International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 3 | JUL-SEPT | 2018 | 1146-1153
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

STUDIES ON OPTIMIZATION OF L-GLUTAMINASE FROM BACILLUS ENDOPHYTICUS RN-7

K. Nagaraju and M.Raghu Ram*

Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh 522510, India

***Corresponding Author Email: mraghuram2002@gmail.com**

ABSTRACT

Cancer is one of the global problems now a days, in particular leukemia is one the threatening diseases. For elimination of cancer, there is a necessity for developing an efficient drug. L-Glutaminase is an amidohydrolase enzyme, which is the choice of interest in the treatment of lymphoblastic leukemia. This study investigates the optimization and production of extra-cellular glutaminase enzyme from forest soil samples. Days of incubation, temperature, pH, supplementary carbon and nitrogen sources and metal ions on the production of L-Glutaminase was studied and accordingly optimum conditions were determined. The isolated soil bacteria Bacillus endophyticus RN-7 is a strain that produces high levels of L-Glutaminase, under optimized culture conditions, viz., on the third day of incubation at an optimum pH of 7 and temperature of 40^{O}C.

KEY WORDS

L-Glutaminase, Bacillus endophyticus RN-7, Enzyme activity, optimum conditions.

INTRODUCTION:

L-Glutaminases belong to the large superfamily of serine dependent lactamases, penicillin binding proteins. They catalyze the hydrolytic deamidation of L-glutamine to L-glutamate. Glutamine, a non–essential amino acid, is a primary substrate for L–glutaminase, which is present in circulating blood and serves as a fuel for cell growth and nucleic acid synthesis [1,2]. In cancer cells, glutamine depletion could accelerate the apoptotic machinery directly or indirectly by inducing the various types of oxidative stress and DNA damage [3]. The enzyme causes selective death of glutamine-dependent tumor cells. The use of enzymes to deprive essential nutrients helps in the treatment of malignancies [4] and as an analytical agent in the determination of glutamine and glutamate [5, 6] and as a bio sensing agent in biosensor [7]. The other important use of L-Glutaminase as a flavor enhancing agent in Chinese foods that replaced the use of monosodium glutamate, which is considered allergic to some individuals [8] and in the production of special chemicals like threonine by gamma-glutamyl transfer reactions [9].

Glutaminase is ubiquitous in microorganisms and it plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes [10, 11]. In recent years, glutaminase has attracted much attention with respect to proposed applications in food industries, pharmaceutical industries and as a model enzyme for the development of new drug delivery system [12]. Commercial importance demands not only the search for new and better yielding microbial strains, but also economically viable bioprocesses for its large-scale production. Studies on the molecular structure, [13] catalysis, [14] clinical aspects [15] genetic determinants involved in regulation [16] and stabilization to enhance biological half-life [17] of L-glutaminase have been reported.

Almost all living cells produce L-glutaminase, but only certain microbial strains have the potential for industrial production of this enzyme. Microbial production of L-glutaminase has attracted more attention by industries because of the cost-effective and eco-friendly process. A Wide range of microorganisms such as filamentous fungi [18, 19], bacteria [20-22], yeast [23,24] and actinomycetes [25] have been reported in the literature for production of this enzyme.

Improvement in microbial metabolite production is generally attempted by manipulating the physical and nutritional parameters of the organism. Combinatorial interactions of medium components with the cell metabolism towards the production of the desired compound are plentiful, and the optimum processes may be developed using an effective experimental design procedure. The literature reports suggested that the enzyme produced by different microbial strains differed in some physiological, biochemical, catalytic and immunological properties. So the continuous screening program is necessary for the isolation of novel microbial strains that could produce an effective enzyme.

To our knowledge, reports on the production of L-glutaminase from *Bacillus endophyticus* is very precious. In the present investigation, the one-factor-at-a-time approach was used to select the best combination of incubation period, pH, temperature, carbon, nitrogen and Metal salt sources.

