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PRODUCTION AND CHARACTERIZATION OF L-GLUTAMINASE BY *BACILLUS* SP.

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Key words:

Batch fermentation, L-Glutaminase, Inducer, Zobell's medium, *Bacillus* sp., Double Reciprocal Plot

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ABSTRACT: L-Glutaminase (EC.3.5.1.2) is an amidohydrolase which catalyses the hydrolytical deamination of L-glutamine resulting in the production of L-glutamic acid and ammonia. L-Glutaminase is gaining marked importance due to its application potential in cancer therapy, food industry and of high value chemicals like theronine. A variety of microorganisms, including bacteria, yeast, and filamentous fungi, from both soil and marine habitat have been reported to produce L-glutaminase .The optimization of L-glutaminase using one of the prominent bacterial isolate (NRBT2822) in batch mode of cultivation showed that a pH of 7.0, temperature of 37°C and agitation rate of 150 rpm are most suitable parameters for maximum enzyme synthesis in presence of 1.0 g/L of L-glutamine as an inducer. The kinetic studies revealed that the Km and V_{max} for the enzyme were 66.83 mM and 0.755 mM/min respectively. It was also found that the enzyme showed highest activity at 37oC with a half life of 3 hours.

INTRODUCTION: L-glutaminase is an enzyme (L-glutamine amidohydrolases E.C. 3.5.1.2) which catalyses the hydrolysis of L-glutamine to glutamic acid and [1]. In recent years, this enzyme has gained much attention due to its potential application as anti-cancer agent since it inhibits the proliferation as well as causes the death of leukemic cells by depriving them from L-glutamine supply [2]. A parallel interest on microbial L-glutaminase resulted from its applications in food flavoring, especially in the soy sauce and preparation of high value chemicals like theronine has initiated the quest for searching novel enzyme which can be exploited for large scale production [1].

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Goldstein and Schooler (1967) found that changes in activity as well as level of L-glutaminase are important in the control of ammonia synthesis in the kidney [3]. Other promising applications of L-glutaminase is its usage in the treatment of human immunodeficiency virus (HIV) [4] and as biosensors to monitor L-glutamine level in mammalian and hybridoma cell lines [5].

As the demand of L-glutaminase is increasing due of its vast applications in various industrial sectors therefore, it is necessary to produce L-glutaminase to reach its demand in various fields. There are several factors like search for new isolate with high production capacity, media designing and optimization for maximum production or genetic manipulation to achieve a hyper-producing microorganism etc. Certain other factors which govern the production of L-glutaminase enzyme include applications of solid state fermentation and submerged fermentation using synthetic media and agro based by-products for enhancing the productivity [4, 6].

As far as the occurrence of L-glutaminase is concerned, it is widely distributed in animal tissues, plants and in microorganisms including bacteria, fungi and yeast. A variety of microorganisms, including bacteria, yeast,and filamentous fungi, from both soil and marine habitat have been reported to produce L-glutaminase [7, 8]. The production of L-glutaminase under submerged fermentation process by *Aspergillus wentti* [9], *Zygosaccharomyces rouxii* [7], *Pseudomonas aeruginosa* [10], *Pseudomonas fluorescens* [11], *Bacillus subtilis* [12], *Bacillus amyloliquefaciens* [13], *Bacillus cereu s*[14], *Streptomyces canaries* [15] have been reported.

In the present communication, the production and characterization of L-glutaminase was investigated using a soil bacterial isolate (NRBT2822) at various process parameters.

MATERIALS AND METHODS:

Cultural conditions:

The Zobell's agar media (ZM) was prepared for screening and maintenance of bacterial cultures. The composition of medium (g/l) include, beef extract 1.0; peptone5.0; sodium chloride 5.0; yeast extract 2.0; L-glutamine 1.0; phenol red 0.012; and agar powder 20 where phenol red acts as pH indicator.Glutamine-utilizing microorganisms were isolated by enrichment method in which 1.0g of soil was aseptically transferred to 50ml of sterile Zobell's medium in absence of phenol red and agar and incubated at 37ºC under agitation of 150 rpm for 24h. Dilutions of broth were made and 50 ml was pour pated on ZM incubated at 37ºC for 24h to observe the isolates with positive utilization of glutamine and glutamic acid production as indicated by a decrease in pH (change of color of the plate) [16]. The L-glutaminase producing bacterial isolates were purified by several streaking and maintained till further use.

