Production, purification and characterization of L-asparaginase from bacterial endophytes

R.D. Joshi and N.S.Kulkarni

Microbiology Research Laboratory,

Department of Microbiology,

R.A.College, Washim-444505, MS, India.

ABSTRACT

L-asparaginase is a well-recognized as asparagine degrading enzyme. It is recommended as therapeutic agent due to an antineoplastic activity. The present work deals with partial purification and characterization of L-asparaginase enzyme from bacterial endophytes like *Bacillus licheniformis, Bacillus pseudomycoides* & *Paenibacillus denitriformis* isolated from *Withania somnifera*, *Ocimum sanctum*, *Alovera*, *Murraya koenigii* and *Catharanthus roseus.* The enzyme was partially purified by ammonium sulphate precipitation followed by dialysis. Enzyme specific activity was calculated for purified samples. The maximum enzyme specific activity was observed in *Paenibacillus denitriformis* in which the specific activity of L-asparaginase recorded was 87.5 U/mg followed by *Bacillus licheniformis* 84.48 U/mg and *Bacillus pseudomycoides* 80.95 U/mg. The enzymes characterization exhibited maximal enzyme activity at pH 8 and temperature 40°C and 30 minutes as the optimum time course .The enzyme was positively modulated by $MgCl_2$ and inhibited by EDTA.

Key words: L-asparaginase, bacterial endophytes, specific activity

INTRODUCTION

L-asparaginase received attention in recent years for its anti carcinogenic potential (Siddalingeshwara and Lingappa, 2010). Its action depends upon the fact that tumor cells are deficient in aspartate-ammonia ligase activity, which restricts their ability to synthesize the non-essential amino acid asparagine. Therefore, the tumor cells are forced to extract it from body fluids. The action of asparaginase does not affect the functioning of normal cells, which are able to synthesize enough for their own requirements, but reduce the free exogenous concentration, and so induce a state of fatal starvation in the susceptible tumor cells. Deamidation of L-asparagine by extracts of *E. coli* was first reported in 1957. Later, Mashburn and Wriston observed that L-asparaginase (L-asparagine amidohydrolase, Enzyme Commission 3.5.1.1) purified from cell extract of *E. coli* has an antitumor activity similar to that of guinea pig serum. Although other microorganisms such as *Aerobacter*, *Bacillus*, *Erwinia*, *Pseudomonas*, *Serratia*,

Xanthomonas, *Photobacterium*, *Streptomyces*, *Proteus Vibrio* and *Aspergillus* have a potential for asparaginase production, purified enzyme from *E. coli* has been supplied and employed in the clinical application for acute leukemia and other malignant neoplasms in human. *Erwinia* L-asparaginase exhibited less allergic reactions compared to the *E. coli* enzyme. However, *Erwinia* asparaginase had a shorter half-life than *E. coli* (Asselin *et al*., 1993) suggesting the need to discover new L-asparaginases that are serologically different but have similar therapeutic effects. This may require the screening of soil samples from various sources for isolation of potential microbes, which have the ability to produce the desired enzyme. Hence, studies are continued and focused on abatement of immune reactivity either by modifying the L-asparaginase or by exploring the exotic environment L-asparaginases with novel properties. Various medicinal plants were also used as a source of L-asparaginase (Oza *et al*., 2009). The present study focused on extraction of enzyme from bacterial endophytes isolated from medicinal plants recommended for cancer therapy and continued to partially purification and characterization of the same to explore newer sources of L-asparaginase.

MATERIALS AND METHODS

Isolation and identification of bacterial endophytes: The medicinal plants with the rational as recommended for cancer therapy were selected after proper ancient literature survey and used for isolation of bacterial endophytes. The selected plants were *Withania somnifera*, *Ocimum sanctum*, *Alovera, Murraya koenigii* and *Catharanthus roseus.* The bacterial endophytes were isolated adopting method suggested by Ahmed *et al*., (2012). All isolates were screened for their L-asparaginase activity, followed by genomic identification by 16S rRNA sequencing.

