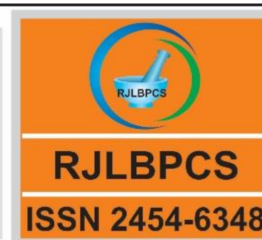

Original Research Article **DOI: 10.26479/2019.0504.12**

BACILLUS CEREUS RN-6, POTENTIAL L-GLUTAMINASE PRODUCING BACTERIA FROM FOREST SOILS

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ABSTRACT: L-Glutaminases have admitted significant vigilance recently owing to their potential applications. L-glutaminase is an amidohydrolase which is produced by a variety of micro-organisms including bacteria, yeast, fungi. The enzyme L-glutaminase is a green anticancer agent that can be piloted topically quite easily and acts on amino acid L-glutamine. Clinical studies currently report that this enzyme is a auspicious agent for the treatment of most of the neoplastic cell cancers in humans. In this study, production of L-glutaminase by *Bacillus cereus* RN-6 was evaluated under different parameters. The highest enzyme production was observed after 72 hr at 45ºC pH 7.0. The highest enzyme production recorded was 27.6 U/ml, 47.9 U/ml and 18.8 U/ml respectively, when Sucrose, Ammonium sulphate and $CuSO_4$ were added as carbon, nitrogen and metal ions source to the production medium.

KEYWORDS: L-glutaminase, *Bacillus cereus* RN-6, Enzyme assay, Carbon source, Nitrogen source, Metal ion.

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1.INTRODUCTION

L-Glutaminase (EC.3.5.1.2) is an amidohydrolase which catalyses the hydrolytical deamidation of L-glutamine resulting in the production of L-glutamic acid and ammonia. L-Glutaminases are pervasive in the biological world [1, 2] and organisms ranging from bacteria to human beings have this enzyme in their organization. L-Glutaminase has a central role in mammalian tissues [3]. Enthusiasm on amidohydrolases initiated with the innovation of their antitumor properties [4-7] and

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since then a lot of efforts have gone into broad studies on microbial L-glutaminases with the objective of developing them. Another most promising application of L-glutaminase is its prescription in the remedy of human immunodeficiency virus (HIV) [8]. L-Glutaminase can bring about degradation of L-glutamine and thus can act as feasible aspirant for enzyme therapy [9]. Although almost all living cells produce L-glutaminase, microbial L-glutaminase has received the greater attention because of its apparent advantages in production at large scale in addition to its antitumor property. However, source-dependent biochemical properties, productivity yields, medium component requirements, etc., play a vital role in its economic production and application potential at pharma sector level. Various reports are available in purification and antitumor studies on glutaminase enzyme from many microbes like *Bacillus pasteurii* [10], *Pseudomonas acidovorans* [11], *Pseudomonas stutzeri* [12], *Pseudomonas aeruginosa* [13], *Bacillus subtilis* [14], *Escherichia coli* [15], *Achromobacter* sp. [8] and *Xanthomonas sp*. [16]. L-Glutaminase has inculcated significant buzz in food industry as well, a potential flavor modulating agent, imparting a savory flavor as it increases the foods glutamic acid content. It finds application in food fermentation by hydrolyzing L-glutamine to produce highly savory amino acid L-glutamic acid imparting a unique taste called umami and thereby regarded as a key enzyme that controls the delicious taste of fermented foods [17, 18]. Thus the present study, focuses on the L-glutaminase production from a potential and novel isolate *Bacillus cereus* RN-6 (MG271913) under submerged fermentation and optimization of the process parameters and nutritional factors of fermentation for enhanced enzyme production. In this study, bacteria isolated from pulichinthala forest soils were investigated for their ability to produce L-glutaminase.

2. MATERIALS AND METHODS

Micro-organism and culture maintenance conditions

The *Bacillus cereus RN-6* (MG271913) identified by 16s rRNA sequence (Macrogen, South Korea) used in this study was isolated from soil sample collected from Pulichinthala forest area. The culture was maintained in nutrient agar slant at 4°C and was periodically sub-cultured.

