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Optimization studies on L-asparaginase production from endophytic Bacteria

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Abstract
L-asparaginase is a well-recognized as amino acid degrading enzyme. It is recommended as therapeutic agent due to an antineoplastic activity. Hence, with the focus to optimize the enzyme production of a novel anticancer enzyme an attempt for the optimized production of L-asparaginase was done from the bacterial endophytes of selected medicinal plants. Total Twenty five endophytic bacteria were isolated from ethno medicinal plants specifically recommended for cancer therapy. Pure cultures of isolated bacterial endophytes were examined qualitatively and quantitatively, adopting spot inoculation and submerged fermentation respectively, for their optimum ability to produce L-asparaginase using M9-medium. Out of total, 25 different isolates, 3 cultures of endophytes namely Bacillus *licheniformis, Bacillus pseudomycoides* & *Paenibacillus denitriformis* showed efficient qualitatively and quantitatively enzyme activity. The optimization studies reviled that the maximum enzyme was produced at PH.8.Temp, 40 ^{0}C, & in presence of glucose as most suitable carbon source, after incubation of 48 hrs. The substrate concentration showed direct proportional relation with an enzyme activity up to 1%w/the study indicates the bacterial endophytes as a possible source of L-asparaginase as the production strain.

Keywords: L-asparaginase, Bacterial endophytes, submerged fermentation

Introduction

Interest in L- Asparaginase (EC 3.5.1.1.) has grown considerably since this enzyme was found to have antitumor activity (Kidd, 1953; Broome, 1961). Asparaginase (L- Asparagine amino hydrolase) is an enzyme which is responsible for conversion of L- asparagine to aspartic acid and ammonia. The therapeutic use of this enzyme was responsible for remission in most patients suffering from acute lymphoblastic leukemia (ALL) [22]. There was also application of L- Asparaginase enzyme in many other clinical trials of tumor therapy in combination with chemotherapy. L- Asparaginase is an oncolytic enzyme that degrades non-essential amino acid like l- asparagine. Tumor cells are unable to synthesize L-asparagine and hence extract it from body fluids; by contrast most normal cells can synthesize their own amino acids. The effective depletion of L- asparagine results in cytotoxicity for leukemic cells (figure 1).

L-asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings [18] since extraction of L-asparaginase from mammalian cells is difficult, microorganisms have proved to be a better alternative for L-asparaginase extraction, thus facilitating its large scale production. The L- asparaginase enzyme is also used as a processing aid to reduce levels of free L- asparagine, which is a major precursor in formation of food contaminant acrylamide. Specifically in baking industry during baking of starchy food material there was formation of acrylamide which will reduce due to presence of L-asparaginase.

Endophytes are microorganisms present in internal tissues of living plants without causing any symptoms & harm to host plants [3]. Endophytes are present ubiquitously in all plant species & get importance for their ability to produce various bioactive compounds. It has been earlier reported that some of the endophytic bacteria belongs to members of diverse genera namely *Pseudomonas* and *Bacillus*. These genera are well-known for production of their secondary metabolites namely antibiotics, anticancer agent, antiviral compound and some immune suppressant.

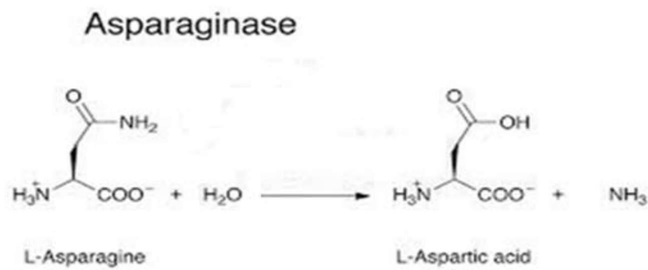


Fig 1: Schematic representation of the reaction mechanism of the L – asparaginase

Research about the use of endophytic microbes as a source of L-asparaginase with anticancer activity is limited. Hence, taking into consideration the potential of microbial endophytes of some medicinal plants used in cancer therapy like *Withania somnifera*, *Ocimum sanctum*, and *Catharanthus roseus* with their L-asparaginase producing ability, the present work has been intended to carry out.