The Production media contained (g/l), malt extract 10.0; peptone 10.0; sodium chloride 5.0; and L-glutamine 3.0. The pH of medium was adjusted to 7.0 before sterilization. One loop full cells from well grown plate was transferred to the 50ml of sterile liquid medium and incubated at 37ºC for 24h in an orbital shaker set at 150 rpm. The enzyme production broth was harvested by centrifugation at 10,000 rpm under refrigeration for 10 min and cell pellets as well as supernatant were tested for L-glutaminase assay. The cell pellet was washed further with phosphate buffer (0.1M pH 7.0) and stored in 5.0 ml till further use.

Assay of L-Glutaminase:

The activity of L-glutaminase was assayed by detecting the amount of ammonia released during the hydrolytic deamination of L-glutamine using modified protocol of Imada *et.al* (1973) [17]. The enzyme substrate reaction mixture was prepared in 1.0 ml which contains 0.1 ml of 10mM L-glutamine, 0.8 ml of 0.1M buffer (phosphate, pH 7.0) and 0.1 ml of enzyme preparation and incubated at 37oC for 30 min. The reaction was stopped by adding 0.25 ml of trichloroacetic acid. Control tubes were also prepared by adding the enzyme preparation after the addition of trichloroacetic acid.

The reaction mixture was centrifuged and supernatant was used for nesslerization reaction. To 3.7 ml of distilled water, 0.1ml of the above mixtures and 0.2 ml of nessler's reagent were added. The solution was kept at room temperature for color development for 20 min and optical density was measured at 450 nm. The L-glutaminase activity (U/ml) was defined as the amount of ammonia liberated per ml of enzyme preparation per min and calculated from the standard plot of ammonium chloride. All the reactions were performed in triplicates and mean value was recorded.

Optimization of process parameters for maximum production of L-glutaminase:

The optimization of different process parameters were carried out by one factor at a time analysis keeping other factors constant. For evaluating the effect of pH on the production process, the pH of the production medium was varied in the range of 6-8 keeping other parameters constant and activity was calculated. Similarly, the effect of incubation temperature in the range of 28-45ºC and concentration of L-glutamine in the range of 0.5-2.0 g/l were evaluated for maximum activity of L-glutaminase. The thermal stability, maximum reaction velocity (V_{max}) and Michelis Menten constant (K_m) were also investigated.

Biochemical tests and 16s r-RNA analysis of most prominent isolate:

Biochemical tests were performed as given in Bergey's Manual of Systemic Bacteriology. 16S rRNA sequencing was conducted at the BIOGENE, Gandhi Nagar, Gujarat, India. The sequence obtained was searched against the GenBank database, and homology studies were undertaken to identify the isolate.

The sequence was initially analyzed at the NCBI server (http://www.ncbi.nlm.nih.gov) using the BLAST (blastn) tool, corresponding sequences were 2downloaded, and the sequence homology analysis performed. A phylogenetic tree was constructed using clustalw2 program (http://www.ebi.ac.uk/ Tools/msa/clustalw2/).

RESULTS:

Screening of L-glutaminse producing bacterial isolates: From a number of different bacterial strains obtained from soil after enrichment method, few isolates showed colour change on the agar-plate (**Fig. 1**) and hence, selected for optimization of enzyme production in liquid culturing conditions and evaluation of kinetic properties. The change of colour from yellow to pink is due to the enzymatic reaction, where acid (glutamic acid) is produced from the substrate L-glutamine lowering the pH of the agar plate medium [16].

