International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 9 | Special Issue 2- ICESMT| 2019 | 99-106
International Conference on "Empowering Society with Microbial Technology"
Held at Tuljaram Chaturchand College of Arts, Science and Commerce, Baramati - 413 102,
Dist. Pune, Maharashtra, India, 7-9 February 2019
| Research Article | Biological Sciences |Open Access |MCI Approved|
|UGC Approved Journal|

Studies on Isolation, Screening of L-Asparginase Producing Bacteria, Optimization and Purification of L-Asapraginase Production

Komal Jagtap, Shivaji Sathe, Nilofer Bagwan, Swapna Tathwadakar and Yogini Mulay*
Tuljaram Chaturchand College of Arts, Science& Commerce, Department Of Microbiology Baramati-413102, Maharashtra, India.

Received: 30 Jan 2019 / Accepted: 20 Feb 2019 / Published online: 01 Apr 2019
Corresponding Author Email: mailtoyogini@yahoo.com

Abstract
L-asparginase has received increased attention for its anti- carcinogenic potential. There is tremendous scope in screening a novel source and studying their properties and application. In the current study L-asparginase producing bacteria were screened from rhizosphere soil of Baramati. Modified M9 medium with a phenol red indicator was used for screening purpose. Total 20 isolates were L-asparginase positive. Among these isolates, RS-2 produced maximum L-asparginase activity (6.60U/ml). L-asparaginase assay was done by Nesslerization method. The factor such as pH, temperature, NaCl, MgSO4, and $CaCl_2$ affecting production of L-asparginase were optimized in present study. The optimum pH, temperature, NaCl, $MgSO_4$ and $CaCl_2$ for enzyme production were 7, 37°C,0.5%,2%,2% respectively. The enzyme production was induced by different carbon and nitrogen sources Glucose (20%) and L-aspargine (2%) proved to be best carbon and nitrogen source respectively. At optimum condition RS-2 has shown L-asparginase activity (17.3U/ml) which is twofold higher than that of before optimization. L-asparginase has been partially purified with ammonium sulphate precipitation (80%). The enzyme was purified13.59 fold and showed a final specific activity 811 IU/mg with an 84.39% yield, hence could find potential application in medical and agricultural field.

Keywords
anti- carcinogenic; L-asparginase; Nesslerization

*****

INTRODUCTION
L-asparagine is an important amino acid used as nutritional factor for the growth of both normal cells and cancer cells. Tumor cells require high amount of asparagine for rapid growth whereas normal cell growth is independent of it. [1] Low levels of the nonessential amino acid asparagine only affect the viability of abnormal cells as these cells have abnormally high requirements for asparagine. This is because normal cells produce enzyme asparagine

synthetase, which is able to synthesize asparagine from aspartic acid, whereas, in cancer and tumor cells this enzyme is present in low levels. [16]L-Asparaginase has received increased attention for its anti-carcinogenic potential. Anti-cancer action of this enzyme is attributed to reduction L-asparagines, hence tumor cells unable to synthesis this amino acid are selectively killed by L-asparagine deprivation. Asparaginase (EC 3.5.1.1) is responsible for formation of aspartic acid and ammonia from substrate L-asapargine [1] .L-Asparaginase is widely distributed in large number of microorganism. *E.coli*, *Pseudomonas aurigenosa*, *Vibrio succinogenes* and *Staphylococcus* sp. have the potential of L-Asparaginase production among fungi- *Aspergillus*, *Fusarium* and *Penicillum* were reported to produce L-Asparaginase[2]. Microorganisms are better
Source for the production of L asparginase, because they can easily culture, extraction and purification as well as the methods of this process from them is also convenient.[3] For production of L -Asparginase for industrial use, isolation and characterization of new promising strains is a continuous process. Two types of bacterial L-asparaginases have been identified: type I and type II. Type I L-asparaginases are expressed constitutively in the cytoplasm and catalyze the hydrolysis of Lasparaginase, whereas type II L-asparaginase was expressed under anaerobic conditions in the periplasmic space of the bacterial membranes and exhibit higher specificity for L-asparaginase hydrolysis Campbell HA,1967.[1] In the present study, we report the isolation, screening and characterization of L-Asparginase producing bacteria from the soil samples collected from different sites of Baramati area. Culture conditions were optimized (temperature, pH, etc.) to achieve maximum enzyme production and better enzyme activity.