Materials and Methods

1) Isolation and identification of endophytic bacteria:

a) Surface sterilization of plant material: The parts such as stem cuttings, leaves, fruits, and roots of selected ethno medicinal plants viz; *Withania somnifera*, *Ocimum sanctum*, *Alovera, Murraya koenigii* and *Catharanthus roseus* were used for isolation of endophytes. The plant samples were initially subjected for surface sterilization as per the methodology given by [1] along with some modifications.

b)Isolation of the endophytes: surface sterilized plant parts viz. leavesandfruitswerefurthergroundwith6ml 0.9% NaCl solution using sterile pestle and mortar and kept asepticallyfor15-20 min.for the release of endophytic bacteria from host tissue. The tissue extract was diluted with 0.9% NaCl solution and plated on tryptic soy agar medium. The plant parts viz. Inner bark, roots were cut into thin pieces with sterile knife to excise inner tissues. The excised inner tissues were further inoculated on tryptic soy agar medium plates. All the plates were incubated at 37^0C for 3-5 days. Followed by incubation, the colonies were selected showing different morphological and growth characters. Simultaneously control plate without plant tissue inoculation was maintained to check sterility.

c) Identification of endophytes: Identification of isolated bacterial cultures was done on the basis of its growth characteristics on differential media and biochemical properties such as Grams nature, motility, lactose fermentation, indole production, methyl red Voges proskauer (VP) reaction, citrate utilization, H_2S production, catalase and urease tests using a standard protocol. The isolated organisms were identified by 16 s rRNA sequencing method.

Production of L- Asparaginase

L- Asparaginase production by bacterial isolate was carried out by submerged fermentation. TheM9, fermentation media was composed as (gm/ lit)-Na_2HPO_4-6.0KH_2PO_4-2.0, L-Asparagine-6.0, 1M $MgSO_4.7H_2O$-2.0, NaCl-0.5, 0.1M $CaCl_2.2H_2O$- 1.0 ml, and 20% glucose stock solution-10 ml (P^H-7) was inoculated with a 1% of 24 hrs, enriched bacterial culture and further incubated in rotary shaker at 37^0C at 200rpm for 24 hours.

Analytical studies

Determination of L- Asparaginase activity

L- Asparaginase enzyme activity of culture filtrate was determined by quantifying ammonia formation in a spctrophotometric analysis using Nessler's reagent (Wriston and Yellin, 1973).The reaction mixture containing 0.5 ml of 0.04 M L-asparagine, 0.5 ml of 0.5M Tris HCl buffer (pH 8.5), 0.1 ml of an enzyme and 0.9 ml distilled water to make up the total volume of 2 ml. The tubes were incubated at 37ºC for 10 minutes. The reaction was terminated by adding 0.5 ml of 1.5 M Trichloroacetic acid (TCA). The blank was prepared by adding enzyme after the addition of TCA. To the 3.7 ml of distilled water and 0.2 ml of Nessler's reagent 0.1 ml from the above mixture was added. After incubating the mixture at 20ºC for 20 minutes the OD was checked at 450 nm with Spectrophotometer [Systronics make]. The activity was determined using an ammonium sulfate reference standard. 1 unit of L- Asparaginase (IU) is defined as the amount of enzyme capable of producing 1mole of ammonia per minute at 37ºC.

Estimation of protein:

The amount of protein was estimated by the method of [9] using bovine serum albumin as standard.