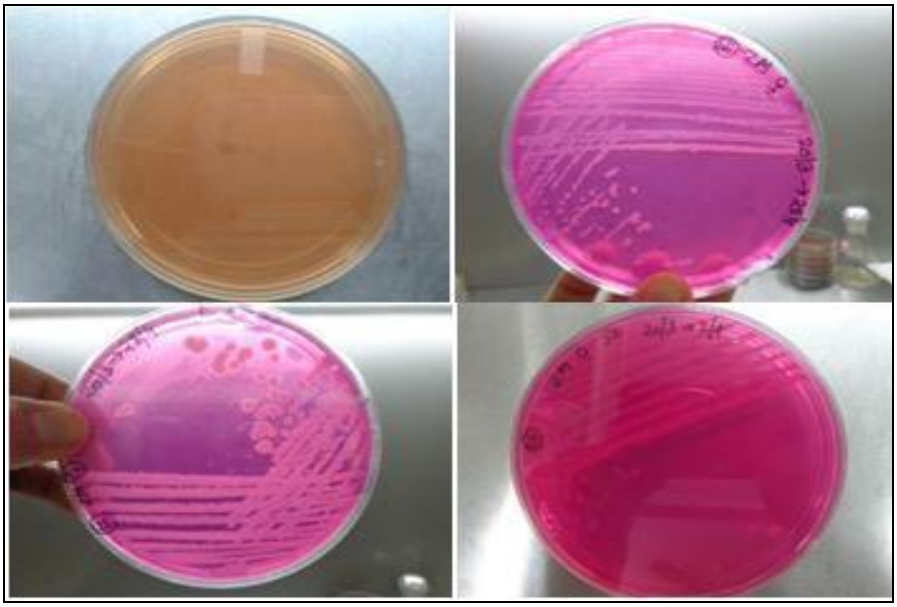

FIG. 1: CHANGE OF COLOUR OF PLATE BY CERTAIN L-GLUTAMINASE PRODUCING BACTERIAL ISOLATES (IN CLOCKWISE DIRECTION: CONTROL, NRBT001, NRBT002, NRBT2822).

The isolates showed positive results on agar plates were further allowed to grow in batch mode of cultivation in production medium at optimal conditions and the activity obtained are expressed in **Table 1**.

TABLE 1: L-GLUTAMINASE ACTIVITY OF DIFFERENT BACTERIAL ISOLATES

S. No.	Name of Isolates	Activity of L-glutaminase (U/ml)
1	NRBT001	1.83
2	NRBT002	3.071
3	NRBT2822	4.231
4	NRBT007	0.976
5	NRBT009	2.752
6	NRBT010	1.521
7	11A	2.854

It is observed from the **Table 1** that three isolates NRBT002, NRBT2822 and 11A showed high level of L-gluamninase production as compared with the other bacterial isolate. Among these three, NRBT2822 showed relatively highest enzyme production. It has also been noticed during the present work that no enzyme activity was obtained in cell free liquid, confirming the intracellular nature of enzyme. In certain studies, it has been reported that some of the bacterial strain secreted L-glutaminase in extracellular environment [4, 18]. Based on the activity profiling of L-glutaminase by different bacterial isolates, NRBT2822 was further selected for optimization of enzyme production and characterization.

Effect of pH on production of L-glutaminase:
The effect of pH on the production of L-glutaminase by NRBT2822 was investigated by varying the pH of production medium from 6.0 to 8.0 and the activity of enzyme at different pH is shown in **Fig.2**.

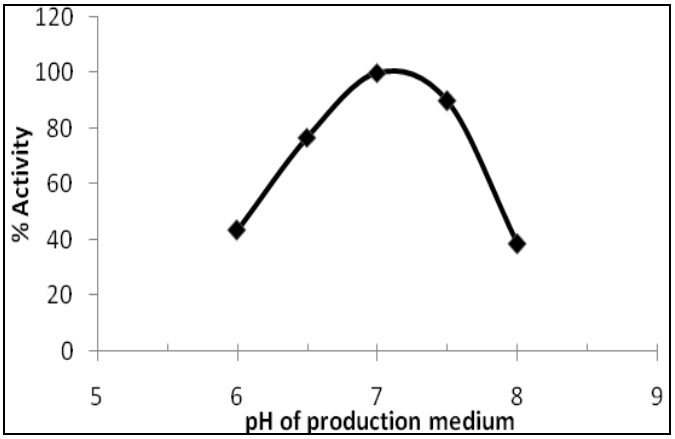

FIG. 2: EFFECT OF pH ON L-GLUTAMINASE PRODUCTION BY NRBT2822.

It is observed from the **Fig. 2** that production of L-glutaminase increases from pH 6.0 and reached to a maximum level at neutral pH 7.0 (optimal) and thereafter the activity of enzyme decreased. The decreased activity at higher pH might be due to denaturation of enzyme protein.