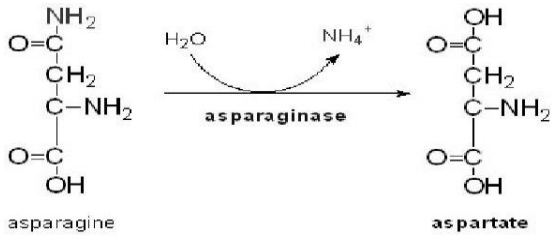

Fig 1 Hydrolysis of L-asapargine to aspartic acid and ammonia

MATERIALS AND METHODS

Sample Collection:

Soil samples were collected from different sites such as rhizosphere soil from Tuljaram Chaturchand College, Baramati, Dairy waste soil (DS) from Malegaon (Nandan dairy).Airflora at Tuljaram Chaturchand college campus (AS). Slaughterhouse waste (SS) from Baramati was collected, with the help of sterile spatula, and then transferred into sterile plastic bags under aseptic conditions [2].

Isolation and Screening of L-Asparginase producing bacteria

One gram of soil sample was mixed with 9 ml of sterile distilled water. Serial dilution was done up to 10^{-5} and spread onto M 9 medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-asparagine: 0.5g; 1mol l^{-1} $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l^{-1} $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml; agar 2 g; p^H 7). Plates were incubated at 37°C for 24 hrs, Colonies showing pink zone were selected for further studies. [6]

Preparation of Inoculum

RS-2 was grown M 9 medium for 24 h at 37°C and was used as inoculum (1% V/V) after adjusting the cell count to10^6cells/ml in all the experiments.

Enzyme Production

L-asparaginase production was carried out by submerged fermentation in250mL Erlenmeyer flask containing 50ml of sterilized medium was used for production. A loop full of exponential phase culture was inoculated into the medium. The flask was incubated on shaker at 120 rpm at 37°C for 24 hrs. Un-inoculated medium served as control. Centrifugation was carried out at 10000 rpm for 10 min at 4°C. The supernatant was used as crude enzyme source to determine the enzyme activity. [3]

L-Asparaginase Assay

0.5 ml asparagines of 0.04 M was taken in a test tube, towhich equal volume of 0.5 M acetate buffer of pH 5.4, enzyme solution and distilled water was added to put together the volume up to 2.0 ml. The reaction mixture was incubated for 30 min. After theincubation period the reaction was stopped by adding 0.5 ml of 1.5 M Trichloroacetic acid. 0.1 mlwas taken from the above reaction mixture and 3.7 ml distilled water and to that 0.2 ml Nesseler'sreagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was prepared by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in International unit.[7]

International Unit (IU)

One International Unit (IU**)** of L-asparaginase is the amount of enzyme which liberates 1 µmol of ammonia per minute per ml (µmole/ml/min). [7]

Protein Estimation

Proteins were estimated using the method of Lowry et al. Bovine serum albumin (BSA) was used as standard. [8]

Growth Curve

Growth curve was determined in terms of absorbance at 600nm as described earlier. [8].

Effect of Different cultural condition on production of L-asparaginase

The effect of pH (4 to 10), incubation temperatures (25 to 55°C), NaCl concentration (1 to5%), $MgSO_4$. $7H_2O$ (0.1to 0.5%) and $CaCl_2$. $2H_2O$ (0.1to 0.5%), glucose (5 to 30 %) and L asparagine (0.5 to 3%) was estimated in shake flask of 500 ml capacity containing 100 ml of medium. The culture filtrate was separated by centrifugation at 10000 rpm for 10 min at 4°C. [4]

Purification of L-asparaginase

The purification was carried out at 4ºC on the crude extract, according to the modified method of Distasio *et al*.1976.[5] Ammonium sulfate fractionation was carried out by adding fine powder of ammonium sulfate to 80% saturation in culture broth. The mixture was left for 24h at 4°C, followed by centrifugation at 8,000 rpm for 20 min at 4°C. The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5and dialyzed against the same buffer for 24 h at 4°C.

Results and Discussion-

Isolation and Screening of L-asparaginase bacteria

From four soil samples, 20 different bacteria were isolated and screened for L-asparaginase production. The results of 20 isolated strains are shown in fig 1. Microbial strains RS-2, DS-3, AS-4, and SW-6 with pink colored colony were selected. Among these isolates, RS-2 produced maximum L-asapraginase activity with respect to zone of hydrolysis.