Effect of physicochemical parameters on enzyme production: The effect of five different Physico-chemical parameters as pH, temperature, incubation time, different carbon sources,& substrate concentration on enzyme production was studied. Production of L- asparaginase was optimized at different pH values as from pH 5-9 by submerged fermentation. The optimum temperature for the enzyme production was determined by incubating the inoculated culture medium at temperature as 30, 37, 40,& 50^0 C. After incubation specific enzyme activity of crude enzyme extract was calculated by L-asparaginase assay method. An effect of incubation time was optimized by extracting enzyme from production medium after time intervals of 24, 48, 72, 96 & 120 hours of incubation & then proceed for enzyme assay. Carbon sources as Glucose, Lactose, Mannitol, and Fructose& Starch were examined to study enzyme production. Optimization effect of L-asparagine, substrate for L-asparaginase was also studied.

Results

Identification and screening of microorganisms

In the present study during the primary screening, total twenty five (25) endophytic bacterial isolates was observed showing the pink zone around the colony and were screened the strains with L- asparaginase producing ability. Where as in case of secondary screening, adopting submerged fermentation only three (3) potential strains with maximum enzyme activity were recorded. The efficient strains were identified on the basis of morphological and biochemical characterization (Table 1& 2.) according to Bergey's manual of bacteriology and further subjected to 16S rRNA gene sequencing. The Isolate (IS-1) was identified as *Bacillus pseudomycoides* with 99.50 % homology, IS-2 as *Bacillus licheniformis*& IS-3 as *Paenibacillus denitriformis* both with 100 % homology.

Table 1: Cultural characteristics of L- asparaginase producing bacterial endophytes

Sr.No.	Colony characters	Observations		
		Is-1	Is-2	Is-3
1	color	Dirty white	Creamy white	white
2	size	2mm	3mm	1mm
3	shape	Circular	Circular	Circular
4	Margin	Entire	Entire	Entire
5	Opacity	Opaque	Opaque	Opaque
6	Elevation	Convex	Convex	Convex
7	Consistency	Sticky	Sticky	Sticky
8	Surface	Smooth	Smooth	Smooth
9	Motility	Actively motile	Actively motile	Actively motile
10	Grams nature	Gram positive	Gram positive	Gram positive

Table 2: Biochemical characteristics of L- asparaginase producing bacterial endophytes

Sr.No	Biochemical characters	Observations		
		IS-1	IS-2	IS-3
1	Indole Production	Positive	Positive	Negative
2	Methyl red	Positive	Negative	Negative
3	Citrate utilization	Positive	Positive	Negative
4	Voges Proskauer	Positive	Positive	Negative
5	Starch hydrolysis	Positive	Positive	Positive
6	Gelatin liquefaction	Positive	Positive	Positive
7	H_2S Production	Positive	Positive	Positive
8	Nitrate reduction	Negative	Negative	Positive
9	Catalase	Positive	Positive	Positive
10	Glucose	Positive	Positive	Positive
11	Mannitol	Positive	Positive	Positive
12	Sucrose	Positive	Positive	Positive