Effect of temperature on production of L-glutaminase:
The effect of temperature on the production of L-glutaminase was studied by incubating the production medium in presence of NRBT2822from 28ºC to 45ºC and the activity recorded at different temperature is given in **Fig.3.**

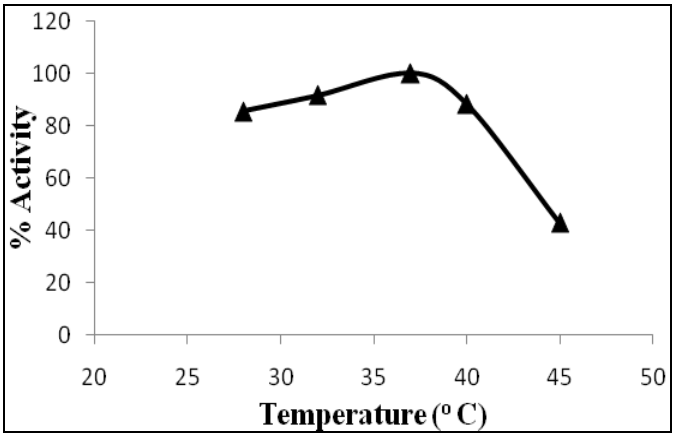

FIG.3: EFFECT OF TEMPERATURE ON L-GLUTAMINASE PRODUCTION BY NRBT2822.

The observation of **Fig. 3** revealed that 37ºC temperature is the optimum temperature of incubation for maximum production of L-glutaminase. At 45ºC, almost 65% activity was lost due to denaturation of enzyme protein.

Effect of concentration of inducer on production of L-glutaminase:
It has been reported that the L-glutamine plays role of inducer for synthesis of L-glutaminase. The production of enzyme by NRBT2822 was performed at different concentrations of L-glutamine (0.5 g/l to 2.0 g/l) in the production medium and enzymatic activity recorded at different concentrations is represented in **Fig. 4.**

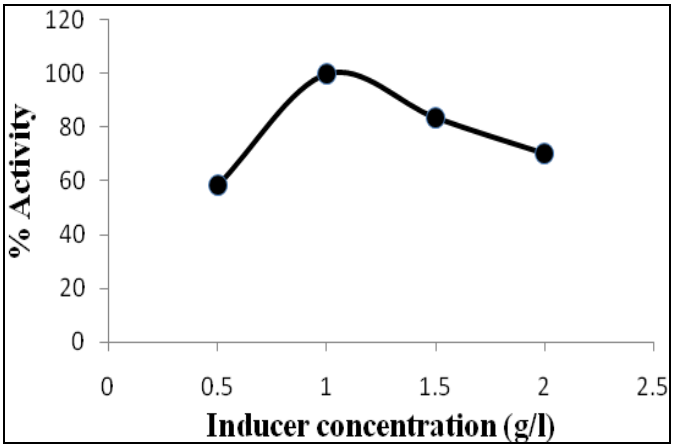

FIG.4: EFFECT OF L-GLUTAMINE CONCENTRATION ON PRODUCTION OF L-GLUTAMINASE BY NRBT2822.

It is observed that 1.0 g/l of L-glutamine induced maximum enzyme synthesis and there was a sharp decrease in synthesis of enzyme when the concentration of glutamine in the production medium was decreased. No activity was observed in the production medium devoid of L-glutamine and hence, it is playing the role of inducer for synthesis of L-glutaminase.

After optimization of various process parameters on the production of enzyme from NRBT2822, the characterization of L-glutaminase was carried out by measuring the activity using whole cells as enzyme source at different process variables.

Effect of different pH of reaction buffer on the activity L-glutaminase
The activity of L-glutaminase in different pH (6.0-8.0) of Phosphate buffer (0.1M) was evaluated and shown in **Fig. 5**.

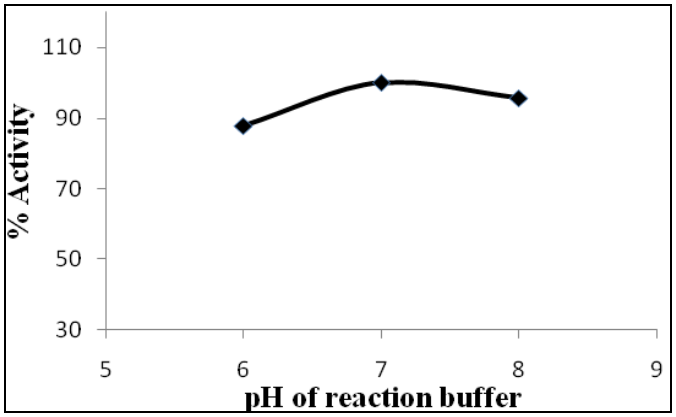

FIG. 5: EFFECT OF DIFFERENT pH OF REACTION BUFFER ON ENZYME ACTIVITY.