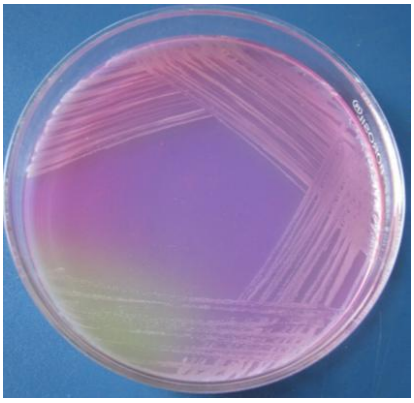

Fig 1: Isolation & Screening of L-asparaginase producing bacteria using M9 medium containing L-asparagine and phenol red indicator. Plate showing positive L-Asparginase activity by RS-2 isolated from rhizoshpere soil.

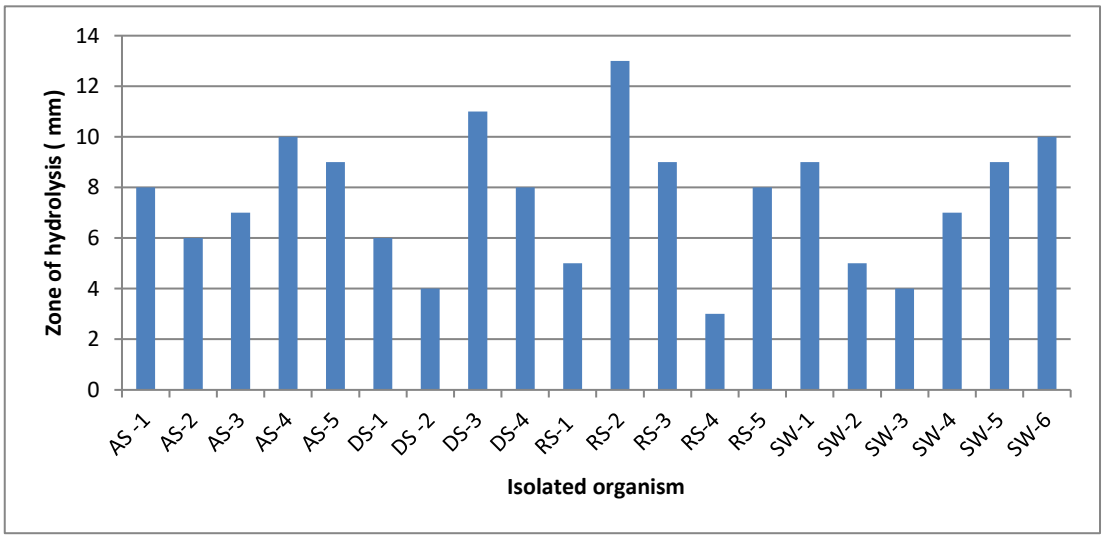

Fig2. The L-asaparginase activity by different isolates on medium.

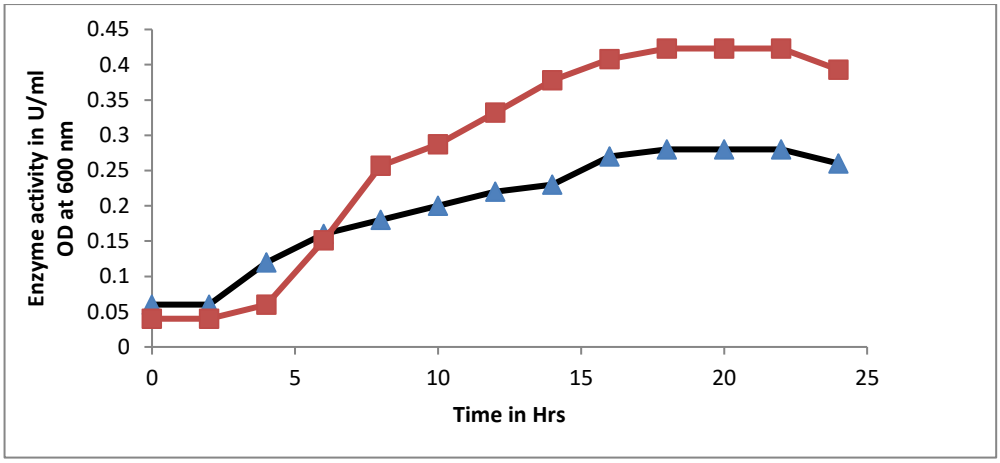

Fig 3. Time course of L-asparaginase production by RS-2cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol l^{-1} $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l^{-1} $CaCl_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml ; agar 2 g; pH7)for 24 hrs.(■)L- Asaparginase activity,(▲) Absorbance.