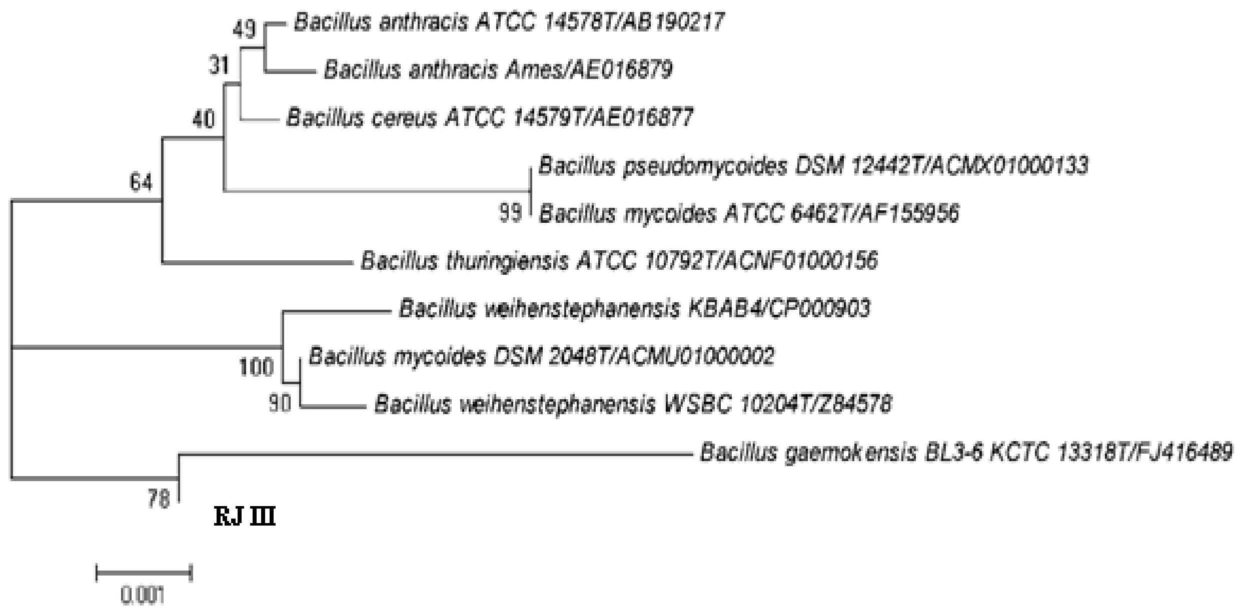


Fig 2: Identification report of IS-1

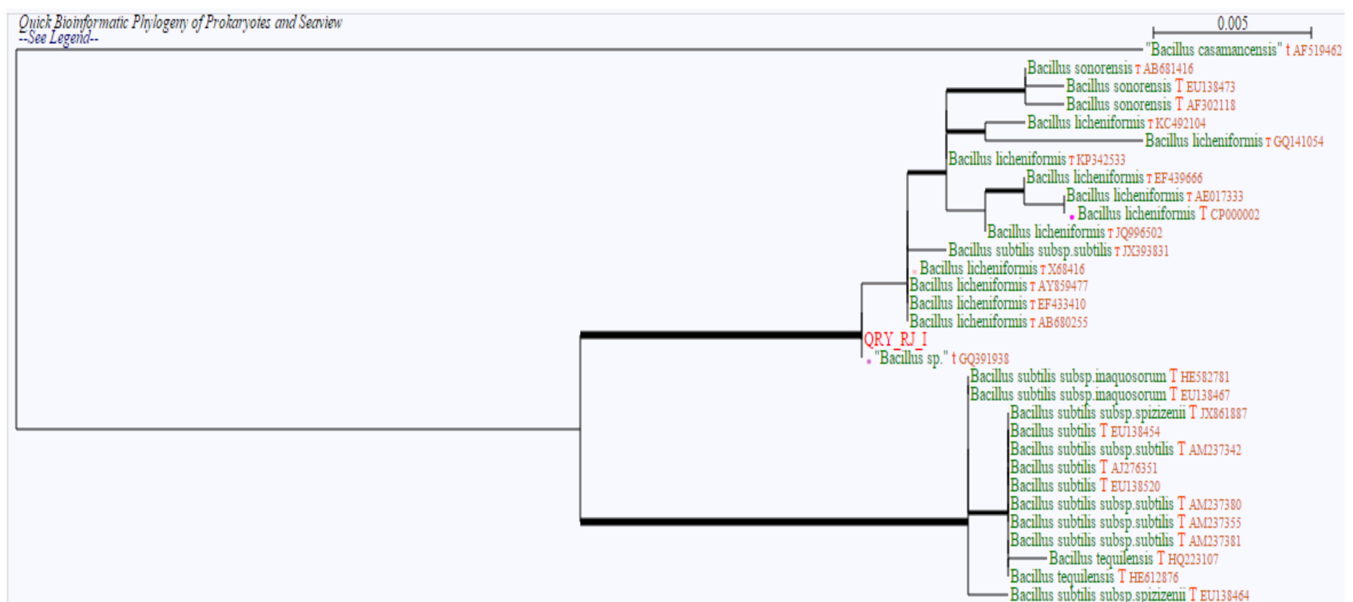


