European Journal of Biotechnology and Bioscience
Online ISSN: 2321-9122, Impact Factor: RJIF 5.44
www.biosciencejournals.com
Volume 4; Issue 10, October 2016; Page No. 01-05

Optimum conditions for L-asparaginase production by *Rhodococcus erythropolis* VLK-12 isolated from Marine habitats

Krishna Naragani, Vijayalakshmi Muvva

Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India

Abstract

The aim of present study includes the production and optimization of L-asparaginase from *Rhodococcus erythropolis* VLK-12 isolated from marine soil samples of Visakhapatnam, Andhra Pradesh. Among the 20 actinobacterial strains screened for enzyme production, one isolate found to be potent was identified as *Rhodococcus erythropolis* VLK-12 using morphological, cultural, physiological, biochemical characteristics and 16S rRNA analysis. Attempts were made to optimize the cultural conditions affecting the production of L-asparaginase by the strain. Maximal yields of L-asparaginase were recorded from 3-day-old culture grown in modified asparagine-glycerol salts broth (ISP-5) with initial pH 7.0 at temperature 30°C.

Keywords: *Rhodococcus erythropolis* VLK-12, Asparaginase, Optimal conditions

1. Introduction

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1) constitutes one of the most important groups of therapeutic enzymes accounting for about 40% of the total worldwide enzyme sales [1]. It has been widely exploited for the treatment of cancer especially acute lymphoblastic leukaemia since the time it was obtained from *Escherichia coli* and its anti-neoplastic activity demonstrated in guinea pig serum [2]. It avails three different forms that have been used as a drug for the treatment of acute lymphoblastic leukaemia (ALL) including asparaginase (*E.coli*), Erwinia asparaginase (*Erwinia caratovora*) and Pegasparaginase (L-asparaginase from *E.coli* attached to polyethyleneglycol) [3].

Actinomycetes act as potential source for the production of L-asparaginase. *Streptomyces griseus* [4], *S. karnatakensis*, *S. venezuelae* [5], *S. longsporusflavus* F- 15 [6], *S. phaeochromogenes* FS-39 [7], *Streptomyces* ABR2 [8], *Streptomyces halstedii* [9], *Streptomyces ginsengisoli* [10], *Streptomyces albus* CN-4 [11], *Streptomyces griseus* NIOT-VKMA29 [12] *Nocardia asteroides* [13], *Pseudonocardi endophytica* VUK-10 [14] and *Nocardia sp*.[15] were proved to be potential producers of this enzyme. However, very little information is available on the production of L-asparaginase by the genus *Rhodococcus.* While screening the actinomycetes for asparaginase production, a strain able to produce asparaginase was identified as *Rhodococcus erythropolis* VLK-12 by 16S rRNA analysis. In the present study, an attempt is made to study the optimum conditions for L-asparaginase production by *Rhodococcus erythropolis* VLK-12.

2. Material and Methods

2.1 Microorganism

The actinobacterial strain was isolated from south coast of Andhra Pradesh, India and identified as *Rhodococcus erythropolis* VLK-12 by using the morphological, cultural, physiological, biochemical and 16S rRNA analysis. The strain was deposited in the GenBank database of NCBI with the accession number KC106730 [16].

2.2 Screening of L-asparaginase

Preliminary screening test for the production of L-asparaginase was carried out by using modified ISP-5 agar medium (L-asparagine @1% and phenol red @ 0.03% with initial pH 7.0.) Sterilized medium was poured into Petri plates and allowed to solidify. The plates were inoculated with the strain and incubated at 30°C for 48-72 h. Change of dye color around the strain from yellow to pink indicates the positive reaction while no color change interprets the test as negative [17].

2.3 Production Profile of L-Asparaginase

To determine the production of L-asparaginase by the strain, culture suspension was inoculated into asparagine-glycerol salts (ISP-5) broth containing asparagine (1%), glycerol (1%), K_2HPO_4 (0.1%) and trace salt solution (0.1%) with initial pH 7.2. The inoculated flasks were incubated at 30°C for 7 days in order to estimate the growth as well as L-asparaginase production by the strain at every 24 h interval. Growth was expressed in terms of dry weight of biomass (mg/mL).