It is observed that no significant decrease in activity of L-glutaminase was noticed at pH 6.0 while at pH 8.0 almost 95% activity was retained by the whole cells at operating conditions.

Effect of temperature on activity of L-glutaminase:

For optimization of temperature on maximum activity of enzyme, the enzyme reaction mixture was incubated at 28°C, 37°C, 45°C, and 55°C respectively and the result obtained is shown in **Fig.6**.

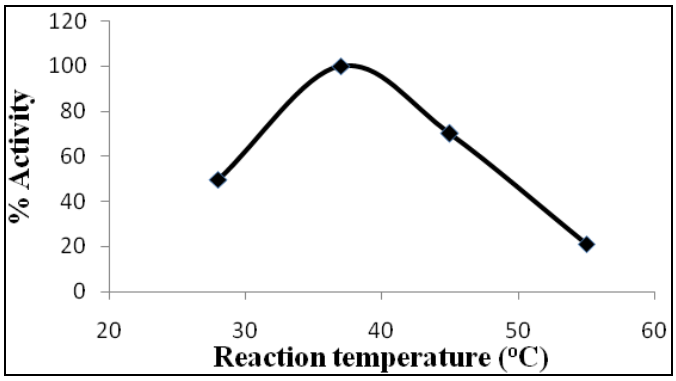

FIG. 6: EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

The observation of **Fig. 6** showed that the optimum temperature for L-glutaminase activity is at 37ºC. At high temperature, there was significant loss in enzymatic activity.

Thermal stability of L-glutaminase:

The stability of any enzyme provides its commercial applicability and it has been found that the enzyme with stability at high temperature has better stability of its protein and could be used for reactions performed for longer duration. The stability of the L-glutaminase produced by NRBT2822 was tested by incubating the whole cells (enzyme) at 37ºC for 5 h and L-glutaminase activity was calculated at different time intervals as shown in **Fig.7.**

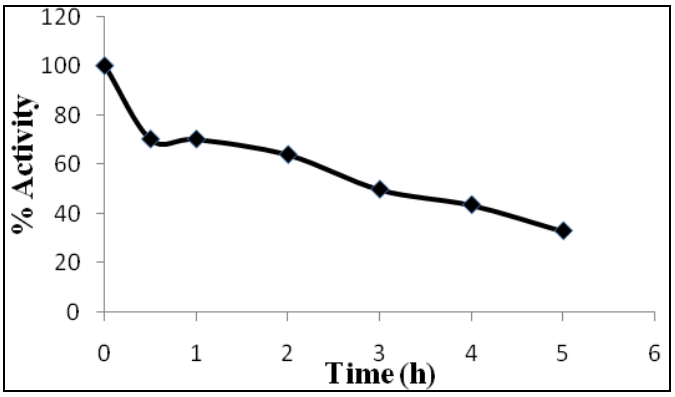

FIG.7: THERMAL STABILITY OF L-GLUTAMINASE.

It was observed from the **Fig. 7** that the activity of enzyme decreased with the increase of incubation of enzyme suspension and half life ($t/_2$) of L-glutaminase synthesized by the isolate was 3 h. This type of studies using L-glutaminase is rarely discussed in literature and the enzyme from NRBT2822 is quite stable and could be exploited for biotransformation of L-glutamine to L-glutamic acid.

Effect of substrate concentration on L-glutaminase activity

The enzyme activity for the L-glutaminase producing isolate was examined by varying the concentration of the substrate i.e., L-glutamine in the range of 5 mM to 180 mM during the reaction assay. The Double Reciprocal Plot between 1/S and 1/V at different concentrations of L-glutamine is presented in **Fig.8**.

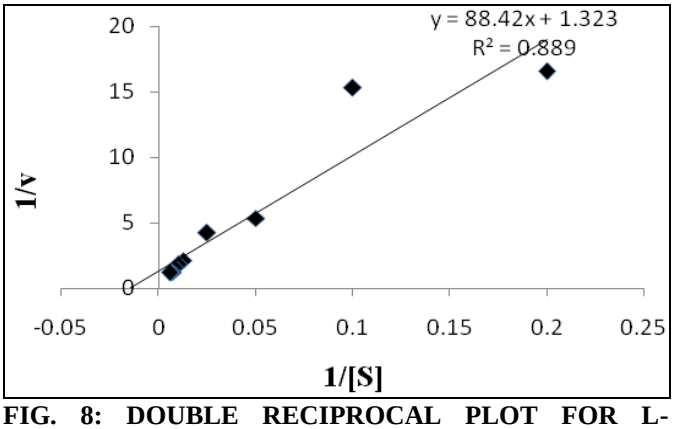

FIG. 8: DOUBLE RECIPROCAL PLOT FOR L-GLUTAMINASE.

