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SCREENING AND PRODUCTION OF EXTRACELLULAR α – AMYLASE, PROTEASE AND GLUCOSE ISOMERASE FROM MESOPHILIC *BACILLUS LICHENIFORMIS* GINM-3 ISOLATED FROM AGRICULTURAL SOIL

Sanjay Parihar and Harshada Joshi*

Molecular Microbiology Laboratory
Department of Biotechnology, Vigyan Bhawan, Block "B", Mohanlal Sukhadia University
Udaipur, Rajasthan, India.

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***Corresponding Author**
Harshada Joshi
Department of Biotechnology, Vigyan Bhawan, Block "B", Mohanlal Sukhadia University Udaipur, Rajasthan, India.

ABSTRACT

Microbial enzymes have proven their importance in bio-industries such as food, animal feed, leather, textiles as well as in bioconversions. The present study was conducted for extracellular screening and production of various microbial enzymes such as α-amylase, protease and glucose isomerase. An attempt was made to isolate mesophilic bacterial strains producing α-amylase, protease and glucose isomerase enzymes. Isolate GINM3 showed high yield production of these enzymes and the strain was later identified as *Bacillus licheniformis* by 16S rRNA partial genome sequencing. The production of enzymes was assayed in submerged fermentation condition. The maximum enzyme activity was found after 48 hours at pH 7. Enzymes activities for α- amylase was (0.316U/ml), protease (0.762U/ml), and glucose isomerase (0.369 U/ml).

KEYWORDS: Protease, α-amylase, glucose isomerase, mesophilic, enzyme activity.

1. INTRODUCTION

Microbial enzymes have gained recognition worldwide for their widespread applications in industries and medicine owing to their stability, catalytic activity, and comfort of production and optimization than plant and animal enzymes (Singh *et al.*, 2016). Fermentations mainly are bio-catalytic processes which occur as a result of microbial metabolism. The global market for industrial enzymes reached nearly $4.6 billion and $4.9 billion in 2014 and 2015,

respectively. This market is predictable to increase from nearly $5.0 billion in 2016 to $6.3 billion in 2021 at a compound annual growth rate (CAGR) of 4.7% for 2016-2021. Food industrial enzyme market is expected to grow from nearly $1.5 billion in 2016 to $1.9 billion in 2021 at a CAGR of 4.7% from 2016 through 2021. Animal feed industrial enzyme market is expected to grow from $1.2 billion in 2016 to nearly $1.6 billion at a CAGR of 5.2% from 2016 through 2021(BCC Research Report, Jan.2017).There is an increased interest in using mesophilic bacteria for the production of enzymes from polysaccharides due to their moderate operating temperatures and substrate range (Naidu and Saranraj, 2013). Extracellular enzymes are of particular interest because they are generally more stable and are easier to purify than intracellular enzymes, thereby reducing the cost of production. *Bacillus* species are the main producers of extracellular enzymes, and industrial sectors frequently use *Bacillus licheniformis* for the production of various enzymes.

Alpha-amylase (1,4-α-D-glucanglucanohydrolase, EC 3.2.1.1) is the furthermost essential carbohydrate degrading enzyme for starch based industrial companies. Microbial amylases are enzymes which hydrolyze polysaccharide such as starch to give various products including dextrin, maltose and gradually minor polymers composed of glucose units. These enzymes are of countless importance in recent applied fermentation biotechnology with uses ranging from food, fermentation and textile to paper industries (Gupta *et al.*, 2003).

Microbial protease is an enzyme that catalyses proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein (Gupta and Khare, 2007). Although protease-producing microorganisms, plants and animals have cosmopolitan distribution in nature; microbial community is preferred over the others for the large scale production of proteases due to their fast growth and simplicity of life for the generation of new recombinant enzymes with desired properties. Microorganisms account for a two-third share of commercial protease production in the enzyme market across the world. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries (Gurung *et al.*, 2013).