L-Asparaginase Assay was done by the method of Peterson and Ciegler [18]. Cells were harvested by centrifuging the culture broth at 10,000 rpm for 15 min and the cell free extract (0.2mL) obtained was mixed with 0.8mL of 0.05M Tris-HCl buffer and 1mL of 0.04M L-asparagine. After incubating the reaction mixture for 15min at 35 °C in a water bath, the reaction was terminated by the addition of 0.5mL of 15% trichloroacetic acid (w/v). Precipitated proteins were removed by centrifugation and the liberated ammonia was determined spectrometrically at 500 nm by nesslerization. Tubes were kept at zero time incubation served as control. The liberation of ammonia was calculated with reference to a standard curve of ammonium sulphate. One L-asparaginase unit (IU) equals to that amount of enzyme which releases 1 μM of ammonia

(Ammonium sulphate as standard) in 1 min at 35ºC. The cell dry weight was recorded simultaneously after centrifugation in an oven at 90ºC for 24h.

2.4 Optimization of cultural parameters for the enhanced production of L-asparaginase

Optimization of different cultural conditions such as initial pH, temperature, carbon and nitrogen sources on the production of L-asparaginase was determined.

2.5 Effect of Initial pH and Temperature

The effect of initial pH on the production of L-asparaginase was examined by culturing the strain in ISP-5 broth adjusted to various pH levels ranging from 5.0 to 10.0. The most favorable pH achieved at this step was used for further study. To determine the optimum temperature for L-asparaginase production, the strain was cultured in ISP-5 broth at different temperatures, ranging from 20 to 40ºC for 72 h of incubation.

2.6 Influence of Carbon Sources

To study the influence of carbon sources on L-asparaginase production by the strain, ISP-5 broth was supplemented with different carbon sources such as dextrose, maltose, lactose, fructose, xylose, sorbitol, starch, sucrose and cellulose each at a concentration of 1% (w/v). Impact of different concentrations of best carbon source (1–3.5%) on L-asparaginase production of the strain was studied.

2.7 Impact of Nitrogen Sources

To determine the impact of nitrogen sources on L-asparaginase production, ISP-5 broth was supplemented with various nitrogen sources such as ammonium oxalate, ammonium sulfate, ammonium nitrate, beef extract, malt extract, yeast extract, potassium nitrate, peptone, tyrosine, urea and tryptone at a rate of 0. 2% (w/v), containing an optimal amount of superior carbon source. In addition, the optimal concentration of nitrogen source (0.1–2% w/v) supporting high yields of L-asparaginase production was determined.

Statistical analysis

Data obtained under different culture conditions are statistically analyzed by using One-way Analysis of Variance (ANOVA).

3. Results and Discussion

3.1 Identification of the Strain

The strain VLK-12 exhibited typical morphological, cultural, physiological and biochemical characteristics of the genus *Rhodococcus* spp. The strain showed a close relation with *Rhodococcus erythropolis* based on the 16S rRNA gene sequence. The 16S rRNA sequence was deposited in the GenBank database of NCBI with the accession number KC106730. Basing on the morphological, physiological, biochemical characteristics and molecular characterization by 16s rRNA sequencing, the strain has been identified as *Rhodococcus erythropolis* VLK-12 [16].

3.2 Screening for L-asparaginase by the strain VLK-12

Screening test for the production of L-asparaginase was carried out on asparagine-glycerol salts (ISP-5) agar medium. The production of L-asparaginase was evidenced by change of color from yellow to pink (Plate 1).