MATERIALS AND METHODS:

Collection of Soil Samples

Soil samples were collected in sterile polythene bags [26] from the forest area of Pulichinthala, Guntur district, A.P, India. The collected samples were air-dried at room temperature, powdered by grinding in mortar and pestle and stored at 4^{o}C for further studies. One gram of each sample was suspended in 10 ml of distilled water and shaken for 15 mins. Later, 0.1 ml of this suspension was spread on the Nutrient agar medium plats. The plates were incubated at 30º C for 24 hours (h) [27].

Identification of Bacterial strains

The bacterial strains were identified by using the morphological, cultural, physiological, biochemical and 16S rRNA analysis. The morphological and physiological properties of the isolates were investigated according to Bergey's Manual of determinative bacteriology [28]. The isolate was identified up to the genus level by morphological and biochemical characterization *viz*., Gram staining, Motility, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Catalase test, Oxidase test and hydrolysis of casein, starch, urea and Carbohydrate fermentation test.

Screening for L-glutaminase production

Microorganisms isolated from the soil samples were tested for L–Glutaminase activity by rapid plate assay method [29]. All bacterial isolates were evaluated for their ability to produce L- glutaminase by culturing in the M9 medium [30] containing L-glutamine with phenol red as an indicator and incubating at 37 º C for 24h. L-glutaminase producing colonies were selected on the basis of formation of the pink zone around the colonies of the medium [31].

Determination of L-Glutaminase activity

The active isolate was cultured on M9 broth supplemented with L-glutamine and phenol red as an indicator, pH 6.8 was incubated at 37 ºC with shaking at 125 rpm for 72 h. L-glutaminase activity was measured, following the method of Drainas et al., 1977 [32]. The culture broth was centrifuged at 10000 rpm for 15 min. This method utilizes the determination of ammonia liberated from L-glutamine in the enzyme reaction by the Nessler's reaction. The reaction was started by adding 0.1 ml supernatant into 1.7 ml of 0.01 M L-glutamine and 0.2ml of 0.05M tris-HCl buffer, pH 8.6 and was incubated at 37ºC for 10 min. The reaction was stopped by the addition of 0.5ml of 1.5 M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined calorimetrically by adding 6.5 ml of distilled water and 1.0 ml Nessler's reagent into tubes and was incubated at room temperature for 10 min. The samples were shaked well and the enzymatic activity was estimated spectrophotometrically at 480nm. The blank was run by adding enzyme preparation after the addition of TCA. One unit (U) of L-Glutaminase is defined as the amount of enzyme which liberated 1 µmol of ammonia/min/ml under the above assay conditions. A standard curve was drawn with various concentrations of ammonia and was used as reference to measure enzyme production.

Optimization of medium and cultural conditions for L–Glutaminase Production

Optimization of different cultural conditions such as incubation period, pH, temperature, carbon, and

nitrogen sources on the production of L-glutaminase was determined.

Effect of incubation period on L-Glutaminase production:

To study the effect of incubation period on L-Glutaminase production, the enzyme production by RN-7 was studied for every 24 hours up to 96 hours. For this study, the RN-7 culture was inoculated into 500 ml conical flask, containing sterile M9 broth and was incubated from 24 hours up to 96 hours at 37°C and triplicates were maintained. The optimum time, showing maximum production was taken for further experiments.

Effect of pH on L-Glutaminase Production

To study the effect of different pH on L-Glutaminase production, the isolate RN-7 was grown in sterile M9 broth with various pH levels ranging from 5.0 to 9.0. and was maintained by using phosphate buffer. 0.5 ml of the 24-hour old inoculum was transferred into the sterile M9 broth at different pH and incubated at 37°C for 48 hrs [33] and assayed for enzyme activity. The most favorable pH achieved at this step was used for further study.

Effect of Temperature on L-Glutaminase Production

To study the effect of temperature on L-Glutaminase production, the isolate RN-7 was grown in sterile M9 broth at different temperatures, ranging from 30 to 55^{O}C for 48 hrs of incubation and temperature was maintained by using respective incubators. 0.5 ml of the 24-hour old inoculum was transferred into a sterile M9 broth and was incubated and further assayed for enzyme activity.