Screening of bacterial isolates for L-glutaminase productivity

Screening of bacterial isolates for L-glutaminase activity was performed using the M9 medium (glucose- 3 g, L-glutamine- 10 g, KH_2PO_4 - 0.1 g, KCl- 0.5 g, $MgSO_4.7H_2O$- 1.0 g, $FeSO_4.7H_2O$- 0.1 g, $ZnSO_4$ - 0.1, NaCl- 0.5 and Agar- 20 g, and distilled water- 1000 mL). For the plate assay, 3 mL of 2.5% stock solution of phenol red (pH 6.8) was added to 1000 mL of M9 medium. The M9 media plates were inoculated with test bacteria at the center of the plate and incubated at 37 ºC fro 72 hrs. After incubation, the appearance of a pink zone around the bacterial colony indicated the L-glutaminase activity. The bacteria which show highest zone was selected for this study.

Enzyme Assay

The activity of L-glutaminase is determined by estimating the amount of NH_3 liberated from

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glutamine. For 0.5 ml of 0.04 M L-glutamine, 0.5 ml of enzyme preparation was added and 0.5 ml of distilled water and 0.5 ml of 0.1 M phosphate buffer (pH 8) were also added and then incubated at 37 °C for 30 min. After incubation, 0.5 ml of 1.5 M trichloroacetic acid was added to stop the enzymatic reaction. Blank was run by adding the enzyme preparation after the addition of trichloroacetic acid. 0.1 ml of above mixture was taken and added to 3.7 ml of distilled water followed by addition of 0.2 ml Nessler's reagent. Absorbance was measured at 450 nm using a visible spectrophotometer. One international unit of L-glutaminase (U) was defined the amount of enzyme that liberates 1 μmol of ammonia from glutamate under optimum assay conditions. The enzyme yield was expressed as units/ml according to Imada et al [19].

Optimization of process parameters for L-glutaminase production

The M9 medium was used as the basal medium and the process parameters were optimized one after another. After optimization of each parameter, it was included in the next study at its optimal level. The pH of the medium (5-9), incubation temperature (30-55 °C), incubation time (24-96 h), additional carbon source (fructose, sucrose, galactose, maltose, starch and cellulose at 1% w/v), additional nitrogen sources (peptone, yeast extract, malt extract, tryptone, ammonium sulphate, and urea at 1% w/v), and different metal ions ($MgCl_2$, $MnCl_2$, $FeSO_4$, $ZnCl_2$, $NiCO_4$, Li_2SO_4, $CuSO_4$, $CaCl_2$, and $Ba(OH)_2$ 1% w/v) were optimized for L-glutaminase yield. All experiments were conducted in triplicates.

Statistical Analysis

Statistical analysis was 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 as statistically significant. The results were presented as mean values (± SD, standard deviation).

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3. RESULTS AND DISCUSSION

The sequence homology studies indicated that strain RN-6 showed 99.0% relation with *Bacillus cereus* (strain KF687028) based on the 16S rRNA gene sequence. Basing on the morphological, physiological, biochemical characteristics and molecular characterization by 16s rRNA sequencing, our strain was identified as *Bacillus cereus* RN-6 (Fig. 1). The 16S rRNA sequence was deposited in the GenBank database of NCBI with the accession number MG271913.