(A) Control (without inoculation). (B) L-asparaginase production by the strain VLK-12

Fig 1: Screening of the strain VLK-12 for the production of L-asparaginase

3.3 Production Profile of L-asparaginase by the strain VLK-12

Growth pattern as well as L- asparaginase production of the strain was determined in modified ISP-5 broth. The production of L- asparaginase by the strain began after 24 h of incubation, increased progressively, peaked at 72 h and then started declining (Fig. 1). Maximum levels of L-asparaginase production as well as growth were observed after 72 h of incubation in *Arthrobacter kerguelensis* VL-RK_09 [19], *Nocardia levis* MK-VL 113 [20] and *Amycolatopsis* CMU-H002 [21]. A positive correlation between the growth and L-asparaginase productivity was reported in *S. albidoflavus* [22].

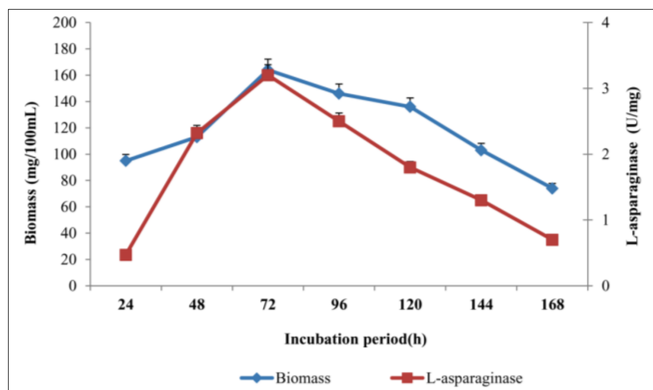

Fig 1: Growth and production of L-asparaginase by *Rhodococcus erythropolis* VLK-12 cultured in modified ISP-5 broth (Values are the means of three replicates ± SD).

3.4 Impact of initial pH on L- asparaginase production by the strain VLK-12

The influence of initial pH levels from 5 to 10 was examined to find out the optimal pH. The optimal production of L-asparaginase by the strain VLK-12 was found at pH 7.0 (Fig. 2). These results are in conformity with those reported for *Arthrobacter kerguelensis* VL-RK_09[19], *Nocardia levis* MK-VL_113 [20] and *Streptomyces* AQB VC67 [23]. Sahu *et al.* [24] also recorded optimal L-asparaginase production at pH levels between 7 and 8 for *S. aureofasciculus*, *S. chattanoogenesis, S. hawaiiensis, S. orientalis, S. canus* and *S. olivoviridis.* In the present study, the strain showed maximum L-asparaginase production when cultured in modified ISP-5 broth for 72 h with an initial pH 7.

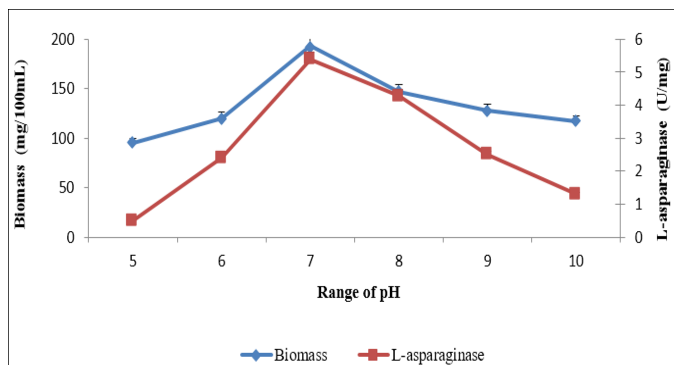

Fig 2: Effect of initial pH on growth and L-asparaginase production by *Rhodococcus erythropolis* VLK-12 cultured in modified ISP-5 broth (Values are the means of three replicates ± SD).

3.5 Effect of temperature on L- asparaginase production by the strain VLK-12

The influence of temperature on L-asparaginase production by the strain is presented in Fig. 3. The enzyme production is enhanced with increase in temperature from 20°C to 30°C. Further increase in temperature resulted in decline of enzyme production. The optimal temperature recorded for the production of L-asparaginase by the strain VLK-12 was 30 ºC. Similar results were recorded in *Arthrobacter kerguelensis* VL-RK_09 [19], *S. ginsengisoli* [25], *S. acrimycini* NGP [26] and *Nocardia levis* MK-VL_113[20].The study revealed that L-asparaginase production by the strain VLK-12 was high when grown in modified ISP-5 broth with initial pH 7.0 for 72 h at 30ºC.