Glucose isomerase (also known as xylose isomerase, EC.5.3.1.5) catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, *in vitro* and *in vivo* respectively (Bhosale *et al.*, 1996). Glucose isomerase is an essential process for the industrial production of high fructose corn syrup (HFCS), main sweetener in many soft drinks

and food. The growth in food, healthcare, fuel ethanol, animal feed is prompting the demand for GI (Bhasin and Modi, 2012).

2. MATERIALS AND METHODS

2.1. Chemicals: Chemicals which were used during this study were purchased from Himedia and Sigma- Aldrich India and glassware were purchased from Borosil.

2.2. Agricultural soil collection: The agricultural soil samples were collected from agricultural land of Badgaon, 24.6366' and 73.6801' longitude; district Udaipur, Rajasthan, India. The samples were collected in sterile polythene bags and transferred to the laboratory. The temperature of the soil sample during sample collection was 30˚C.

2.3. Isolation and identification of bacteria: Isolation was done on nutrient agar using pour plate method. The identification of isolates was done on the basis of partial 16S rRNA sequencing. It was maintained on nutrient agar slant and sub cultured every 3 week.

2.4. Preparation of inoculum: Bacterial inoculum was prepared in 250 ml conical flask containing 50 ml of sterilized nutrient broth and was aseptically inoculated with a loopful of bacteria from a fresh slant and allowed to grow at 37°C for 24 h in a rotary shaker. After 24 h of growth, the bacterial culture was used as inoculum.

2.5. Screening and enzyme assay of α-amylase: *Bacillus licheniformis* GINM-3 was tested for α-amylase production by starch hydrolysis. The 24h old culture was transferred in to starch agar medium (peptone – 0.5 g, beef extract – 0.15, yeast extract – 0.15, NaCl – 0.5g, starch – 1g, Agar – 2g, and distilled water- 100 ml) and the plate were incubated at 37° C for 48h. After 48h the pates flooded with iodine solution (iodine – 0.2%, KI- 0.4%, distilled water - 100ml). When starch was fragmented down into sugars, there were clear zones nearby streaked lines which indicate starch hydrolysis. Negative control was also streaked on plate. The α-amylase activity was determined by quantifying the formation of reducing sugars released during starch hydrolysis. The reaction mixture containing 0.5 mL of appropriately diluted enzyme and 0.45 mL of 1.0 % (w/v) soluble starch in 50 mM phosphate buffer (pH 6.5) was incubated at 50 °C for 15 min. The amount of liberated reducing sugar was determined by the dinitrosalicylic (DNS) acid method (Miller, 1959). One unit of α-amylase activity was defined as the amount of enzyme that released 1 µmol of reducing end groups per min at 50 °C. D-Glucose was used to construct a standard curve.

$$Alpha\ amylase\ enzyme\ activity\ \frac{\Delta E \times Vf}{\Delta t \times \sum \times Vs \times d}$$

ΔE = Absorbance at 540 nm, Vf = Final volume including DNS, Vs = Volume (mL) of α-amylase used ,Δt = Time of hydrolysis, Σ = Extinction coefficient, d = Diameter of cuvette (1 cm for standard cuvette).

2.6. Screening and enzyme assay of protease

Production of protease was detected. The 24h old culture was transferred in to skimmed milk agar medium (g/l) (skim milk powder-28, casein enzymic hydrolysate-5, yeast extract-2.5, dextrose-1, NaCl-0.5, agar-15) with pH 9.0 (using phosphate buffer) and incubated at 37°C for 48 h. The development of clear zone nearby the colonies confirms the production of protease. The colonies that had formed a clear zone around the growth were considered as protease positive isolates. Negative control was also streaked on plate.