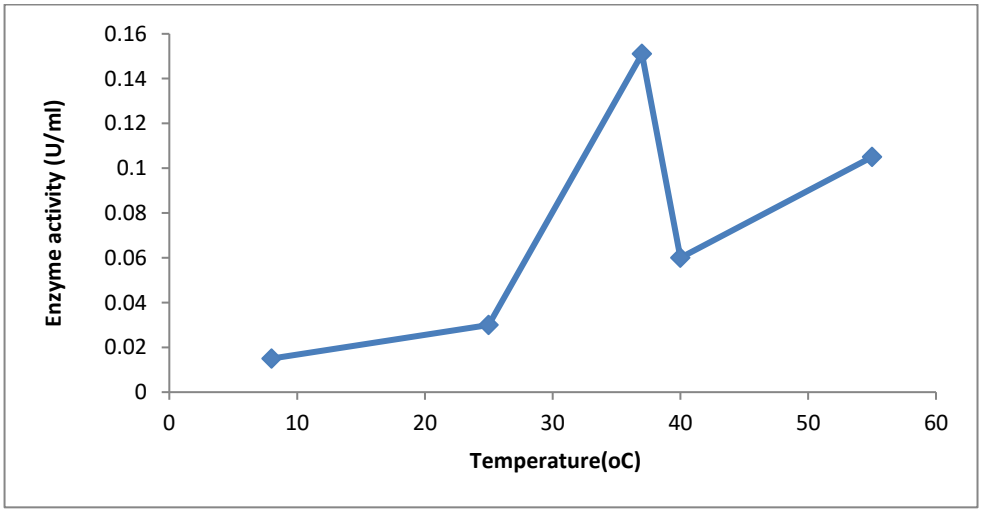

Fig 4 Effect of temperature on L-asparaginase production by RS-2.cells were cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol l^{-1} $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l^{-1} $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml ; agar 2 g; pH 7)for 24 hrs.

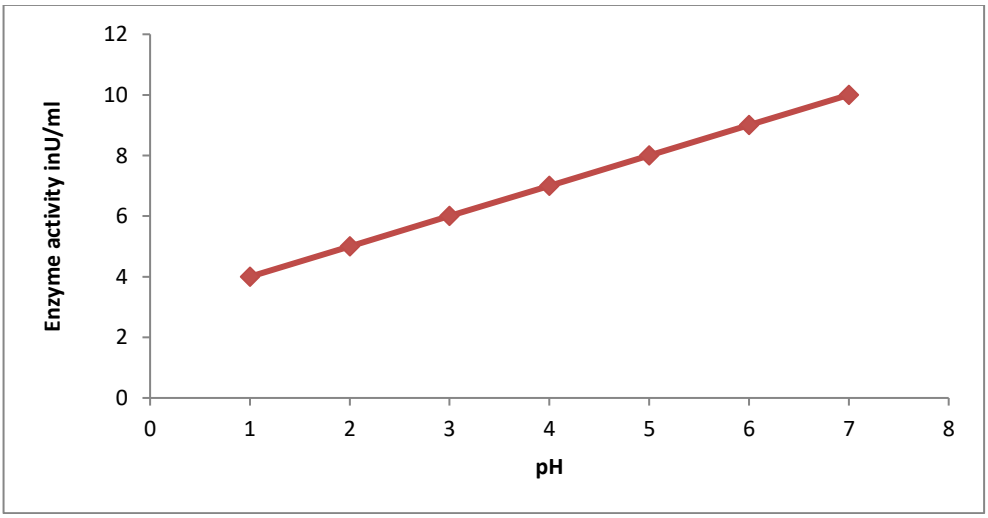

Fig.5 Effect of pH on L-asparaginase production by RS-2cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol l^{-1} $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l^{-1} $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml; agar 2 g; pH 7) for 24 hrs.

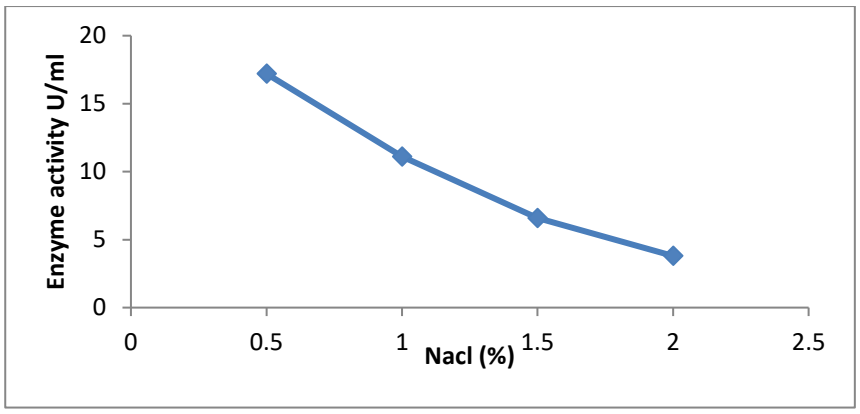

Fig.6 Effect of NaCl on L-asparaginase production by RS-2cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol^{-1} $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l $^{-1}$ $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml; agar 2 g; pH 7) for 24 hrs.