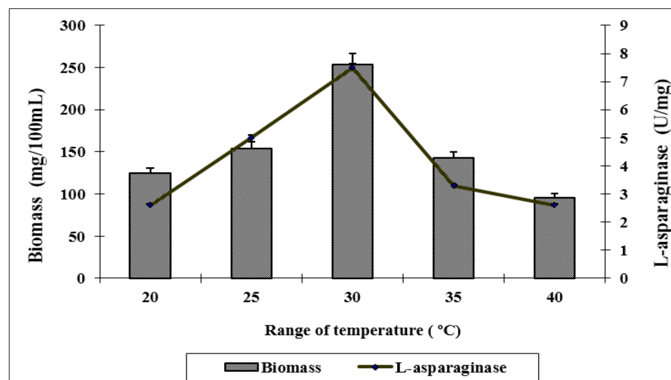

Fig 3: Effect of temperature on growth and L-asparaginase production by *Rhodococcus erythropolis* VLK-12 cultured in modified ISP-5 broth (Values are the means of three replicates ± SD).

3.6 Influence of Carbon Sources on L- asparaginase Production by *Rhodococcus erythropolis* VLK-12

The effect of different carbon sources amended in modified ISP-5 broth on L-asparaginase production by the strain VLK-12 is presented in Fig.4. As compared to the other carbon sources tested, L-asparaginase production by *Rhodococcus erythropolis* VLK-12 was high in the basal medium containing glycerol. Also noted that the glycerol as the one of the best carbon source for L-asparaginase production by *Nocardia levis* MK-VL_113 [20] and *Streptomyces albus* [27]. Studies on the effect of different levels of glycerol (1–3.5% w/v) revealed that optimal yields of L-asparaginase were recorded in medium amended with glycerol @ 1.5% (Fig.5). Further increase in concentration led to decline in the production of the enzyme.

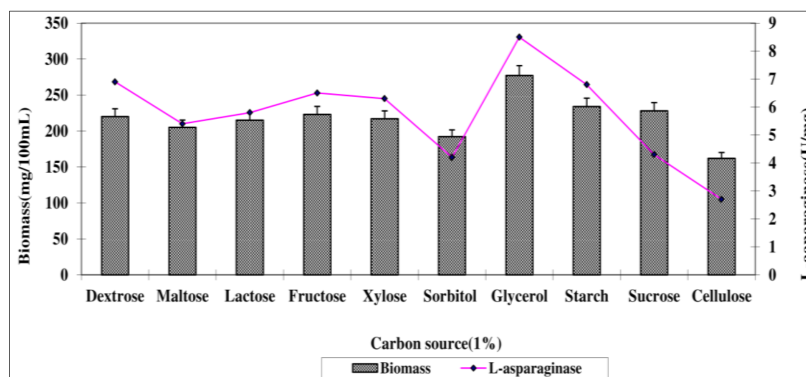

Fig 4: Effect of carbon source on growth and L-asparaginase production by *Rhodococcus erythropolis* VLK-12 cultured in modified ISP-5 broth (Values are the means of three replicates ± SD).

