubowy R, Ritchey AK, Land V, Stueber CP and Weinstein H, Acute myeloid leukemia _AML in Down's syndrome is highly responsive to chemotherapy: experience on Pediatric Oncology Group AML Study 8498, Blood, 8, 1992, 221-2214.

[9] Moola ZB, Scawen MD, Atkinson T and Nicholls DJ, *Erwiniachry santhemi* L-asparaginase: epitope mapping and production of antigenically modified enzymes, Biochem. J., 32, 1994, 921-927.

[10] El-Bessoumy AA, Sarhan M and Mansour J, Production, isolation, and purification of L-asparaginase from Pseudomonas aeruginosa 571 using solid-state fermentation, J. Biochem. MolBiol., 37 (24), 387-393.

[11] Sarquis MIM, Oliviera EMM, Santos AS and Da- Costa GL, Production of L-asparaginase by filamentous fungi, Mem. Inst. OswaldoCruz., 99, 24, 489-492.

[12] Elzainy TA and Ali TH, Detection of antitumor glutaminase asparaginase in the filamentous fungi, J. Appl. Sci., 6, (26), 1389-1395.

[13] Ferrara MA, Severino NMB, Mansure JJ, Martin AS, Oliveira EMM, Siani AC, Jr NP, Torres FAG

and Bon EPS, Asparaginase production by a recombinant *Pichiapastoris* strain harbouring *Saccharomyces cerevisiae ASP3* Gene, Enz. Microbial Technol., 39 (26), 1457-1463.

[14] Borkotaky B and Bezbaruah RL, Production and properties of asparaginase from a new *Erwinia* sp., Folia Microbiol., 47, 22, 473-476.

[15] Prakasham RS, Rao CS, Rao RS, Lakshmi GS and Sarma PN, L-asparaginase production by isolated *Staphylococcus* sp.-6A: design of experiment considering interaction effect for process parameter optimization, J. Appl. Microbiol., 12, 27, 1382-1391.

[16] Siddalingeshwara KG and Lingappa K, Key fermentation factors for the synthesis of l-asparaginase - an anti tumour agent through SSF methodology, Pharma Sci. Monitor an Int. J. Pharmaceu. Sci., 1 (21).

[17] Albanese E and Kafkewitz K, Effect of medium composition on the growth and asparaginase production of *vibrio succinogens*, Appl. Environ. Microbiol., 36, 1978, 25-3.

[18] Mostafa SA and Salama MS, L-asparaginase producing Streptomyces from the soil of Kuwait, Zentralbl Bakteriol Naturwiss, 134, 1979, 325-34.

[19] Radcliffe CW, Kafkewitz D and Abuchowski A, Asparaginase production by clinical isolates of *vibrio succinogens*, Appl. Environ. Microbiol., 38, 1979, 761-2.

[20] Imada A, Igarasi S, Nakahama K and Isono M, Asparaginase and glutaminase activities of microorganisms, J. Gen. Microbiol., 76, 1973, 85-99.

[21] Lowry OH, Rosebrough, NJ, Farr A L and Randall RJ, Protein measurement with the folin phenol reagent, J. Biol. Chem., 193, 1951, 265-275.

[22] Wade HE, Robinson HK and Philips BWM, Automated determination of bacterial asparaginase and glutaminase, Anal. Biochem., 44, 1971, 189-199.

[23] Arima K, Igarasi S, Nakahama K, Isono M, Production of extracellular L-asparaginases from microorganisms, Agr. Biol. Chem., 36, 1972, 356-361.

[24] Rani SA, Sundaram L and Vasantha PB, Isolation and screening of l-asparaginase producing fungi from soil samples, Int. J. Pharm. and Pharmaceut. Sci., 4, 212, (1).

[25] Koshy A, Dhevendaran K, Georgekutty MI and Natarajan P, L–asparaginase in *Streptomyces plicatus*isolated from the alimentary canal of the fish, *Gerresfilamentosus*(Cuvier), J. Marine Biotechnol., 1997, 181-185.

[26] De Angeli LC, Pochiari F, Tonolo A, Zurita VE, Ciaranfi E and Perin A, Effect of L-asparaginase from *Aspergillus terreus* on ascites sarcoma in the rat, Nature (London) 225, 197, 549-55.

[27] Jayaramu M, Hemalatha NB, Rajeshwari CP, Siddalingeshwara KG, Mohsin SM and Sunil Dutt PLNSN, A Novel Approach for Detection, Confirmation and Optimization of L-Asparaginase from *Emericella nidulans*, Curr. Pharma Res., 1 (21).

[28] Narayana KJP, Kumar KG and Vijayalakshmi M, L-asparaginase production by *Streptomyces albidoflavus*, Ind. J. Microbiol., 48, 27, 331-336.

[29] Baskar G and Renganathan S, Optimization of L-asparaginase production by *Aspergillus terreus MTCC 1782* using response surface methodology and artificial neural network-linked genetic algorithm, Asia-Pac. J. Chem. Eng., 1 (21), 52.