The basal culture medium for the protease production contained (g/1): K_2HPO_4, (4g); Na_2HPO_4, (1g); $MgSO_4 \cdot 7H_2O$, (0.1g); Na_2CO_3, (6g), pH 7.5. Sodium carbonate solution was sterilized individually, and then added to the medium. The medium (50 ml) was inoculated with three loopfull of a 24-hour-old isolate culture in 250 ml flask, and incubated at 37°C with shaking at 200 rpm for 96 h. The cell-free supernatant was recovered by centrifugation (8,000 g, 4°C, 20 min), and used for determining the protease enzyme activity.

2.7. Substrate screening and enzyme assay of glucose isomerase

The isolates were screened qualitatively for GI by plate assay method. The screening strategy was designed according to the method described by Manhas and Bala, 2004. The production of glucose isomerase was checked on medium containing xylose as a sole source of carbon and wheat bran medium. Three different media combinations for screening, X+P+ medium containing (g/L) Xylose (10), Peptone (0.3), KNO_3 (2), K_2HPO_4 (2), NaCl (2), $MgSO_4.7H_2O$ (0.5), $FeSO_4$ (0.1), $CaCO_3$ (0.2), Agar (20), pH (7) were used. Another medium named X+P- contained all above ingredients without peptone. Third one was wheat bran medium, it contained all above ingredients present in X+P+ but xylose was replaced with wheat bran. The organisms producing glucose isomerase can isomerize xylose to xylulose besides glucose to fructose. Xylose has to be first converted to xylulose which is further channelized into pentose phosphate pathway for generation of energy. The organisms possessing very low or negligible GI activity might not grow on such a media. The cultures were spot inoculated on

all the media combinations and incubated at 37°C. The plates were observed daily. The early responders were considered as probable GI producers. The isolates developing early on the plates and giving luxurious growth were picked up as GI producers.

The centrifuged supernatant of fermented broth was used as crude enzyme extract for determination of GI activity by assay method described by Chen *et al.,* (1980). The reaction mixture for GI assay contained 500 µl of 0.2 Molar sodium phosphate buffer, 200 µl of 1 M glucose, 100 µl of 0.1 M magnesium sulphate, 100 µl of 0.01 M cobalt chloride and 200 µl of crude enzyme extract. The final volume of assay mixture was made up to 2 mL. This reaction mixture was incubated in water bath at70°C for 60 minutes. The reaction was stopped by adding 2 mL of 0.5 M perchloric acid. To 0.05 mL aliquot of above 0.95 ml of distilled water was added. To this 200µl of 1.5% cysteine hydrochloride, 6mL of 70% sulphuric acid and 200 µl of 0.12% alcoholic Carbazole is added. The intensity of purple colour so developed was estimated spectrophotometrically at 560 nm (Dische and Borenfreund, 1951). One unit of glucose isomerase activity was defined as the amount of enzyme that produced 1µmol of fructose per minute under the assay conditions described. All the experiment performs in triplets.

2.8. Phylogenetic tree Analysis

All the nucleotides in the selected sequences were corrected according to the homologous sequences searched by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and/or other 16S rRNA sequences of the type strain(s). The phylogenetic trees were constructed using the neighbor-joining (NJ) algorithm with Phylogeny.fr**. (**Dereeper *et al*., 2010**).**

3. RESULT AND DISCUSSION

The first footstep was to isolate the desired microorganism that produces α-amylase, protease and glucose isomerase in proper yield form. Soil sample were collected in proper sterilized condition and serially diluted in nutrient agar plate. A total five isolates were recovered, out of which only isolate number GINM-3 was found positive for all three enzymes, therefore isolate GINM-3 was further used for screening and enzyme assay. Identification of isolate GINM-3 was done using 16s rRNA partial sequencing and identified as *Bacillus licheniformis* GINM-3. The sequence was deposited to NCBI and accession number KY492396 provided for the isolate.

3.1. Screening of Amylolytic bacteria

The amylolytic (starch degradation) activity of *Bacillus licheniformis* GINM-3 was evaluated using starch agar and it was expressed as appearance of clear zone (zone of starch hydrolysis) around bacterial streaked line. The result for starch hydrolysis is shown in figure1. For negative control *E.coli* (MTCC No. 443) was streaked on another half of petriplate.