Optimization of Nutritional Parameters for L-Glutaminase Production

Effect of Different Carbon Sources on L-Glutaminase production:

To study the effect of carbon sources on L-Glutaminase production by growing the bacterial strain RN-7 on M9 broth, the broth was supplemented with different carbon sources such as Galactose, Maltose, Fructose, Starch, Sucrose, and Cellulose, each at a concentration of 1% (w/v). 0.5 ml of the inoculum was added to a sterile M9 broth with different carbon sources, incubated at 37°C for 72 hrs and assayed for L-Glutaminase activity using the method of Shah *et al* [33].

Effect of Different Nitrogen sources on L-Glutaminase production:

Different nitrogen sources like Yeast extract, Tryptone, Ammonium sulphate, Urea, Peptone and Malt extract were added to the M9 broth separately at a rate of 1% (w/v), and 0.5 ml of the inoculum from the bacterial isolate RN-7 was added to sterile M9 broth, incubated at 37°C for 72 hrs. and was assayed for enzymatic activity [33].

Effect of different metal ions on L-Glutaminase production:

Metal ions like Magnesium chloride, Manganese chloride, Ferrous sulphate, Zinc chloride, Nickel carbonate, Lithium sulphate, Copper sulphate, Cobalt chloride and Barium hydroxide were added to the M9 broth separately at the rate of 0.5%. 0.5ml of the inoculum from the bacterial isolate RN-7 was added to the sterile basal broth, incubated at 37°C for 72 hrs and assayed for enzymatic activity [33].

Statistical Analysis:

All measurements were carried out in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range tests. Differences at $P < 0.05$ were considered statistically significant. The results were presented as mean values (±SD, standard deviations).

RESULTS AND DISCUSSION

Identification of the Strain

The strain RN-7 showed a close relation with *Bacillus endophyticus* based on the 16S rRNA gene sequence. The 16S rRNA sequence was deposited in the GenBank database of NCBI with the accession number MG271914. Basing on the morphological, physiological, biochemical characteristics and molecular characterization by 16s rRNA sequencing, the strain was identified as *Bacillus endophyticus* RN-7.

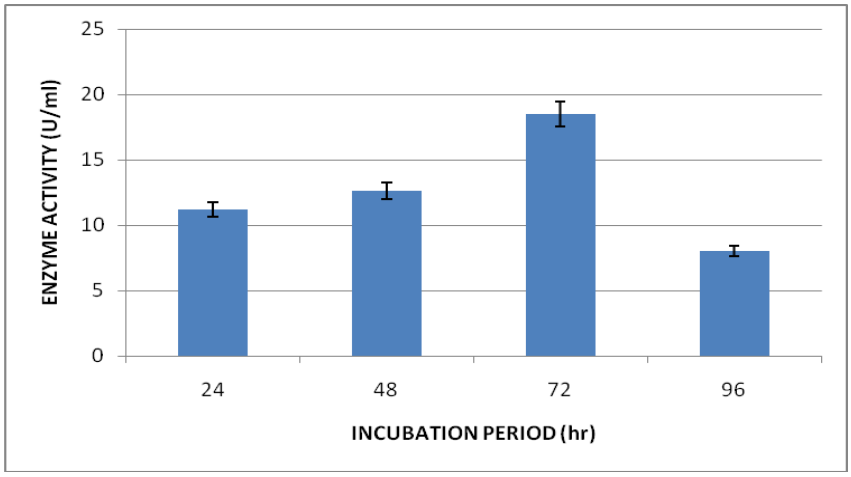
Optimization of medium and cultural conditions for L–Glutaminase Production

Optimization of different cultural conditions such as incubation period, pH, temperature, carbon, and nitrogen sources on the production of L-glutaminase were determined.

Effect of incubation period on L-glutaminase production:

Enzyme activity was measured for every 24 hrs of time intervals. The maximum enzyme production of 18.5 U/ml was observed at 72 hours of incubation and activity was gradually decreased afterwards. The

decrease in enzyme production was due to poor growth of the organism with limited nutrients. Therefore, the incubation period of 72hrs was considered as maximum for L-glutaminase production by *Bacillus endophyticus* RN-7 (fig. I). Similar results were also reported from *Bacillus sps* SFL-15 by Prakash *et al.,* (2009) and *Escherchia coli* SFL-1 by Poorani *et al.,* (2009).