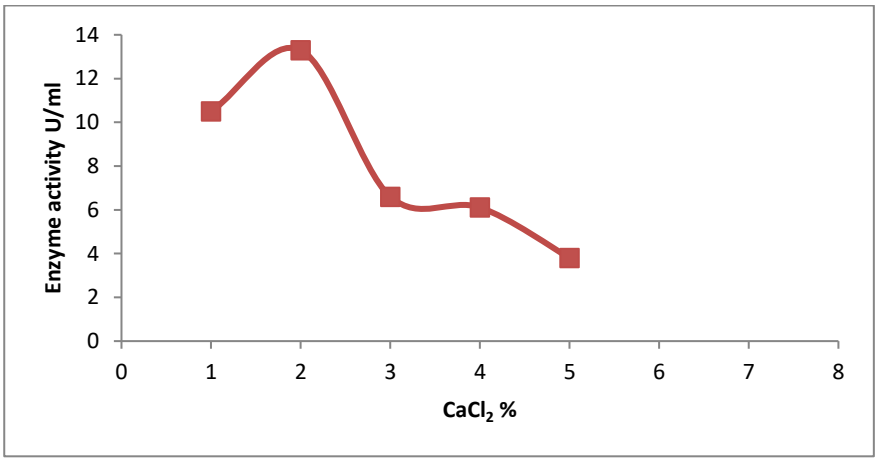

Fig 8 Effect of$CaCL_2$on L-asparaginase production by RS-2cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol l $^{-1}$ $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l $^{-1}$ $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml; agar 2 g; pH 7)for 24 hrs.

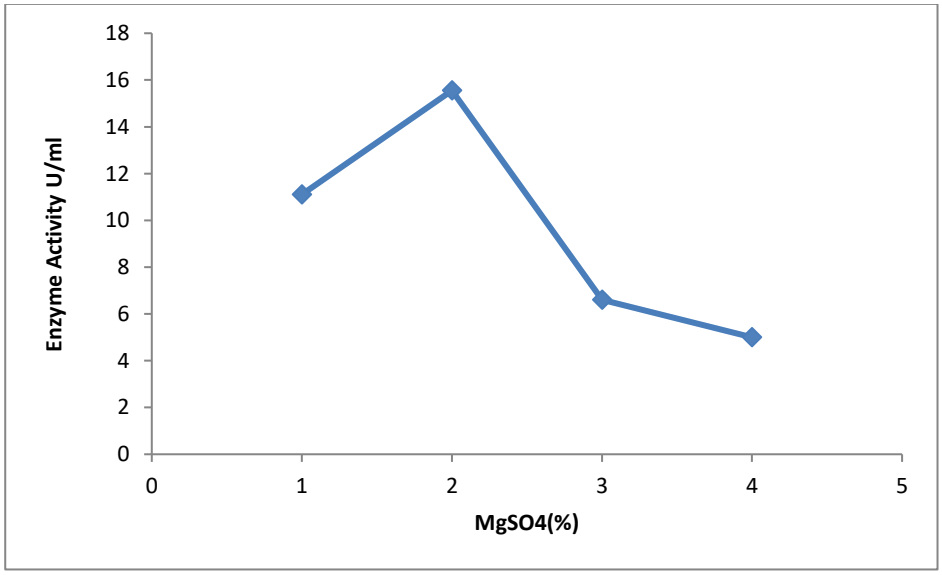

Fig 7 Effect of $MgSo_4$ on L-asparaginase production by RS-2cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol l $^{-1}$ $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l $^{-1}$ $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml; agar 2 g; pH 7) for 24 hrs.