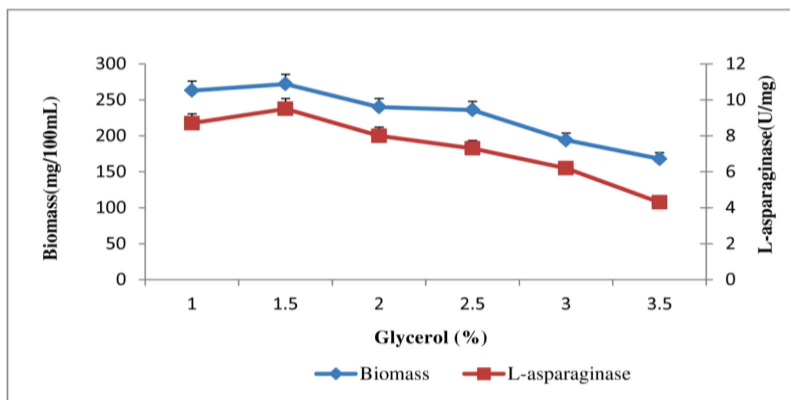

Fig 5: Effect of concentration of optimized carbon source on L-asparaginase production by *Rhodococcus erythropolis* VLK- 12 cultured in modified ISP-5 broth (Values are the means of three replicates ± SD).

3.7 Impact of nitrogen sources on L-asparaginase production by *Rhodococcus erythropolis* VLK- 12

The impact of nitrogen sources on the production of L-asparaginase by the strain VLK-12 was studied by incorporating different nitrogen compounds to ISP-5 broth containing 1.5% glycerol. L-asparaginase production by the strain varied with different nitrogen compounds tested (Fig.6). Among the nitrogen sources used, yeast extract supported high yields of L-asparaginase by the strain VLK-12 followed by beef extract. Yeast extract was reported as an excellent nitrogen source for L-asparaginase production by *Arthrobacter kerguelensis* VL-RK_09 [19] and *Nocardia levis* MK-VL 113 [20]. Yeast extract is essential for growth and L-asparaginase synthesis, but at high concentrations it inhibits the production of L-asparaginase [28].

The study of different levels of yeast extract (0.2 -2% w/v) revealed that the production of L-asparaginase by the strain VLK-12 was high in medium containing 1% yeast extract (fig. 7). Optimization studies done by Rajesh kumar *et al.* [19] and Kavitha and Vijayalakshmi [20] revealed high L-asparaginase production with yeast extract @ 1.5% by *Arthrobacter kerguelensis* VL-RK_09 and *Nocardia levis* MK-VL 113 respectively. Narayana *et al.* [22] reported 2% yeast extract was good source for the production of L-asparaginase by *Streptomyces albidoflavus.*

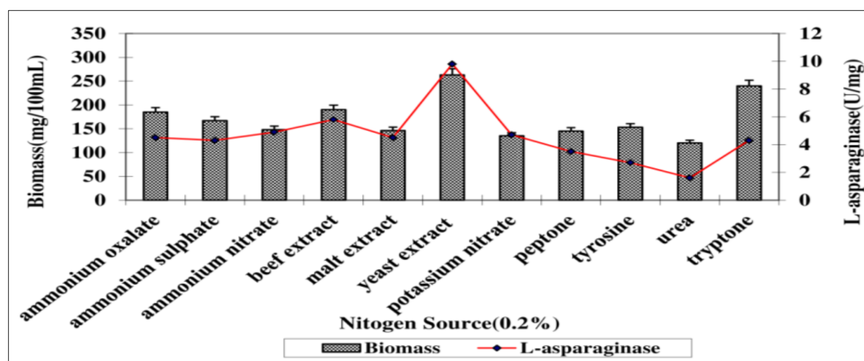

Fig 6: Effect of Nitrogen source on L-asparaginase production by *Rhodococcus erythropolis* VLK-12 cultured in modified ISP-5 broth (Values are the means of three replicates ± SD).