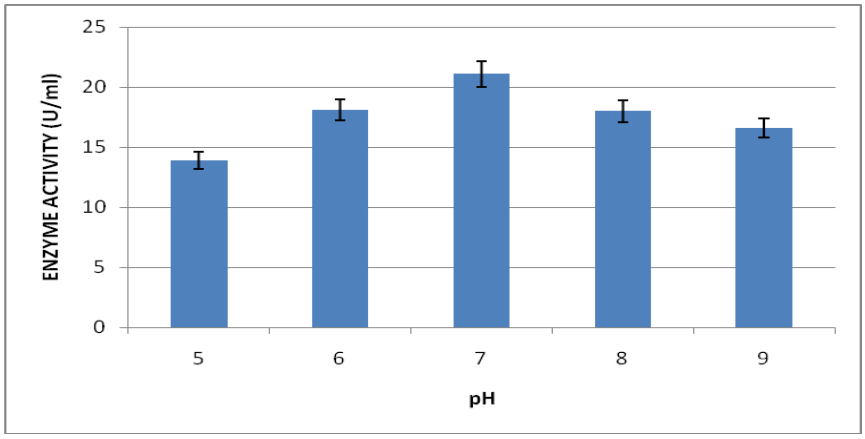


***The F-Value for incubation period and interactions are all significant with p<0.05.**

Fig. I Effect of incubation period for L-Glutaminase production by *Bacillus endophyticus* RN-7

Effect of pH on the L-Glutaminase production:

Generally, pH of the growth medium affects the growth and physiology of microorganisms. It was reported that, L-glutaminase production by most of the bacterial strains, under submerged conditions, was found to be maximum at pH 5.0 to 9.0. The maximum production of L-glutaminase by *B.endophyticus* RN-7 was observed at pH-7 with 21.1 U/ml and reduction of enzyme production was observed with either increase or decrease in pH from 7(fig.II). In the present study *B.endophyticus* RN-7 produced maximum of enzyme than those reported by *Bacillus* sps previously. Optimum pH-7 was also reported by *Bacillus* sps by Sinha *et al.,* (2016), in *Bacillus amyoliquefaciens* DL3 by Lee *et al*., (2008), in *Vibrio* sps by Durai *et al.,* (2014), and in Alkali *genes faecalis* KLU102 by pandian *et al.,* (2014).

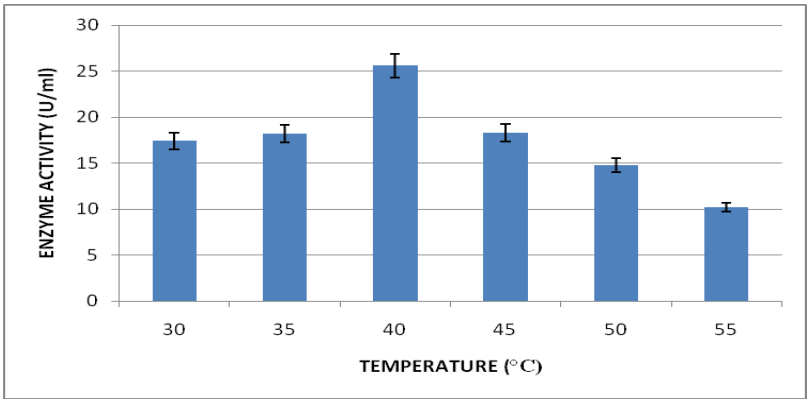


***The F-Value for pH and interactions are all significant with p<0.05.**

Fig. II Effect of Initial pH for L-Glutaminase production by *Bacillus endophyticus* RN-7

Effect of temperature on the L-Glutaminase production:

The effect of temperature on L- glutaminase production by the strain is represented in Fig.III. The enzyme production was enhanced with an increase in temperature from 30°C to 55°C. The optimal temperature for maximum production of L-glutaminase by the strain RN-7 was recorded at 40°C with 25.6 U/ml. However relatively high enzyme production of more than 17.4 U/ml was observed from 30°C onwards before reaching the maximum at 40°C by this species. Further increase in temperature resulted in the decrease of enzyme production. There were no reports on effect of temperature on L-glutaminase production by *Bacillus* sps previously. Similar results were reported from *Pseudomonas stutzeri* ISL B5 by Dutta *et al.,* (2016) and *Corynebacterium glutamicum* by Mesas *et al.,* (1990).