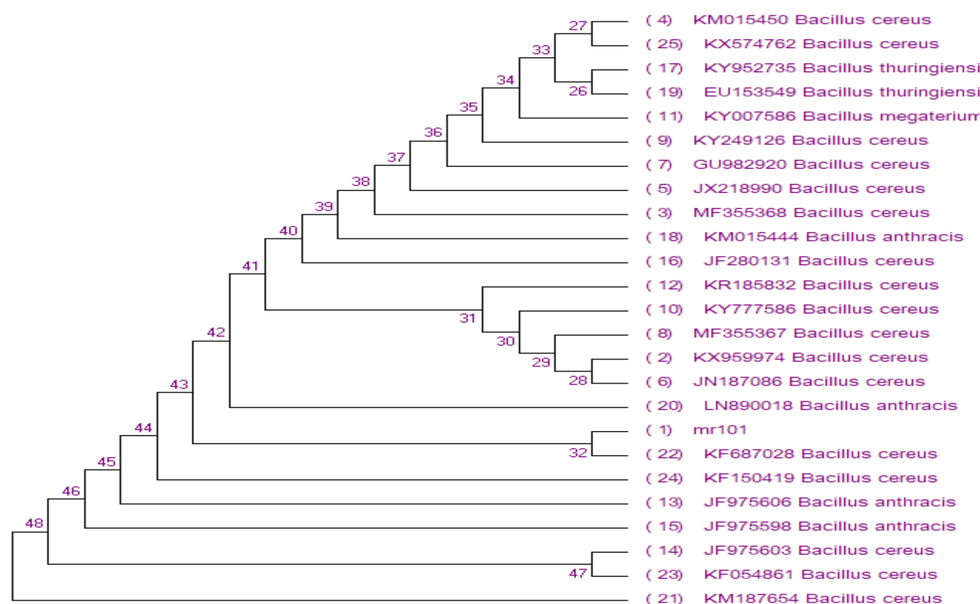

Fig. 1 Phylogenetic analysis of 16s rRNA sequence of *Bacillus cereus* RN-6 (MG271913) with other strains by neighbor-joining method

The microbial production of the enzyme depends on the genetic nature of the organism, the physio-chemical parameters, optimization of the above conditions are important to get maximal yields and to develop effective bioprocess system for industrial application.

Optimization of medium and cultural conditions for L-Glutaminase Production:

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

Effect of incubation period on L-glutaminase production:

Optimization of various process parameters, a time course experiment was carried out to monitor the rate of L-glutaminase production by *B. cereus* RN-6 strain for every 24 hrs of incubation. Results presented in the figure- 2. Maximum enzyme production of 18.3 U/ml was observed at 72 hrs of incubation and activity was gradually decreased afterwards. Similar maximum enzyme production of L-glutaminase at 72 hrs was also reported in *Pseudomonas* sp SFL-3 and *Micrococcus* sp SFL-15[20].

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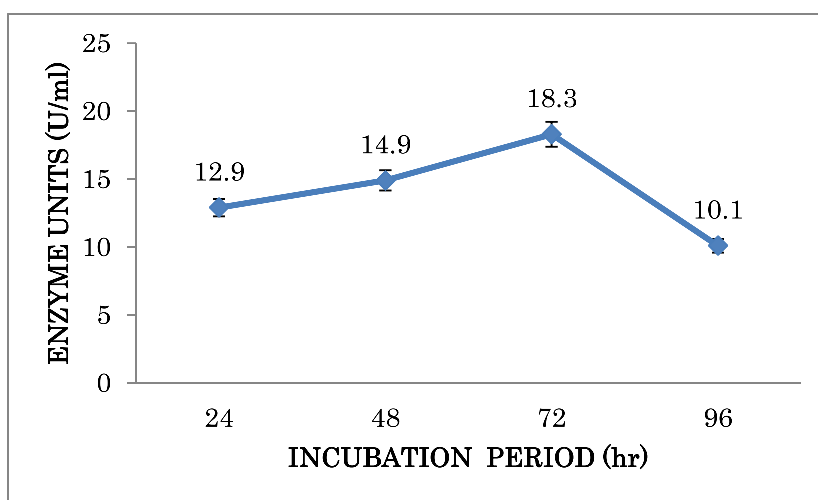
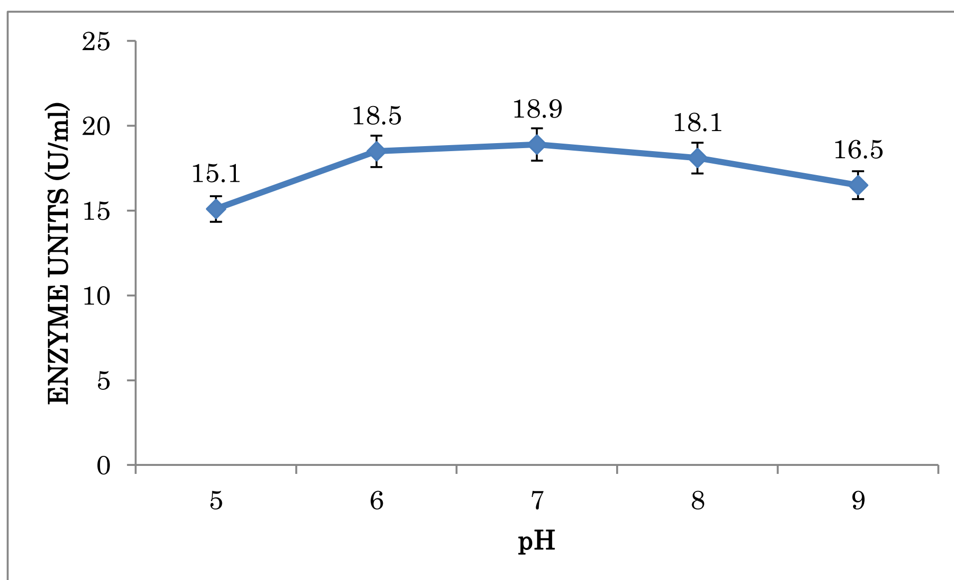


*The F-Value for incubation period and interactions are all significant with p<0.05.