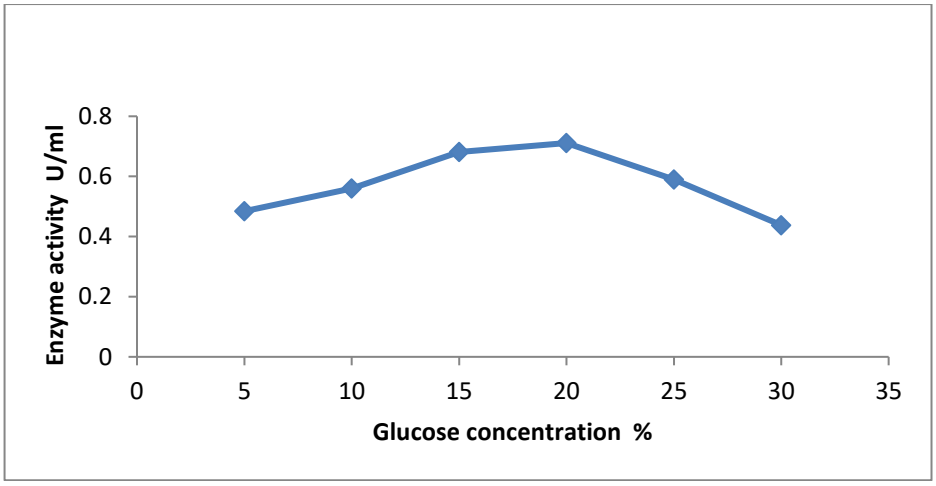

Fig 9 Effect of glucose on L-asparaginase production by RS-2cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol l^{-1} $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l^{-1} $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml; agar 2 g; pH 7) for 24 hrs.

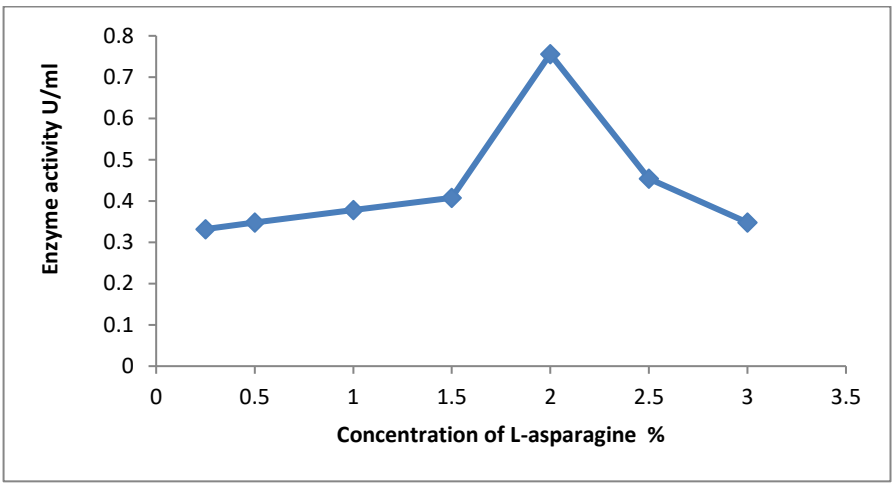

Fig 10 Effect of L-asparagine on enzyme production by RS-2cultivated in production medium supplemented with 2.5% phenol red ($Na_2HPO_4.2H_2O$: 0.6g; KH_2PO_4: 0.3g; L-aspargine: 0.5g; 1mol l^{-1} $MgSO_4$. $7H_2O$: 2ml; 0.2 mol l^{-1} $CaCL_2$.$2H_2O$: 0.1 ml; 20% glucose stock 1ml; agar 2 g; pH 7) for 24 hrs.

The L- asaparginase activity by different isolates was determined by inoculating (100 µl) an equal number of cell(10^^{6} cells/ml) on M9 medium containing 1%L-asapargine.The agar plates were incubated for 24 hrs at 37^{o}C.The diameter of zone of hydrolysis were measured and represented as L-asaparginase activity.

Time course of L-asparaginase production

Fig .3 shows growth of isolated strain RS-2 and time course of L-asparaginase production at 37^{o}C, pH 7 for 24 hrs. L-asparaginase production by RS-2 was detected after six hrs of growth (0.151U/ml) and showed continuous rise till it reached stationary phase (0.423 U/ml) at 22 hrs. After 22 hrs there was bit decrease in enzyme production. Fig 2 reveals that maximum L-asparaginase was produced in logarithmic phase. Alejandra et.al1996 found similar result with *Rhizobium. etli.*[11]. As L-asparaginase is an enzyme it was expected to be produced in log phase. This graph shows that L-asparaginase production is associated with growth of selected strain RS-2. The enzyme production is depending upon the characteristics of culture and growth rate.