Fig 3: Identification report of IS-2

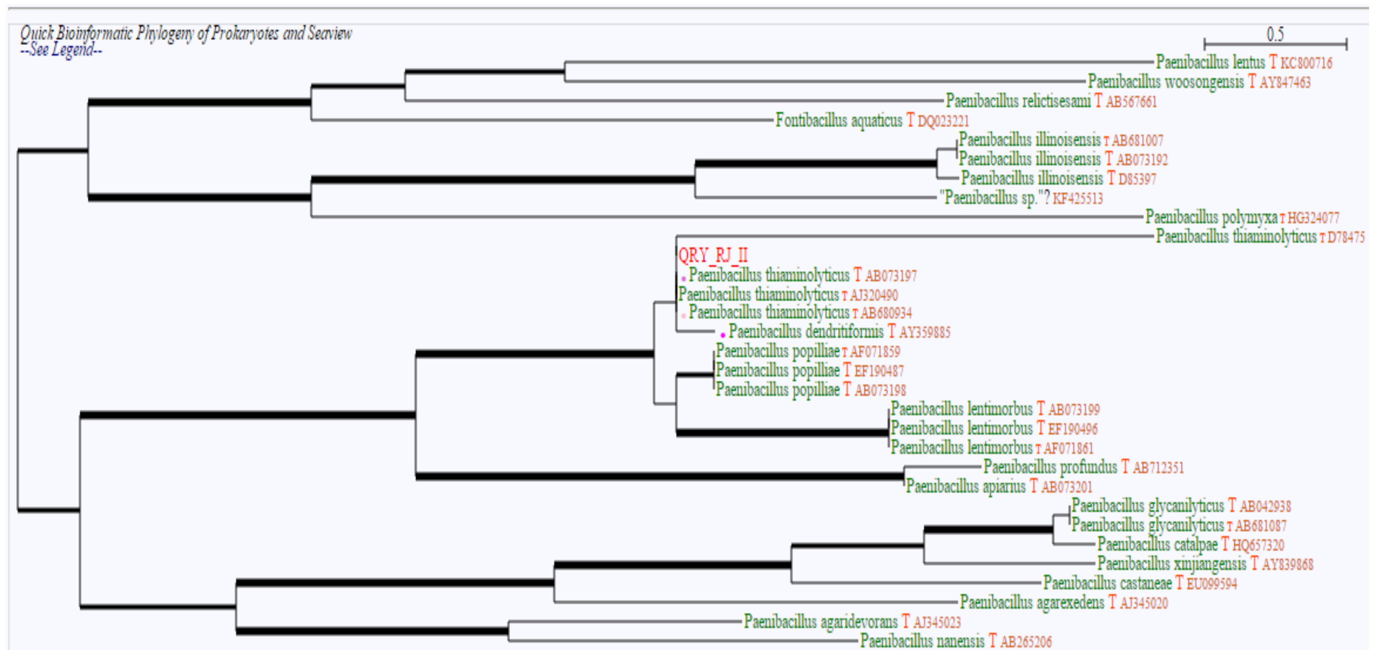
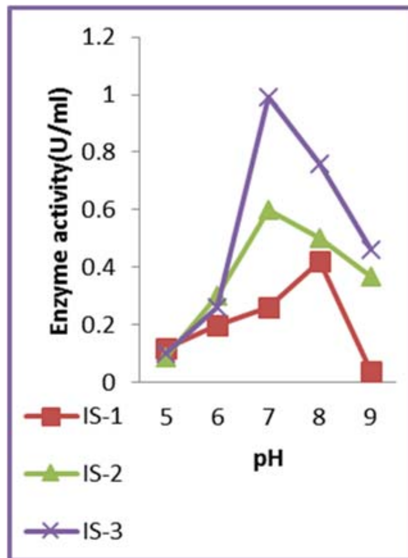