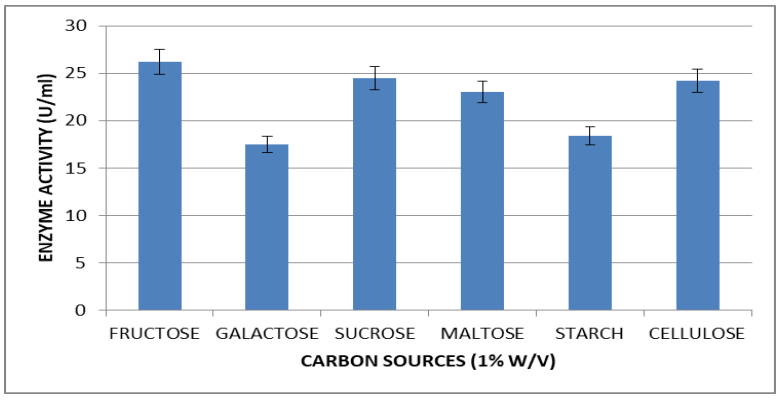


***The F-Value for Temperatures and interactions are all significant with p<0.05.**

Fig. III Effect of Temperature for L-Glutaminase production by *Bacillus endophyticus* RN-7

Effect of carbon sources on the L-Glutaminase production:

Carbon sources represent the energy sources that are available for growth of the microorganism. Carbohydrates and related compounds are superior carbon sources for many micro-organisums [34]. The addition of small amount of external carbon may lead to an increase in enzyme production. Fig.IV shows the effect of additional carbon source on the yield of L-glutaminase by *Bacillus endophyticus* RN-7. Much variation in enzyme production was observed with the different carbon sources used. In the present study maximum of 26.2 U/ml of enzyme production was observed when fructose was used as carbon source. Relatively high enzyme production of 24.2 U/ml was recorded when cellulose and sucrose were used as carbon sources. However, when galactose and starch were used, the enzyme production is very limited. There were no reports on effect of carbon and nitrogen on L-glutaminase from *Bacillus* sps previously. Keerthi *et al.* (1999), observed enhanced enzyme production by *Beauveria bassiana* BMTF S10 and Similarly, Iyer & Singhal (2009, 2010) observed a significant influence of fructose on L-glutaminase production by *Providencia* sp. and *Klebsiella oxytoca.*

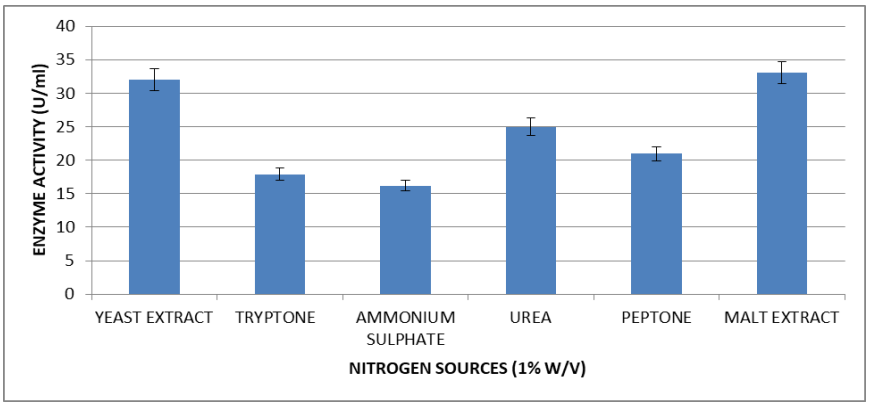


***The F-Value for Carbon sources and interactions are all significant with p<0.05.**

Fig.IV Effect of Carbon sources for L-Glutaminase production by Bacillus *endophyticus* RN-7

