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STUDY ON A NOVEL ACTINOMYCETE, *STREPTOMYCES GANCIDICUS* (MF464014) AND ENHANCING ITS TANNASE PRODUCING ABILITY BY OPTIMIZING GROWTH CONDITIONS IN SUBMERGED FERMENTATION

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ABSTRACT: Tannase (Tannin acylhydrolase E. C. 3.1.1.20) is a ubiquitous enzyme catalyzes the hydrolysis of ester and depside bonds of hydrolyzable tannins, releasing glucose and gallic acid. Gallic acid is used in the food industry as a substrate for the chemical synthesis of food preservatives such as gallates and pyrogallol, propyl gallate being a very important food antioxidant and in the pharmaceutical industry, for the synthesis of antibacterial drugs. Tannase is also utilized for bioremediation of effluents from tanneries. A number of bacteria and fungus has been reported to produce tannase, but tannase-producing actinomycetes have not been reported. So, the present study was undertaken to isolate actinomycetes with the ability to produce tannase enzyme. A total of 30 microorganisms was isolated by enrichment culture technique using tannin as carbon source. The tannase producing strain AT 9 was selected based on the zone of tannic acid hydrolysis. AT 9 was identified as *Streptomyces gancidicus* based on 16s rRNA. *Streptomyces gancidicus* showed maximum tannase production when cultured in mineral medium using 4 plugs of culture were used as inoculum size, 2% tannic acid as carbon source and ammonium chloride (0.6%) as nitrogen source. The optimum temperature and pH required for maximum enzyme production was 30 °C and 6.5 pH, respectively. Ca^{+2} plays a significant role in tannase production by *Streptomyces gancidicus*.

INTRODUCTION: Tannin acyl hydrolase (EC.3.1.1.20), commonly referred to as tannase, is an attractive biocatalyst for tannin biodegradation^{1, 2}. Tannase enzyme hydrolyses tannins as the natural substrate. These (tannins or tannase) are widely distributed in various parts of plants such as in barks, needles, heartwood, grasses, seeds, and flowers. Tannins are a group of complex oligomeric chains characterized by the presence of polyphenolic compounds.

These have a molecular weight higher than 500 and reaching up to 20,000 kDa. One of the major characteristics of tannins is its ability to form strong complexes with protein and other macromolecules such as starch, cellulose and minerals³. Tannase has shown many potential applications in different industrial sectors, like the brewing industry, food industry⁴, leather industry, in environmental pollution treatment⁵ and has been associated with colon cancer allocating the possibility of bacterial tannase as a biomarker for colon cancer⁶.

Many bacteria and fungus has been reported to produce tannase, but tannase producing actinomycetes have not been reported. So, the present study was undertaken to isolate actinomycetes with the ability to produce tannase enzyme.

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An attempt was also carried out to optimize growth medium and enzyme assay conditions for maximum production of crude tannase by *Streptomyces gancidicus*.

MATERIALS AND METHODS:

Chemicals: The chemicals used throughout the study were of analytical reagent grade and obtained from Sigma, Aldrich, and Merck.

Collection of Samples: The tannery effluent sample was collected from the Central Leather Research Institute (CLRI), GIDC (Gujarat Industrial Development Corporation), Ahmedabad, Gujarat, India in sterile polypropylene bottles. Microorganisms were isolated from the soil samples contaminated by dye, catechu, and tea waste as well as from the soil near the roots of Amla and Jamun trees and tannery effluent of CLRI.

Enrichment, Isolation, and Identification of Tannase Producing Microorganisms and Estimation of Tannase Activity: The methods of enrichment, primary and secondary screening for tannase isolates were carried out⁷. The tannase activity was estimated by a modified spectrophotometric method⁸. The potential bacterial strain was characterized by cultural, morphological (Gram Staining and growing on different media (Glucose Aspergine Agar, Nutrient agar, Czapek dox agar, Bennett's Agar, Potato Dextrose agar and Glycerol Aspergine agar)), physiological, biochemical methods. The culture was subjected to genotyping using 16s rRNA sequencing. The sequencing was carried out at Gujarat State Biotechnology Mission (GSBTM). The partial 16S rRNA sequence (1400 bp) of the isolate has been submitted to Genbank. (The highest tannase activity was shown by the strain coded AT 9, and it was identified as *Streptomyces gancidicus*).

Optimization of Tannase production by AT 9 (*Streptomyces gancidicus*) Under Submerged Fermentation: Various cultural and nutritional parameters that influenced enzyme yield during submerged fermentation (SmF) were optimized.