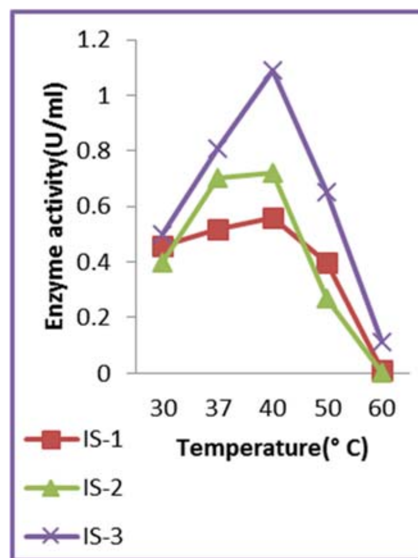
Fig 4: Identification report of IS-3

Optimization of Enzyme Production

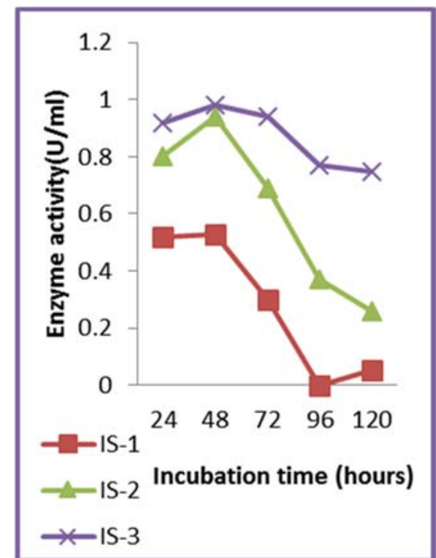
Effect of pH, Temperature, and Incubation time: Optimization study was done to examine the impact of environmental parameters on enzyme production. (Figure - 1.) IS-1, *Bacillus pseudomycoides* showed maximum enzyme production at pH 8+/-1after 48 hours of incubation at 40 ^{0}C+/-3. Is-2, *Bacillus licheniformis* produced maximum enzyme at 37^{0}C after48 hours of incubation in neutral pH condition. Is-3, Paenibacillus *denitriformis* showed similar results. It was observed that by almost all the cultures the parameters were more or less at par with each other in enzyme production.