Effect of nitrogen sources on the L-Glutaminase production:

Effect of different nitrogen sources (Fig.V) showed that the maximum of 33.1 U/ml of the enzyme was produced when malt extract was used as nitrogen source, as malt extract serves as a complex nitrogen source for the metabolic activity. Among the nitrogen sources tested, yeast extract also supported maximum enzyme production with 32 U/ml after malt extract. Very low amount of enzyme (16 U/ml) was recorded when ammonium sulphate was used as nitrogen sources. There were no reports on effect of carbon and nitrogen on L-glutaminase from *Bacillus* sps. Sivakumar *et al., (*2005) reported Similar results from *Streptomyces rimosus.*

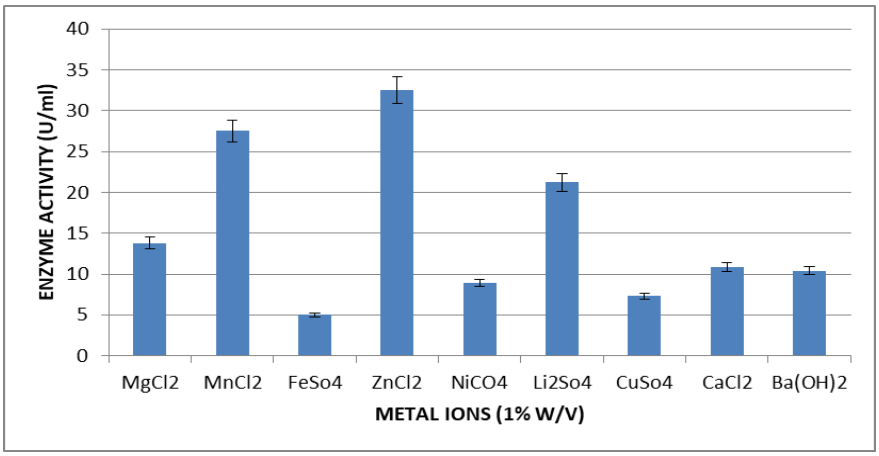


***The F-Value for Nitrogen sources and interactions are all significant with p<0.05.**

Fig.V Effect of Nitrogen sources for L-Glutaminase production by *Bacillus endophyticus* RN-7

Effect of metal ions on the L-Glutaminase production:

All the living organisms need some inorganic nutrient for their growth, that does not usually contain the element carbon and when it dissolves in water they separate into ions. L-glutaminase yield obtained from *Bacillus endophyticus* RN-7 in the presence of different mineral salts (Fig.VI) shows that the maximum yield of 32.5 U/ml was observed in the presence of Zinc chloride [35]. Similar results were reported from *Bacillus sphaericus* by Soda et al., (1972) The other metal ions like manganese chloride and lithium sulphate were showed enzyme production of 27.5 U/ml and 21.2 U/ml respectively. L-glutaminase production was also reported in *Pseudomonas fluorescens* ACMR 171, *V. costicola* ACMR 267 and *V. cholera* ACMR 347 by Yano et al., (1988).



***The F-Value for Metal ions and interactions are all significant with p<0.05**

Fig. VI Effect of Metal ions for L-Glutaminase production by *Bacillus endophyticus* RN-7

CONCLUSION

In conclusion, the results of the present study indicate scope for exploring terrestrial bacterium, *Bacillus endophyticus* RN-7 as a source for L-glutaminase, an enzyme that has gained industrial and pharmaceutical significance recently. Secondly terrestrial bacteria grown in shake flasks can produce extracellular enzyme. Thirdly it was first report from *Bacillus endophyticus* RN-7.

ACKNOWLEDGEMENTS

Authors would like to thank the authorities in Department of Botany and Microbiology, Acharya Nagarjuna university, Guntur for providing the facilities to carry out this work. Authors are also thankful to P. D. Prakash and Mahaboob Ali in Nakoda chemicals limited (Quality control department), Hyderabad.

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Received:01.05.18, Accepted: 10.06.18, Published:01.07.2018

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***Corresponding Author:**
M.Raghu Ram*
Email: mraghuram2002@gmail.com