Effect of different cultural condition physicochemical factors

It was found that RS-2 produced maximum L-asparaginase at 37^{o}C (0.151µmol/ml/min). Amena et al, 2010 found similar result while working with *Bacillus sp* [12].Ekpa Emmanuel et al,2015 showed effect of temperature on L- asparaginase from Hedgehog Serum, was recorded at 39°C.[13]As RS was isolated from soil sample, it was expected that L-asparaginase will have 30^{0}C optimum temperature. The effect of temperature on L- asparaginase activity is shown in Figure 4. Commonly, the outcome of temperature on enzyme-catalyzed reactions is awfully complex. This is because amendin the observed rate

may be due to a variety of causes like stability of the enzyme protein, pH of the buffer system, other factors.

Effect of pH on L-asparaginase production by RS-2

The result of pH on Asaparginase activity is shown in Figure 5. It was found that RS-2 produced maximum L-asparaginase at pH 7 (1.195µmol/ml/min). Moharam and Amena 2010 observed that yield of L-asparaginase increased with increasing the initial pH of medium upto 8 and thereafter it decreased.[12] Ekpa Emmanuel et al,2015 reveals that pH optimum for the partially purified Hedgehog serum L-asparaginase is 7.8.[13] Most L-asparaginases having antitumor activities have optimum pHs of between 7.5-8.5 as reported by Swain et al.

Sidda lingeshwara K.G et al, 2011 partially purified L-asparaginase was active over broad pH ranges (4.0 - 11.0) with an optimum at pH 9.[4] Reason for these observed optimum pH values are due to the fact that medically useful asparaginases normally act within physiological pH that is around the normal pH of blood.

Effect of NaCl on L-asparginase production by RS-2

It was found that RS-2 produced maximum L-asparginase at 0.5 % NaCl concentration (17.2 U/ml). Usha et al. 2011 found 0.05% NaCl concentration optimum for L-asparginase production by *E.coli*, which is less than what we have found. [14]

Effect of $MgSO_4$ (%) on L-asparginase production by RS-2

It was found that RS2 produced maximum L-asparginase at 2% $MgSO_4$(15.55 U/ml) It was observed that increasing in concentration of $MgSO_4$ beyond 0.5% resulted in decreasing the L-asparginase production.

Effect of $CaCl_2$ (%) on L-asparginase production by RS-2

It was found that RS2 produced maximum L-asparginase at 2%$CaCl_2$ (13.33U/ml) beyond that production deceased consecutively.

Effect of different concentration Glucose on L-asparaginase production by RS-2.

It was found that RS produced maximum L-asparaginase at 20% Glucose concentration (0.711µmol/ml/min). Usha et al. 2011 found 15% concentration optimum for L-asparaginase production by *E.coli*, which is less than what we have found. [14]

Effect of different concentration substrate (%) on L-asparaginase production by RS-2.

It was found that RS produced maximum L-asparaginase at 2%. L-asparaginase concentration (0.756µmol/ml/min). Usha et al. 2011 found 0.5 % L-asparaginase concentration optimum for L-asparaginase production by *E.coli, which* is less than what we have found.[14] Renuka D Joshi et al 2016 showed a concentration of 1.0% of L-asparagine in the culture media, all the three endophytic bacterial IS-1, 2, 3 isolates showed maximum L-asparaginase activity.[15].P. V. Kamalkumari et al 2013, observed that L-Asparaginase activity is maximum at 0.04 M concentration from *Streptomyces griseoluteus*WS3. [16] Enzyme activity was increases with increase in substrate concentration.

Partial purification of L-asparaginase

Crude L-asparginase preparation was affected by salt extraction method (80%) and showed that most of the enzyme activity was obtained in the precipitate. The enzyme was purified 13.59-fold with recovery of 84.39%. El shobaky Ahmed et al 2014, L-Asparaginase productions by *Erwinia carotovora* reach to enzyme activity (682.48U/ml) and specific activity (5.6U/mg) after precipitation by Ammonium sulphate.[8]

Ashraf A.et al (2013) found that purification of L-asparaginase from *Pseudomonas aeruginosa* 50071 was achieved by using 80% ammonium sulfate saturation. [8]

Table 1. Partial purification of L-asparginase

Content	Fraction volume ml	Total activity U/ml	Total Protein mg/ml	Specific activity U/mg	Yield %	Purification fold
Crude extract	10	173	2.9	59.65	100	1
Ammonium Sulphate precipitation (80% saturation)	1	146	0.18	811.1	84.39	13.59

CONCLUSION

Rhizosphere soil samples can provide a rich source of L-asparaginase producing bacteria when compared to other soil sample. Looking at the results obtained in the present investigation it can be said that the RS-2Isolates hear can be a good source for the production of L-Asparaginase enzyme. The enzymes production can be optimized to further state by studying various parameters like optimizing the incubation time, optimizing the various carbon and nitrogen sources. In conclusion, maximal L-asparaginase productivity was attained at pH 7.0 and 37ºC. The isolate RS-2 produced significant amount of L-asparaginase (17.3µmol/ml/min). The enzyme was purified 13.59-fold and showed a final specific activity of 811 IU/mg with 84.39% yield. The potential of this isolate is important in medical and agricultural field.