Graph 1: Effect of pH on enzyme

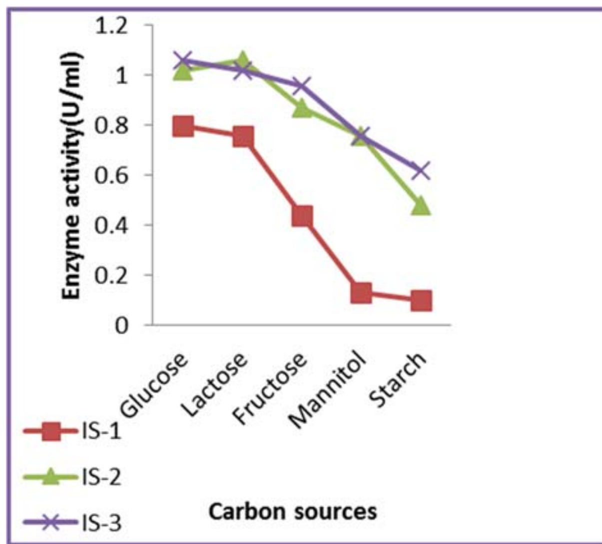


Graph 2: Effect of temperature

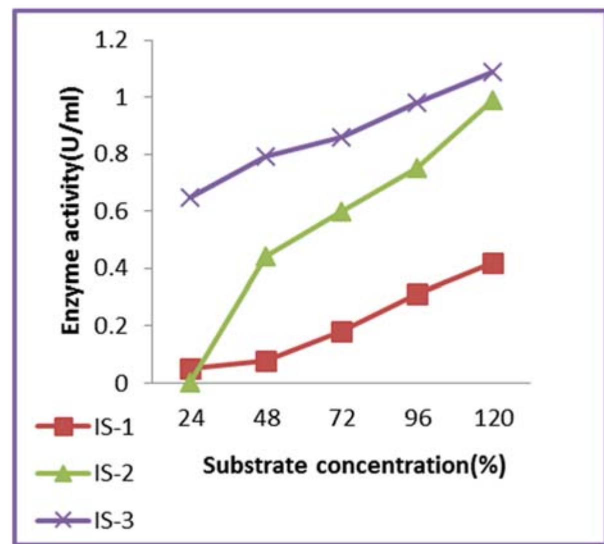


Graph 3: Effect of incubationactivityon enzyme activity time onenzyme activity

Media optimization for enzyme production: The various concentrations of L-asparagine, & various carbon sources were tested as ingredients of medium; one factor was changed while others were kept constant (Table 1). The pH of the media was adjusted at 7.0.Five different carbon sources as Glucose, Lactose, Fructose, and Manitol &Starch were used separately in production medium to check out its effect on enzyme production. Glucose was proven to be the best carbon source for all the three endophytic bacterial isolates at which maximum enzyme production was recorded. *Bacillus licheniformis* showed similar enzyme production in the presence of both glucose & lactose. Utilizing starch as a carbon source in all three isolates showed minimum enzyme activity. Enzyme activity was increases along with increase in substrate concentration from 0.2- 1%w/v.



Graph 4: Effect of carbon sources on enzyme activity



Graph 5: Effect of substrate concentration on enzyme activity

Discussion

In present study endophytic bacteria isolated from ethnomedicinal plants were screened for L-asparaginase production, as done by Pradeep *et al*., 2010. Total of twenty five bacterial endophytes with positive L-asparaginase activity only three will show efficient enzyme production &selected further studies. Three efficient strains were identified by 16SrRNA sequencing as *Bacillus licheniformis, Bacillus pseudomycoides,*& *Paenibacillus denitriformis*. Identification of L-asparaginase producers was also studied byRudrapati & Audipudi in 2015.Optimization of L-asparaginase production was carried out by studying effects of physicochemical parameters on enzyme production. All three endophytic bacterial strains were showed maximum enzyme production at 7-8 pH. Similar observations have been reported for asparaginase from *Pseudomonas stutzeri*MB-405. Dhavegi and Poorani (2009) reported the maximum L-asparaginase activity of *Streptomyces* sp. PDK7 between pH 8.0 and 8.5. Regarding the pH stability, the enzyme retained more than 80% of the activity in the pH range of 7–10. This optimum L-asparaginase production at 40°C is similar to that of *Corynebacterium glutamicum*, reported by Mesas *et al.,* (1990). Glucose &lactose was found to be best carbon sources for L-asparaginase production after 48-72 hours of incubation by all three endophytic strains.

Conclusion: The present study enlightens the medicinal plants as potential sources of endophytic bacteria with the L-asparaginase activity. *Bacillus licheniformis, Bacillus pseudomycoides,*& *Paenibacillus were the predominant bacterial endophytes in Withania somnifera*, *Ocimum sanctum*, and *Catharanthus roseus plants.* The optimization studies indicated the maximum enzyme was produced at PH.8.Temp, 40°C, &in presence of glucose as most suitable carbon source, after incubation of 48 hrs. The substrate concentration showed direct proportional relation with an enzyme activity up to 1%w/the study indicates the bacterial endophytes as a possible source of L-asparaginase as the production strain. Hence this organism may possibly be exploited for industrial production of L-asparaginase which ultimately can be supplied to cancer drug making sectors.

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