Fig. 2 Effect of incubation period for L-Glutaminase production by *Bacillus cereus* RN-6

Effect of pH on the medium

The pH of the medium was reported to influence the growth of any microbial strain and subsequent metabolic product formation. In general, L-glutaminase production by most of the microbial organisms under submerged fermentation conditions was observed to be optimum in the pH range of 5.0 to 9.0. The results presented in Figure- 3 indicate that pH of the fermentation medium influence the enzyme production. Maximal enzyme production was observed at pH 7.0 (18.9 U/ml). Either increase or decrease in the pH of the medium resulted in decreased enzyme production. Results also suggest that this bacterium is neutrophilic in nature. Glutaminase activity from *Bacillus firmus* [21] and *Pseudomonas aeruginosa* [22] was also reported to be active over a broad range of pH (5-9) with an optimum production in pH 7.0.



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

Fig. 3 Effect of Initial pH for L-Glutaminase production by *Bacillus cereus* RN-6

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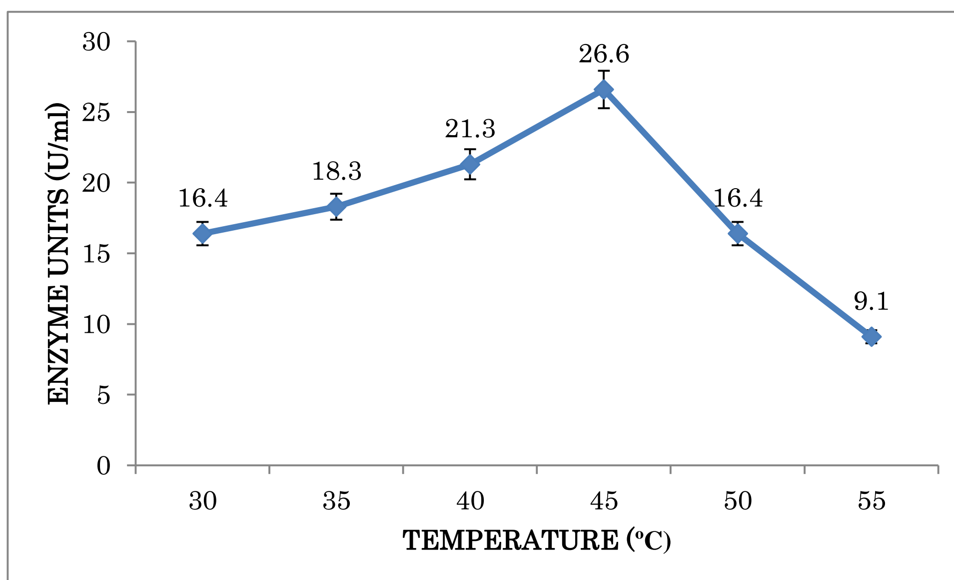
Effect of temperature

Incubation temperature influenced the rate of L-glutaminase production by bacteria *Bacillus cereus* RN-6. Thus the maximal enzyme production (26.6 U/ml) was observed at 45°C. Variation in temperature in either way resulted in decrease of L-glutaminase production (Figure- 4). The loss of activity is more at higher temperature when compared to the lower temperatures. Based on the literature, the optimum temperature for L-glutaminase production is varied with micro-organism studied. It was observed that 45°C was optimum for the enzyme production by *Bacillus circulans* [22].



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

Fig. 4 Effect of Temperature for L-Glutaminase production by *Bacillus cereus* RN-6

Effect of additional carbon sources

Incorporation of additional carbon sources into the enzyme production medium at 1% level, resulted in a significant increase in the enzyme production (Figures- 5). Among the various carbon sources tested, sucrose supported maximal enzyme yield (27.6 U/ml). All the other carbon sources also showed considerable amount of enzyme production. Cellulose was the least effective as a carbon source (16.4 U/ml). From the result, it was observed that all the other carbon sources except Cellulose supported maximum enzyme production in L-glutaminase. Similarly, in *Paenibacillus validus* [22] and *Zygosaccharomyces rouxii* [23] high enzyme production was reported when sucrose used as a carbon source.