It has been calculated from the Figure 8 that affinity of enzyme (Michaelis-Menten constant, K_m) towards specific substrate i.e. L-glutamine was found to be 66.83. Similarly, the maximum reaction velocity i.e. V_{max} was found to be 0.755 mM/min.

Biochemical characterization and Phylogenetic tree construction:

The biochemical characterization of the isolate NRBT2822 based on different properties has been provided in **Table 2**. It has been inferred that the bacterium is gram positive rod shaped and utilizes maltose and glucose for gas production. It does not hydrolyse starch but degraded casein.

TABLE 2: BIOCHEMICAL CHARACTERIZATION OF NRBT2822.

Biochemical tests		NRBT2822
Gram stain		(+), rod
Fermentation	Sucrose	Acid(-), Gas(-)
	Lactose	Acid(-), Gas(-)
	Maltose	Acid(+), Gas(+)
	Glucose	Acid(+), Gas(+)
Casein hydrolysis		+
Starch hydrolysis		-
Methyl red test		-
Urease test		-
Catalase		-

The evolutionary history was inferred using the Neighbor-Joining method [19] and the phylogenetic tree with the sum of branch length (0.20) is shown in **Fig.9**.

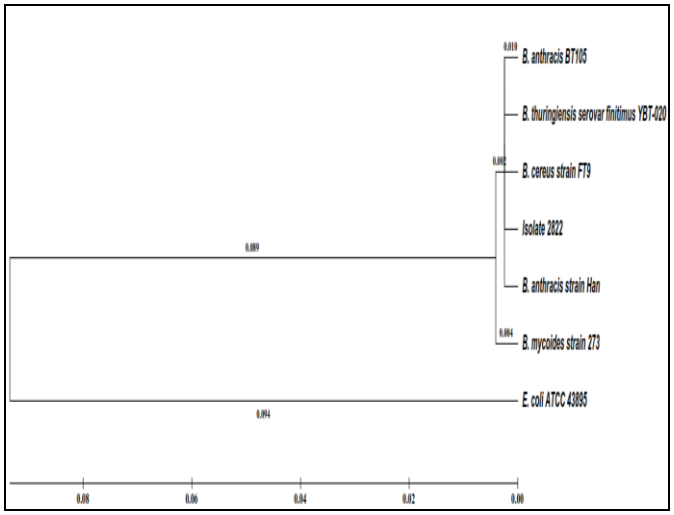

FIG.9: PHYLOGENETIC TREE OF ISOLATE NRBT2822.

The tree is drawn to a scale with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [20] and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences where *E. coli* ATCC 43895 is used as out-group. All positions containing gaps and missing data were eliminated. There were a total of 1374 positions in the final dataset. Evolutionary analyses were conducted in MEGA521 and based on the tree, it has been found that the NRBT2822 is closely related to *Bacillus cereus*.

CONCLUSION: The L-glutaminase producing isolate showed the maximum activity of 4.231 U/ml at optimal conditions of 37 °C, 150 rpm, and pH of production media of 7.0. A parallel work on *Bacillus subtilis* shows similar conditions for maximal production of L-glutaminase [12] Whereas an optimum temperature of 28ºC and optimum pH 7.0 have been reported for *Aspergillus wentti* [9]. Work done on *Bacillus cereus* MTCC 1305 differs since maximum production of L-glutaminse was obtained at 34ºC, pH 7.5 and fermentation time of 40 hours [14]. Work done by Habeeb *et al.*, report that neutral to slight alkaline pH is required from maximal glutaminase production with S*treptomyces* sp., and incubation above 40ºC promoted remarkable inactivation of L-glutaminase [22]. The enzyme we obtained was intracellular in nature and synthesized only in presence of L-glutamine with a half life of 3h at 37°C in the region of neutral pH. Similar result has been reported in glutaminase produced by *E. Coli* where the enzyme is active at neutral and alkaline pH 23.

Identification using 16S rRNA sequencing revealed that the isolate has similarity to *Bacillus cereus* strain FT9. The high stability along with the maximum reaction velocity of enzyme be useful for biotransformation of L-glutamine and could be exploited for development of a drug for treatment of cancer by studying certain other immune-related parameters.

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