Production of L-asparaginase: Laboratory scale studies on L-asparaginase production:

Enzyme productions from all thee bacterial endophytes as *B.pseudomycoides*, *B. licheniformis* and *P.denitriformis* were carried out separately in triplicate maintaining recorded optimized conditions. Stock cultures of three bacterial endophytes were maintained on starch casein agar slants at 4° C and regular sub culturing of the strains was carried out at an interval of every four weeks. For fermentation, to avail the actively metabolizing cells the Spores suspension of isolated Strains were carried out separately by providing unfavorable conditions and use as initial inoculum. Spores suspension was prepared using 5 day old culture by adding 10 ml of sterile distilled water containing 0.01% of tween 80 and mixed vigorously on cyclomixer the suspended spores were considered as inoculum. Fermentations studies were carried out in a 3L capacity glass Fermenter with 1L synthetic M9 medium modified with 1% concentration of L-asparagine as substrate concentration. The medium was further inoculated with 10ml of inoculum and incubated for 48 hr. The pH was controlled to 8.0 prior to sterilization and temperature was maintained at 40° C. Aeration was provided at the rate of 1.0 L/min (0.1 vol of air per vol of medium

per min), agitation rate was maintained as 150 rpm. From each set 5ml of Sample of fermented broth was withdrawn at 48 hr. of incubation and further used for the determination of bacterial growth and L-asparaginase assay. The crude enzyme thus obtained was further subjected for extraction and purification.

Purification of L-asparaginase

The enzyme was purified by the following steps at 4°C. The fermentation broth was centrifuged at 10,000 g for 10 min. Crude enzyme was brought to 45% saturation with ammonium sulphate at pH 8.4 and kept overnight in a cold room at 4^0C. It was thereafter subjected to centrifugation at 8000rpm for 10 min.at 4^0C.Collected precipitate was discarded and supernatant was brought to 85% saturation with ammonium sulphate and again centrifuged at 8000rpm at 4^0C for 10 min.The precipitate was collected from this step dissolved in 0.5M Tris-HCl (pH 8.5) buffer and dialyzed against the same buffer. The dialyzed fraction was used for characterization of enzyme (Basha *et al.*, 2009).

ANALYTICAL STUDIES

Estimation of L-asparaginase activity

L-asparaginase enzyme assay was performed by a colorimetric method according to Wriston and Yellin (1973) at 37°C using a UV Visible spectrophotometer (Systronics) by estimating the ammonia reduced during L-asparaginase catalysis using Nessler's reagent. A reaction mixture consisting of 0.04 M L-asparagine and 0.05 M Tris-HCl buffer (pH 8.6) and 0.1 ml enzyme extract was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1.5 M Trichloroacetic acid solution. The liberated ammonia was coupled with Nessler's reagent and was quantitatively determined using an ammonium sulphate reference standard. 1 unit of the L-asparaginase (IU) is defined as the amount of enzyme capable of producing 1 mole of ammonia per minute at 37°C.

Estimation of protein: The amount of protein was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

ENZYME CHARACTERISATION

Effect of pH and temperature, incubation time and metal ions: The activity of L-asparaginase was evaluated at different pH, temperature and incubation time. The partially purified enzyme was incubated 0.05 M buffers of pH 3 - 11, under assay conditions and the amount of ammonia liberated was determined. Buffers used were potassium phosphate (pH 5.0 - 7.0) and Tris-HCl (pH 8.0 - 9.0). The pre-incubation was carried out for 60 min and then the residual activity was measured. The optimum temperature for the enzyme activity was determined by incubating the assay mixture at temperatures ranging from 30 - 60°C. Thermostability studies were carried out by pre-incubating the enzyme at different temperatures for 60 min (Amena *et al*., 2010). The effect of the incubation time on L-asparaginase activity was studied in the ranges of 5 to 45 minutes (EI-Bessoumy., 2004). In order to

determine enzyme activity in the presence of different metal ions,2 Mm salt solutions of magnesium, copper, zinc, magnous and calcium sulphate were added to enzyme substrate reaction mixture.

RESULTS AND DISCUSSION

Isolation and identification of bacterial endophytes: Collected plant material in present study comprise endophytic bacteria appeared during in vitro cultivation on Trypticase soy agar medium. Surface sterilization scheme for isolation of endophytic bacteria was found to be sufficient as control plate has not shown any growth. Therefore, bacterial colonies appeared on sample plates can be well thought-out as endophytic bacteria. A total of three morphologically diverse isolates were found as endophytes with potent L-asparaginase activity and all three isolates were identified with 16 S r RNA sequencing. The IS-1 as *Bacillus pseudomycoides* with 99.50 % homology, IS-2 as *Bacillus licheniformis* and IS-3 as *Paenibacillus denitriformis* both with 100 % homology.