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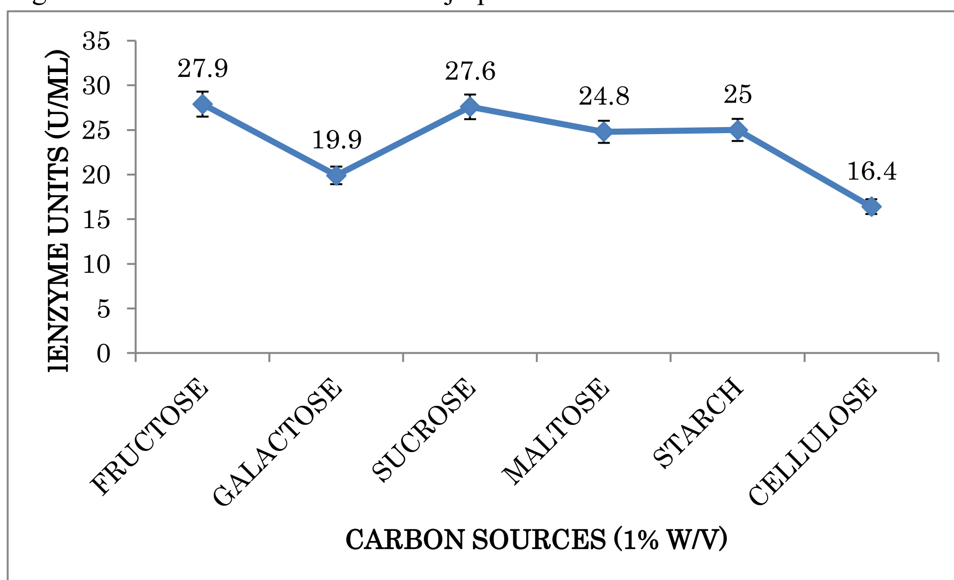
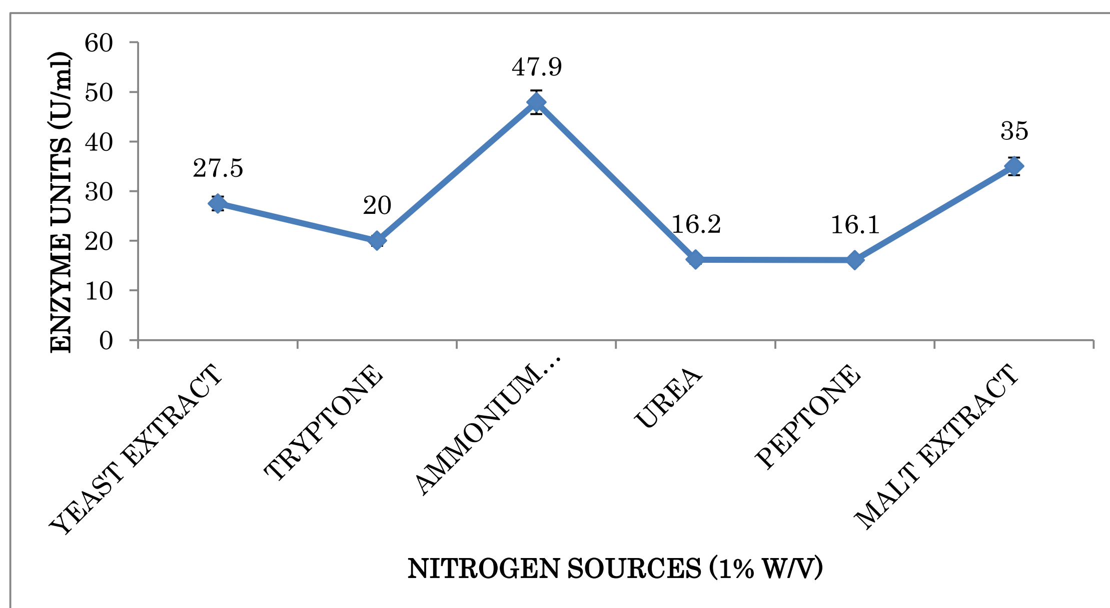


*The F-Value for Carbon sources and interactions are all significant with $p<0.05$.