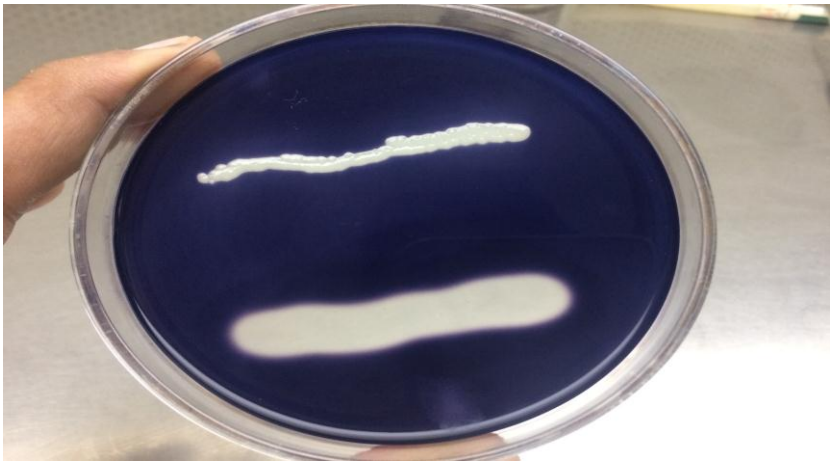

Figure. 1. Amylolytic activity of *Bacillus licheniformis* GINM-3 on starch agar.

3.2. Screening of proteolytic bacteria

The proteolytic activity of *Bacillus licheniformis* GINM-3 was evaluated using skimmed milk agar and it was expressed as appearance of clear zone (zone of casein hydrolysis) around bacterial streak line. The result for casein hydrolysis is shown in figure 2. For negative control *E.coli* (MTCC No. 443) streaked on another half of petriplate.

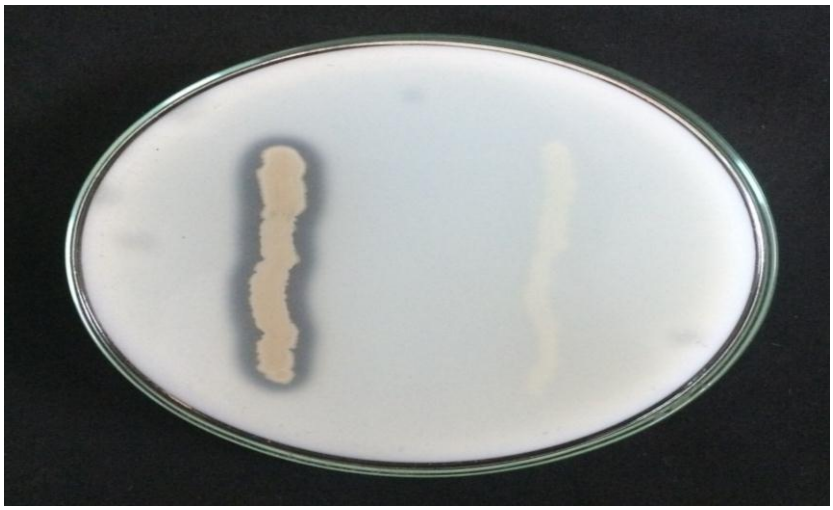

Figure. 2. Casein hydrolysis by *Bacillus licheniformis* GINM-3 on skimmed milk agar.

3.3. Screening of glucose isomerase

The qualitative screening revealed that *Bacillus licheniformis* GINM-3 isolate produced GI. The organism gave early appearance on wheat bran medium and also grew well on other two media. X+P+ and X+P- media gave a clear picture of isolate as GI producer, as former is containing xylose, peptone and mineral salts whereas latter is containing only xylose and mineral salts. The growth of organism on X+P- medium (in absence of peptone) indicated the production of GI for utilizing xylose present in the medium (Figure 3).