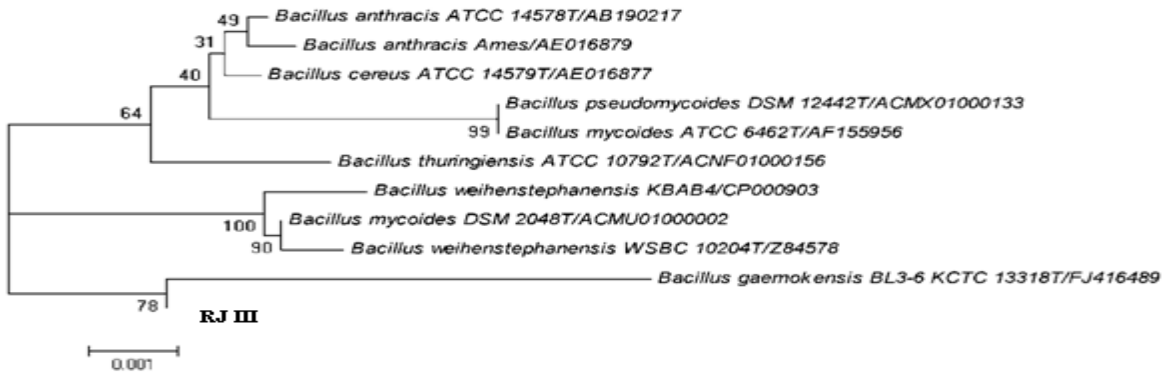


Fig 1: Identification report of IS-1

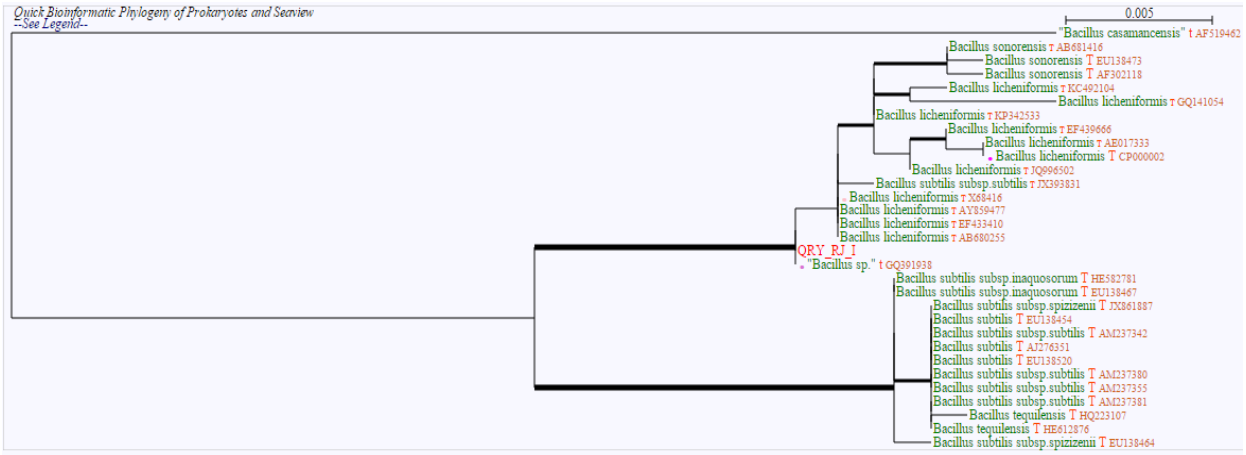


Fig 2: Identification report of IS-2

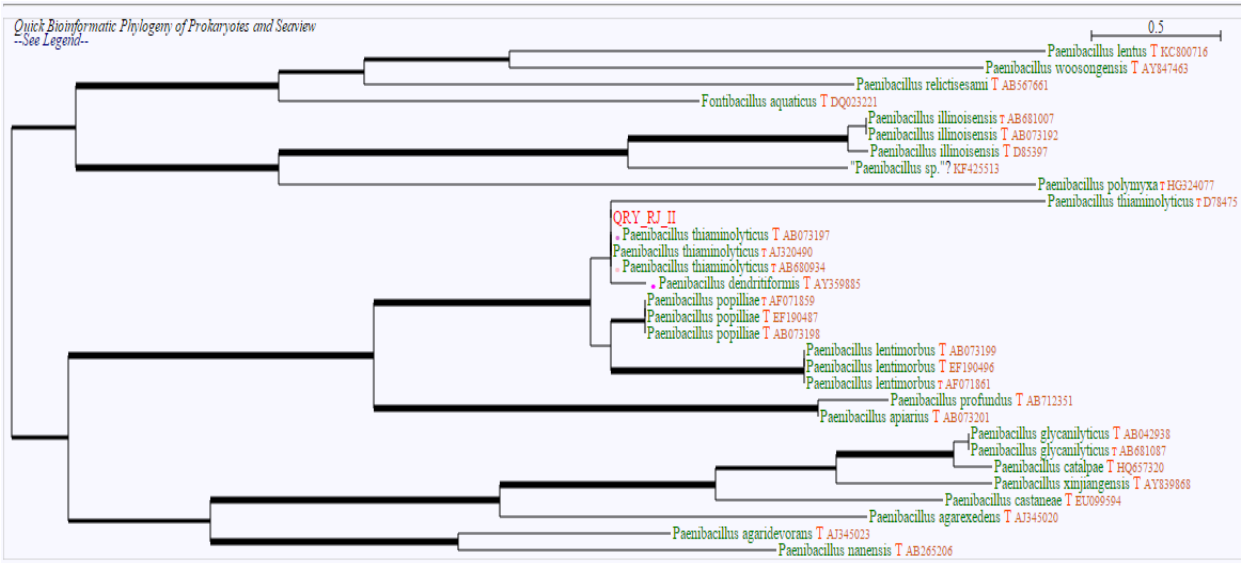


Fig 3: Identification report of IS-3

Production and purification of L-asparaginase

The modified M9 medium was used for production of L-asparaginase, with L-asparagine as a sole source of nitrogen from three efficient endophytic bacterial strains as *B.pseudomycoides, B. licheniformis and P.denitriformis*. The optimized culture conditions for the maximum L-asparaginase yield of were used as pH-8, incubation time of 48 hours and glucose as a carbon source. Partial purification of L-asparaginase from bacterial endophytes was done using ammonium sulphate precipitation followed by dialysis which enhances specific activity of enzyme.

Table 1: Effect of purification steps on Specific activity