Fig. 5 Effect of Carbon sources for L-Glutaminase production by *Bacillus cereus* RN-6

Effect of additional nitrogen sources

The addition of nitrogen sources, along with glutamine in the enzyme production medium influenced the rate of L-glutamine production by bacteria *Bacillus cereus* RN-6. From the results presented in (Figures- 6) it was inferred that among the nitrogen sources tested, Ammonium sulphate supported maximum enzyme production (47.9 U/ml). Among the other nitrogen sources, peptone showed least enzyme production (16.1 U/ml). Similar reports of enzyme production in *Pseudomonas stutzeri* [24] and *Achromobacteraceae* sp [8] were reported when Ammonium sulphate was used as nitrogen source.



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

Fig. 6 Effect of Nitrogen sources for L-Glutaminase production by *Bacillus cereus* RN-6

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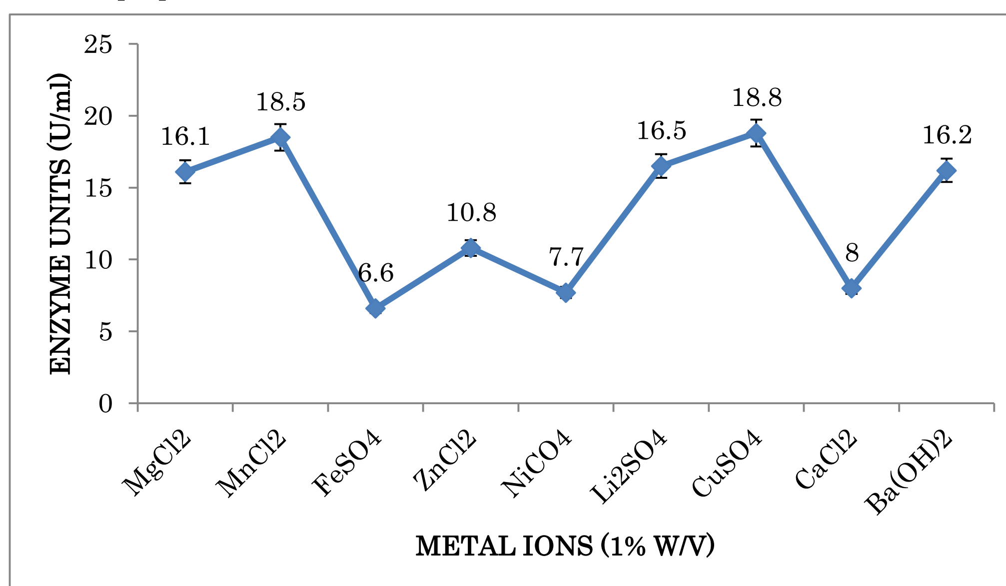
Effect of different metal ions

L-Glutaminase from *Bacillus cereus* RN-6 was affected by all metal salts tested (figure- 7) at 37°C and pH 7. The activity was 20.8 U/ml when $CuSO_4$ was added and 18.5 U/ml when $MnCl_2$ was used. The activity ranged from 6.6 to 16.5 U/ml with the other metal salts. This indicate that the activity was enhanced by $CuSO_4$ and $MnCl_2$. The activity was decreased when $NiCO_4$, Li_2SO_4 and $Ba(OH)_2$ were used. These reports are in accordance with the results on glutaminase from *Pencillium politans* NRC510 [25].



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

Fig. 7 Effect of Metal ions for L-Glutaminase production by *Bacillus cereus* RN-6

4. CONCLUSION

The strain *Bacillus cereus* RN-6 produced maximum enzyme when sucrose and ammonium sulphate were used as carbon and nitrogen sources, in presence of $CuSO_4$ at 7.0 pH and 45 ºC temperature after 72 hrs of incubation. Commercially L-glutaminase was produced by fermentation using wild strains. The production of huge quantities of the enzyme was confined due to the reduced skill of the technique. The clinical application of the drug is limited because of the extremely high expense of the drug. So it is beneficial to search the soil microbes to inspect potential L-glutaminase genes and construct suitable recombinants to over produce the enzyme and to meet the ever increasing clinical demands.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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