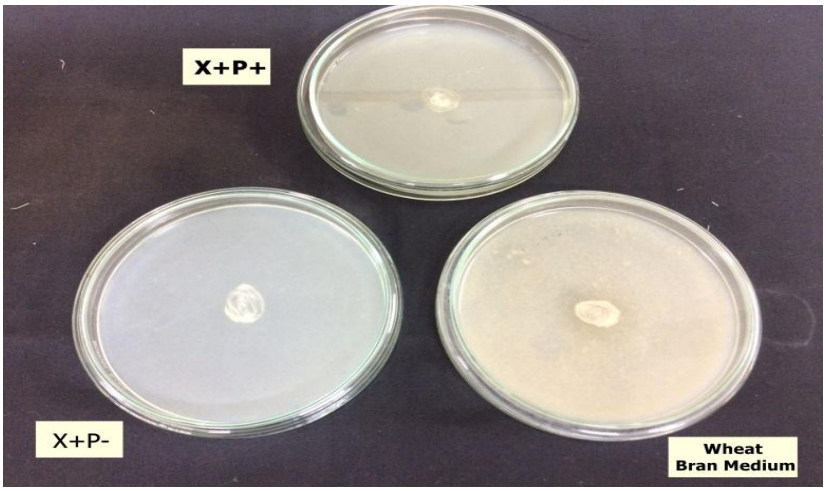

Figure. 3. Screening of *Bacillus licheniformis* GINM 3 glucose isomerase on different media.

3.4. Quantitative assay of α-amylase, protease and glucose isomerase

The methods followed for quantitative estimation are as follow.

Alpha amylase activity of *Bacillus licheniformis* GINM 3

The alpha amylase activity of *Bacillus licheniformis* GINM-3 in submerged culture filtrate was assayed. Alpha- amylase production was investigated at four different incubation period (24, 48, 72 and 96 hour at 37°C). The maximum activity of amylase enzymes was found 0.316U/ml at 37°C after 48 hours at pH 7. Further increase in incubation period did not show any significant increase in enzyme production rather it was decreased. Thus optimum duration of enzyme synthesis was to be 48 h after inoculation. Ramesh and Lonsane, 1990 reported that the enzyme production was initiated at about 6 h in the medium containing 1.0% soluble starch. Locally-isolated strains and cheap substrates can produce inexpensive amylase and reduce the enzyme's production cost. The occurrence of amylolytic organisms in soil agrees with earlier reports of Adebiyi and Akinyanju (1998) and Omemu *et al.* (2005), that

soil is known to be a repository of amylase. The results indicated that there is appreciable high production, activity and stability of the amylase at optimum temperature.

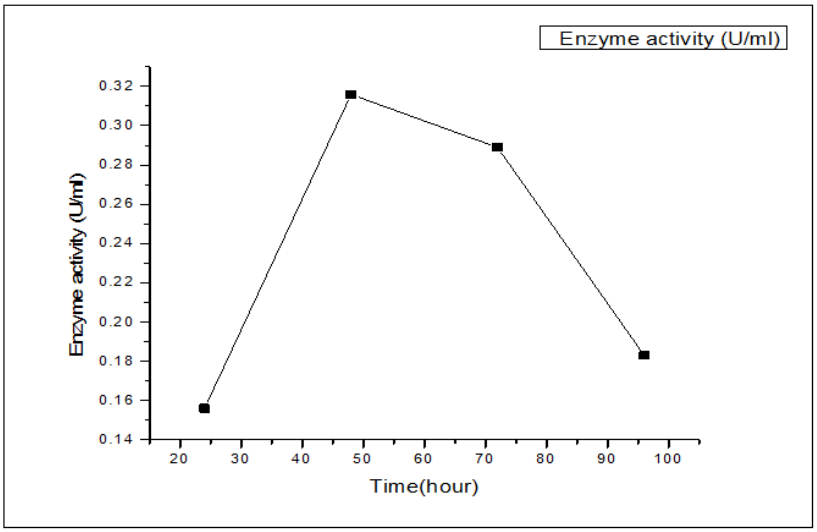

Figure. 4. Alpha amylase enzyme activity of *Bacillus licheniformis* GINM-3.