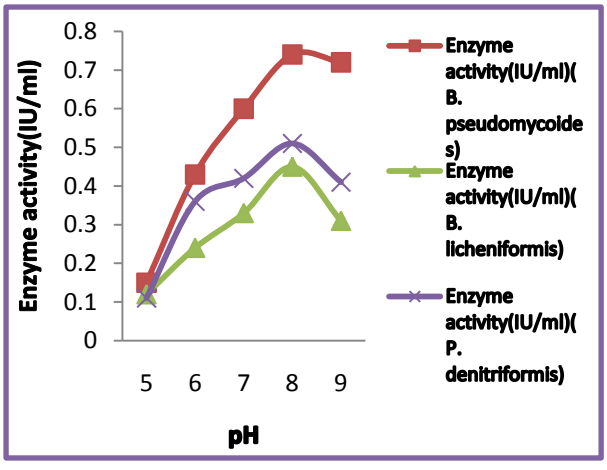
Bacterial endophytes	Purification steps	Volume of medium	Total activity(IU)	Total protein(mg)	Specific activity(IU/mg)	Fold purification	Percent yield
B. psudomycoides	Crude enzyme	100ml	760	10.8	70.4	-	-
	Amm. Sulfate precipitation	50ml	360	4.8	75	1.07	47.36
	Dialysis	20ml	136	1.68	80.95	1.15	17.8
B. licheniformis	Crude enzyme	100ml	560	7	80	-	-
	Amm. Sulfate precipitation	50ml	270	3.3	81.82	1.02	48.21
	Dialysis	20ml	98	1.16	84.48	1.06	17.5
P.denitriforms	Crude enzyme	100ml	1210	15.6	77.56	-	-
	Amm. Sulfate precipitation	50ml	590	7.2	81.94	1.06	45.73
	Dialysis	20ml	224	2.56	87.5	1.12	18.51

Effect of purification steps are summarized in Table1 for three bacterial strains. In case of three bacterial strains with every purification step there was increase in specific activity of enzyme and fold purification