REFERENCE:

1. Balcao V., Mateo C., Fernandez L., Lafuente R., Malcota F. and Guisan J., Structural And Functional Stabilization Of L-Asparaginase Via Subunit Immobilization On To Highly Activated Supports, Journal of Biotechnology progress, 17(3); 537-542, (2001)
2. Akhilesh K., Faivaz A., Jayanand, Pushpendra S., Production &purification of L-asparaginase from bacterial source, International Journal of Universal Pharmacy and Life Sciences 2(2); 2249-6793(2012).
3. Prasad T., Bhavana M., and S. Rajagopal, Isolation and Screening Of L– Asparaginase Producing Bacteria From Visakhapatnam Soil Samples, International Journal Of Pharmaceutical, Chemical And Biological Sciences**,** 3(4); 1121-1125, (2013)
4. Siddalingeshwara K. and Lingappa K., Production and Characterization of LAsparaginase A Tumour inhibitor, International Journal of Pharm Tech Research Vol. 3, No.1, pp 314-319, (Jan-Mar 2011)
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193(1), 265–275, (1951).
6. John D., Ana S., Mehrdad N., Donald D., Glutaminase free aspaginase from Vibrio succinogenes: An anti-lymphoma enzyme lacking hepatotoxicity, International journal of cancer 30 (3); 343-347, (1982)
7. Yogini M., and Rajendra D., Production of amylase from indigenously isolated strain of *Aureobasidium Pullulans* and its hyper producing mutant, Journal of microbiology, biotechnology and food sciences, 7 (3); 287-293 (December 2017)
8. Shobaky A., Mohamad A., Abbas and Shymaa N, Production, Purification And Characterization Of Extracellular L-Asparaginase From *ErwiniaCarotovora,*Aska International Journal of Bioassays,3(12); 3553-3559,(2014)
9. Arima K., Microbial enzyme production in Global Impact of Applied Microbiology, M.P. Starr (eds.), pp. 279-299, JohnWilley, New York, USA. (1964)
10. Ashraf A., El-Bessoumy., Mohamed S., and Jehan M., Production, Isolation, and Purification of L-Asparaginase from *Pseudomonas Aeruginosa* 50071 Using Solid-state Fermentation, *Journal of Biochemistry and Molecular Biology*, Vol. 37, No. 4, pp. 387-393, (July 2004)
11. Alejandra H.,Leobardo O., Gisela D., Socorro D., Alejandro L., Horacio M., and Jorge C, Isolation and Characterization of *Rhizobium etli* Mutants Altered in Degradation of Asparagine, Journal Of Bacteriology, p. 2068–2072, (Mar. 1997).
12. Amena S., Vishalakshi N., Prabhakar M., Dayanand A., Lingappa K., Production, Purification and Characterization of L-Asparaginase from *Streptomyces Gulbargensis,* Brazilian Journal of Microbiology 41: 173-178,(2010).
13. Ekpa E., Nzelibe Hand., Onyike E., Isolation, Partial Purification and Characterization of L-Asparaginase from Hedgehog Serum, Journal of Microbial & Biochemical Technology, 7:6; 404-409(2015)
14. Usha k.,Sudhakar p., and M Vijayalakhsmi, Production and Optimization of L-Asaparginase Actinobacterium isolated from Nizampatam Mangroove ecosystem ,Journal of environmental biology ,(2013)
15. Renuka J., Nikhilesh K.,Optimization studies on L-asparaginase production from endophytic Bacteria ,International Journal of Applied Research; 2(3): 624-629, (2016)
16. P**.**KamalaKumari., Girija S., T.Prabhakar., S.SatyaLakshmi, Purification and Characterization of L-Asparaginase from *Streptomyces griseoluteus*WS3/1, Int. J. Pharm. Sci. Rev. Res., 23(2), 198-202 Nov – Dec 2013.
17. Campbell HA., Two Asparaginase from Escherichia coli B, their separation, purification and antitumor property. Biochemistry, (1967).