Protease enzyme activity of *Bacillus licheniformis* GINM 3

The protease activity of *Bacillus licheniformis* GINM-3 in submerged culture filtrate was assayed. Protease production was investigated at four different incubation period (24, 48, 72 and 96 hour at 37°C). The maximum activity of amylase enzymes was found 0.762U/ml at 37°C after 48 hours at pH 7. Bhuniya *et al.*, 2012 reported that maximum protease production (141.46 U/mg) was obtained in the period of 24 h incubation at pH 8 under 250 rpm compared to the initial enzyme production (89.87 U/mg). A recent investigation by Nwokoro,2017 reported that protease production also increased from the time of incubation at which 0.11 Units /mg protein were produced to 51.4 Units /mg protein after 48 h and decreased after 72 h. Other investigator, reported that both *Bacillus anthracis*, S-44 and *Bacillus cereus* var. mycoides, S-98 exhibited their maximum ability to biosynthesize proteases within 24 h incubation period since the productivity reached up to 126.09 U/ml for *Bacillus anthracis*, S-44 corresponding to 240.45 U/ml for *Bacillus cereus* var. mycoides, S98 respectively. Moreover, Johnvesly *et al*., (2002) found that a high level of extracellular thermostable protease activity was observed after 24 h incubation and hence the results of present study are in complete accordance with earlier reports.

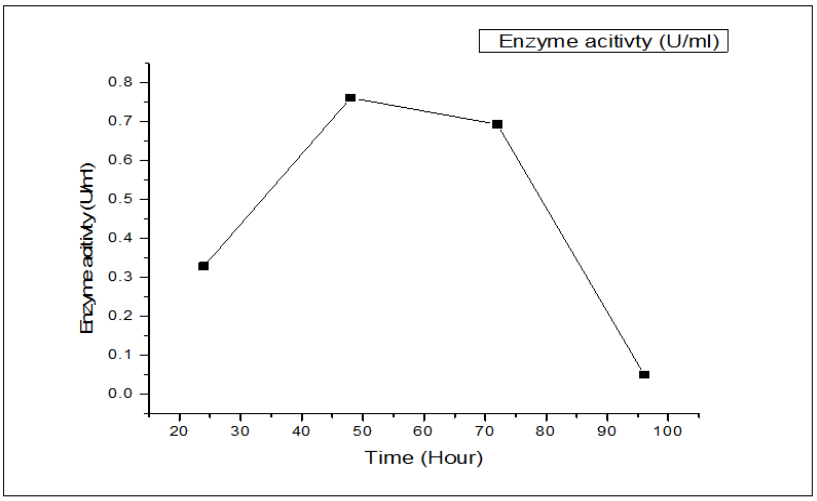

Figure. 5. Protease enzyme activity of *Bacillus licheniformis* GINM-3.

Glucose isomerase enzyme activity of the *Bacillus licheniformis* GINM 3

The glucose isomerase activity of *Bacillus licheniformis* GINM-3 was assayed in submerged culture filtrate was assayed. Glucose isomerase production was investigated at four different incubation period (24, 48, 72 and 96 hour at 37°C). The maximum activity of amylase enzymes was found 0.369U/ml at 37°C after 48 hours at pH 7. Nwokoro, 2015 reported that *Bacillus licheniformis* gave the best enzyme activity of 6.85 U/mg protein while the lowest enzyme activity of 1.02 U/mg protein was produced. The optimum pH for GI production was in the range between pH 7.0–9.0 (Lee and Zeikus, 1991). The optimum pH for GI production was slightly acidic, pH 6.9 for *Streptomyces* species (Dhungel *et al.*, 2007).