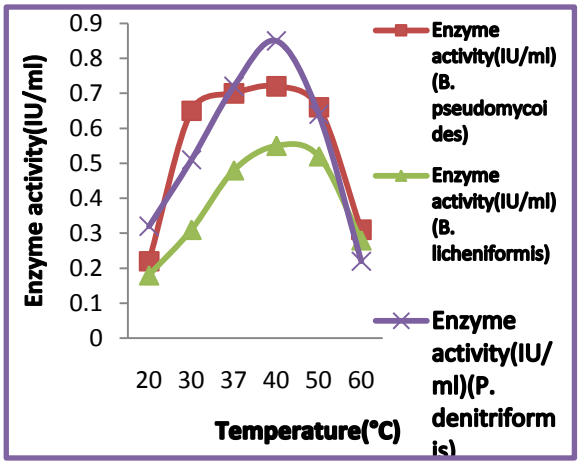
was also improved. Specific activity of L-asparaginase from *B.pseudomycoides, B. licheniformis and P.denitriformis* were 70.4 IU/mg, 80 IU/mg and 77.56 IU/mg in crude enzyme extract which increased up to 189 IU/mg, 84.48 IU/mg and 87.5 IU/mg in dialyzed enzyme extract respectively. Similar results were obtained by Mangamuri *et al.*, 2015 using gel filtration followed by ion exchange chromatography for L-asparaginase purification. The enzyme was purified 96 fold and showed a final specific activity of 702.04IU/mg. In *Streptomyces albidoflavus* L-asparaginase has been purified in CM Sephadex C-50 column up to 99.3 folds (Narayana *et al*., 2008). **Characterization of partially purified enzyme:**

Effect of pH, temperature and incubation time on partially purified enzyme activity

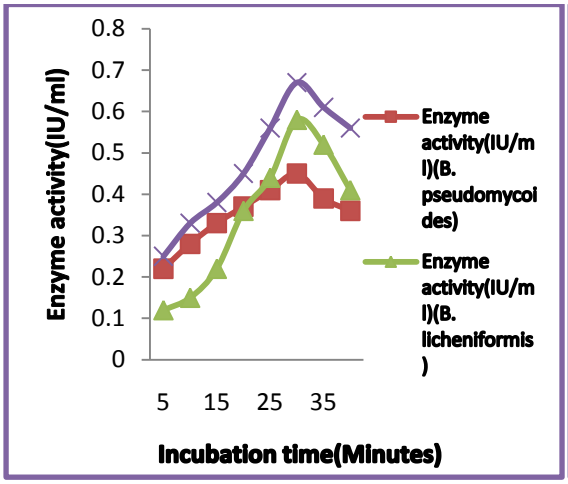
Partially purified L-asparaginase from *B.pseudomycoides, B. licheniformis and P.denitriformis* were active over wide range of pH as 6-9 with maximum activity at pH 8. At acidic pH 5 there was very minimal enzyme activity which increases between pH7-8 and decreases at pH 9. *Streptomyces Sp. PDK2* showed higher enzyme activity at pH 8 (Dhevagi and Poorani, 2006). A temperature profile showed that enzyme activity of three enzymes were gradually increased with increasing temperature up to 40°C and then decreased at 50°C and 60°C temperature. Purified enzyme from *E. carotovora* showed maximum activity at 50°C temperature (Maladkar *et al*., 1993).Similar results were reported for L-asparaginase from *Pseudomonas stutzeri* MB-405(Mannan *et al*., 1995), *Pseudomonas aeruginosa* 50071 (Bessoumy *et al*., 2004).The effects of incubation time on L-asparaginase from bacterial endophytes were studied in the ranges of 5 to 45 minutes. Incubation of L- asparaginase for different time intervals revealed that activity reached its maximum at 30 minutes and decreased as the time increased. Maximum incubation time for L-asparaginase from marine isolate was found to be 35 minutes (Dhevagi and Poorani, 2006).