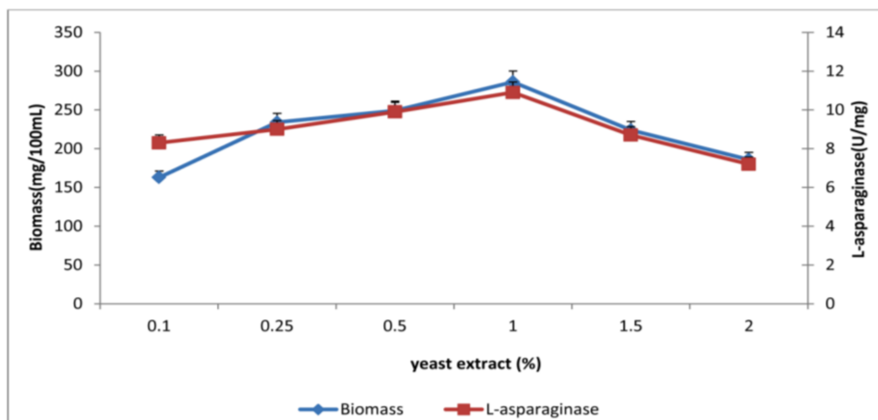

Fig 7: Impact of concentration of yeast extract on L-asparaginase production by *Rhodococcus erythropolis* VLK-12 cultured in modified ISP-5 broth (Values are the means of three replicates ±SD).

4. Conclusion

In the present investigation, the cultural and nutritional parameters for the optimal production of L-asparaginase by *Rhodococcus erythropolis* VLK-12 were recorded. The production of L-asparaginase by the strain improved from initial 3.2 U to 10.9 U when cultured in modified ISP-5 broth adjusted to pH 7.0 supplemented with yeast extract (1%) and glycerol (1.5%) maintained at 30°C for 72 h. This is the first report on the production and optimization of L-asparaginase by *Rhodococcus erythropolis* VLK-12 and further studies on purification and characterization of the enzyme are in progress.