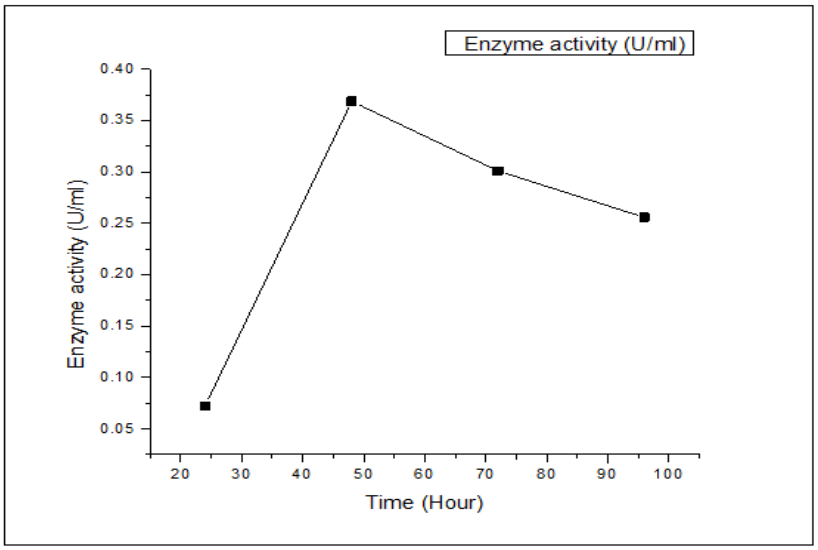

Figure. 6. Glucose isomerase enzyme activity of *Bacillus licheniformis* GINM-3.

3.5. Phylogenic tree

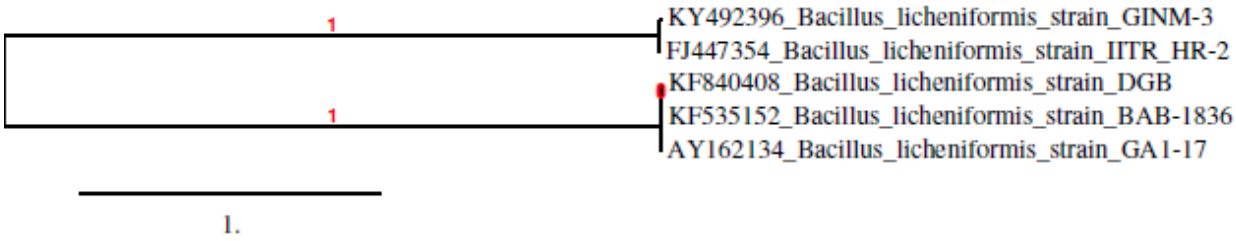

Figure. 7. Phylogenetic tree constructed based on 16S rRNA gene sequence analysis.

The sequences for the closest neighbors were used for constructing phylogenetic tree. Total five sequences available in the NCBI Gen Bank database were aligned and it was found that *Bacillus licheniformis* GINM-3 (KY492396) is clustered in one group and closely related to *B. licheniformis* group (Figure 7). The phylogenetic tree indicated that this bacterial isolate belonged to the genus *Bacillus* and the pattern of the tree determined that the strain is closely related to other *B. licheniformis* strains with 99% 16S rRNA similarity.

4. CONCLUSION

The beauty and charming of enzyme uses in industrial biotechnology are again and again exemplified. Aggregate demand for enzymes in the global market is projected to rise at a fast pace in recent years. *B. licheniformis* GINM-3 has the ability to survive under various industrial conditions and the enzymes produced have now several biological and inorganic chemical processes. Strain is currently being used for the production of different enzyme on large scale as compared to actinomycetes and fungi. Isolation and characterization of predominant *B. licheniformis* GINM-3 capable of producing various enzymes was performed. The results demonstrated that *B. licheniformis* has great potential for producing high titers of different extracellular enzymes of commercial importance.

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