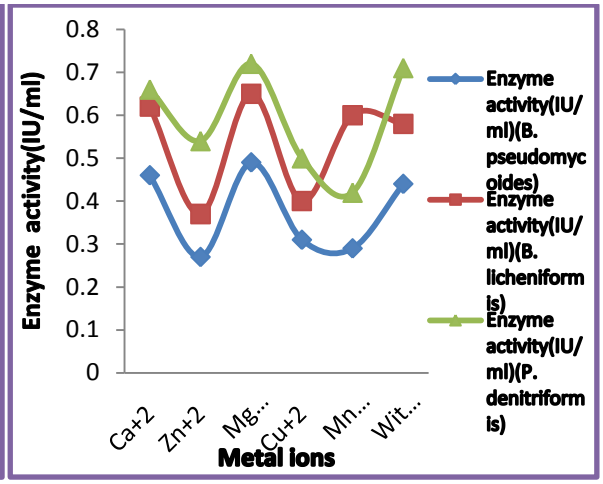
Effect of pH on enzyme activity



Effect of temperature on enzyme activity



Effect of incubation time on enzyme activity



Effect of metal ions on enzyme activity

Effect of metal ions on enzyme activity:

Enzyme partially purified from three bacterial endophytes as *B.pseudomycoides*, *B. licheniformis and P.denitriformis* were showed similar enzyme activity profile in the presence of metal ions. Enzyme activity of all the three enzymes was decreased in the presence of Cu^{+2}, Zn^{+2} and Mn^{+2} and stimulated by Mg^{+2}; Ca+2 not show any stimulatory or inhibitory effect on enzyme. Mohapatra *et al.*, 1995 observed $Cu^{+2}$and Zn^{+2} inhibit enzyme activity of purified enzyme while stimulated by increasing concentration of Mg^{+2} (Moorthy *et al.*, 2010 and Basha *et al.*, 2009).

CONCLUSION

Although L- asparaginase from bacteria has been extensively exploited however, the bacterial endophytes from plants specially recommended for cancer therapy is an untapped resource for L-asparaginase. The present study enlightens the possible L-asparaginase production by submerged fermentation from isolated bacterial endophytes. The enzyme showed considerable activity of partially purified enzyme at pH 8 and temperature 40^0C which is closer to human body parameters makes it highly favorable to be exploited as a potent anticancer agent.

REFERENCES

Asselin BL, Lorenson MY and Whitin JC. (1993). Comparative pharmacokinetic studies of three asparaginase preparations. J. Clin. Oncol., 11: 1780-1786.

El-Bessoumy AA, Sarhan M, Mansour J. (2004). Production, isolation, and purification of L-asparaginase from *Pseudomonas Aeruginosa* 50071 using solid-state fermentation. J. Biochem. Mol. Biol., 37: 387-393.

Narayana KJP, Kumar KG and Vijayalakshmi M. (2007). L-asparaginase production by *Treptomyces albidoflavus*. Ind. J. Microbiol., 48: 331-336.

Dhevagi P, Poorani E (2006). Isolation and characterization of L-asparaginase from marine actinomycetes. Ind. J. Biotechnol., 5, 514- 520.

Amena S, Vishalakshi N, Prabhakar M, Dayanand A. and Lingappa K. (2010). Production, purification and characterization of Lasparaginase from *Streptomyces gulbargensis*. *Brazil J. Microbiol*., 41: 173-178.

Moorthy V., Ramalingam A, Sumantha A and Shankaranaya R. (2010). Production, purification and characterization of extracellular L-asparaginase from a soil isolate of *Bacillus* sp. *African Journal of Microbiology Research*. 4 (18), 1862-1867.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. (1951). Protein measurement with the folin-phenol reagent. *J. Biol. Chem*.48:17-25.

Maladkar, N. K., Singh, V. K. and Naik, S. R. (1993). Fermentative production and isolation of L-asparaginase from *Erwinia cartovora* EC-113. *Hindustan Antibiot. Bull*, **35**, 77-86.

Manna, S., Sinha, A., Sadhukhan, R. and Chakrabaty, S. L. (1995). Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405.Curr.Microbiol., **30**, 291-298.

Basha N S, Rekha R, Komala M and Ruby S (2009). Production of Extracellular Anti-leukaemic Enzyme L-asparaginase from Marine Actinomycetes by Solidstate and Submerged Fermentation: Purification and Characterisation. *Tropical Journal of Pharmaceutical Research*, 8 **(4):** 353-360.

Oza Vishal P. (2009). *Withania Somnifera L (*Ashwagandha): A novel source of L- asparaginase, *J. Integ. Plant. Bio.* 51(**2**): 201-206

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