5. References

1. Warangkar SC, Khobragade CN. Purification, characterization, and effect of thiol compounds on activity of the *Erwinia carotovora* L-asparaginase. Enz. Res., 2010; 1:1-10.
2. Boyse AE, Old LJ, Campbell HA. Suppression of murine leukaemia of various types: comparative inhibition activities of guinea pig serumL-asparaginase and E. coli, Journal of Experimental Medicine. 1967; 125:17-31.
3. Mohana Priya P, Radha Krishnan M, Balagurunathan R. Production and optimization of L-asparaginase from *Streptomyces sp.* (TA22) isolated from Western Ghats, India. J Chem. Pharm. Res. 2011; 3:618-624.
4. DeJong PJ. L-asparaginase production by *Streptomyces griseus*, Applied Microbiology. 1972; 2:1163-1164.
5. Mostafa SA, Salama MS. L-asparaginase producing Streptomyces from the soil of Kuwait, Zentralbl Bakteriol Naturwiss. 1979; 134:325-334.
6. Abdel-Fatah MK. Studies on the asparaginolytic enzymes of Streptomycetes. 1-Culture conditions for the production of L-asparaginase enzyme from *Streptomyces* longsporusflavus (F-15) strain, Egyptian Journal of Microbiology. 1996; 30:247-260.
7. Abdel Fatah MK, Abdel-Mageed AR, Abdel-All SM, *et al.*, Purification and characterization of L-asparaginase produced by *Streptomyces phaeochromogenes* FS-39, Journal of Drug Research. 1998; 22:195-212.
8. Sudhir AP, Dave BR, Trivedi KA, Subramanian RB. Production of an L-asparaginase gene from actinomycete isolate Streptomyces ABR2. Annals of Microbiology. 2012; 62:1609-1614.
9. Sabha Mahmoud El-Sabbagh1, Nadia Hamed El-Batanony, Tarek A. Salem L-asparaginase produced by Streptomyces strain isolated from Egyptian soil: Purification, characterization and evaluation of its anti-tumor African Journal of Microbiology Research. 2013; 7:5677-5686.
10. Neelima Deshpande, Prachi Choubey, Manasi Agashe Studies on Optimization of Growth Parameters for L-Asparaginase Production by *Streptomyces ginsengisoli*. The Scientific World Journal. 2014, 1-5.
11. Chinna Narasaiah BV, Leelavathi, Anil Kumar, Manne G, Swapna, Moparthy John Paul P. Mariya Dasu 4 Screening of Streptomyces Albus CN-4 For Enzyme Production and Optimization of L-Asparaginase. International Journal of Scientific and Research Publications. 2015; 5:1-8.
12. Balakrishnan Meena, Lawrance Anburajan, Thadikamala Sathish, Rangamaran Vijaya Raghavan, Gopal Dharani, Nambali Valsalan Vinithkumar, *et al*. from *Streptomyces griseus* NIOTVKMA 29: optimization of process variables using factorial designs and molecular characterization of L-asparaginase gene Sci Rep. 2015; 5:12404.
13. Gunasekaran SL, Mc Donald, Manavathu M, *et al.*, Effect of culture media on growth and L-asparaginase production in *Nocardia asteroides*, Biomedical Letters. 1995; 52:197-201.
14. Ushakiranmayi M, Sudhakar P, Vijayalakshmi M. Production and optimization of L-asparaginase by actinobacterium isolated from Nizampatnam mangrove ecosystem. 2014; 35:799-805.
15. Chauhan B, Dhaliwal MK. Study of L-asparaginase production in Nocardia spp. isolated from Mangroves. Journal of Chemical, Biological and Physical Sciences. 2014; 4:3476-3484.
16. Naragani Krishna, Rajesh Kumar M, Usha Kiranmayi M, Vijayalakshmi M. Optimization of Culture Conditions fo Enhanced Antimicrobial Activity of *Rhodococcus erythropolis* VLK-12 Isolated from South Coast of Andhra Pradesh, India. *Brit. Microbiol. Res. J.* 2014; 4:63-79.
17. Gulati R, Saxena RK, Gupta R. A rapid screening for L-Asparaginase producing microorganisms. Lett. Appl. Microbiol. 1997; 24:23-26.
18. Peterson RE, Ciegler A. L-asparaginase production by various bacteria. Appl Microbiol. 1969; 18:64-7.
19. Rajesh kumar Munaganti, Vijayalakshmi Muvva, Mani deepa Indupalli. Studies on optimization of L-asparaginase production by arthrobacter kerguelensis VL-RK_09 isolated from mango orchards. International Journal of Pharmacy and Pharmaceutical Sciences. 2015; 7:112-115.
20. Kavitha A, Vijayalakshmi M. A Study on L-Asparaginase of *Nocardia levis* MK-VL 113. Sci World J. 2012, 1-5.
21. Khamna S, Yokota A, Lumyong S. L-asparaginase production by actinomycetes isolated from some Thai medicinal plant rhizosphere soils, International Journal of Integrative Biology. 2009; 6:22-26.
22. Narayana KJP, Kumar KG, Vijayalakshmi M. L-asparaginase production by Streptomyces albidoflavus, Indian Journal of Microbiology. 2008; 48:31-336.
23. Dhevendran, Annie K. Antibiotic and L-asparaginase of Streptomycetes isolated from fish,shellfish and sediments of veli estuarine along Kerala Coast. Indian J Mar Sci. 1999; 28:335-337.
24. Sahu MK, Sivakumar K, Poorani E, Thangaradjou T, Kannan L. Studies on L-asparaginase enzyme of actinomycetes isolated from estuarine fishes, Journal of Environmental Biology. 2007; 28:465-474.
25. Deshpande N, Choubey P, Agashe M. Studies on optimization of growth parameters for L-Asparaginase Production by *Streptomyces ginsengisoli*. Sci World J. 2014; 2014:1-6.
26. Selvam K, Vishnupriya B. Partial purification and cytotoxic activity of L-asparaginase from *Streptomyces acrimycini* NGP. Int J Res Pharm Biomed Sci. 2013; 4:859-69.
27. Krishna Reddy V, Reddy SM. Effect of C and N sources on asparaginase production by bacteria, Indian Journal of Microbiology. 1990; 30:81-83.
28. Verma N, Kumar K, Kaur G, Anand S. L-asparaginase: a promising chemotherapeutic agent, Critical Reviews in Biotechnology. 2007; 27:45-62.