Study on the Effect of Inoculum Size, Temperature, and pH on Tannase Production: The activity of an enzyme is affected by its environmental conditions.

Changing these alter the rate of reaction caused by the enzyme. AT 9 cultures were grown on tannic acid agar medium and incubated for 4 days. The cultures were then transferred to broth by using a sterile cork borer. Culture disc varying from 2,3,4,5 and 6 plugs/discs were inoculated into 5 different flasks containing tannic acid (TA) broth. Broths were then incubated at 30 °C temperature for 72-96 hrs. After incubation, tannase enzyme activity was measured after every 24 h of incubation. To study the effect of temperature, TA broths were inoculated with 4 plugs of isolate AT 9 and incubated at different temperatures like Room Temperature (RT), 30 °C, 37 °C, 40 °C, and 45 °C and the tannase enzyme activity was measured by using rhodanine method. To evaluate the effect of pH on enzyme activity, the pH of the production medium was varied from 5.5, 6.0, 6.5, and 7.0 and was inoculated with 4 plugs of AT 9. The tannase enzyme activity was measured after every 24 h of incubation to 96 h.

Optimization of Nutritional Parameters for Production of Tannase Enzyme: Effect of different concentrations of inducers like Tannic Acid, different carbon and nitrogen sources on Tannase production was carried out. To study the role of inducer tannic acid in the production of tannase, 4 plugs/culture discs of AT 9 from the edge of an actively growing 4 days old colony were inoculated into four different flasks with different concentrations of Tannic acid (1% to 4%) of 100 ml of TA media in 250 ml of a flask. Enzyme activity was measured after every 24 h of incubation. Several Carbohydrates like Glucose, Fructose, Sucrose, and Lactose were incorporated at 0.5% (w/v) concentration into TA broth to study the role of carbon sources. Tannase enzyme activity was measured after every 24 h of incubation to 96 h. The role of organic and inorganic nitrogen sources in the induction of tannase enzyme production was also studied. Different Inorganic nitrogen sources like NH₄Cl, NaNO₃ (0.6%), and organic nitrogen sources like Peptone, yeast extract, and Urea (0.6%) were used in TA broth. Tannase enzyme activity was measured after every 24 h of incubation.

Effect of Metal Ions on Tannase Activity: Various metals play a major role in tannase activity. They act as inhibitors or stimulators. Metal

ions like Mg^{+2} , Mn^{+2} , Ca^{+2} , Na^{+} and K^{+} stimulated the tannase activity, while Cu^{+2} , Fe^{+3} , and Co^{+2} acted as inhibitors of the enzyme. Different metal ions like $ZnSO_4$, $MnSO_4$, $CuSO_4$, and $CaCl_2$ with 1 mM concentration were added in TA broth, and their roles in tannase enzyme produced by the isolate were studied.

RESULTS AND DISCUSSION:

Primary/Qualitative Screening of Bacterial Isolates for Tannase Production: A total of 30 microbes were isolated from the tannery waste by serial dilution and plating on mineral salt agar medium (supplemented with tannic acid as a sole source of carbon) of these 30 isolates, 15 tannase producers were selected on the basis of tannic acid hydrolysis zone formation. The zone formed by isolates on Tannic Acid Agar (TAA) plates were not very clear and was not differentiable between tannase producing and non-tannase producing bacteria. Many researchers^{9, 10, 11} reported about tannase screening methods, but all the methods are time-consuming and less sensitive, which have made these limited towards specificity determination and the isolation and screening of bacteria. To overcome this, the TAA plates were flooded with Gram's iodine instead of $FeCl_3$. Gram's iodine formed a dark brown complex with tannic acid but not with hydrolyzed tannic acid and giving a sharp, distinct zone around the

tannase producing microbial colonies even in cases of low levels of tannase production within 3-5 minutes. In the present technique, gram's iodine reacted with the tannin protein complex, yielding a brown to black color, providing a dark background allowing clear visibility of zones of tannic acid hydrolysis **Fig. 1A**. Actinomycetes isolate AT 9 which showed a maximum (19 mm) diameter of zone of hydrolysis, was selected for further studies.

Effect of Incubation Period on the Production of Tannase: The incubation period is one of the most critical parameters in the optimization state of affairs. Tannase enzyme production by AT 9 was determined by tannase enzyme assay. Higher tannase production of the isolate AT 9 was observed after 72 h of incubation period. The enzyme activity of AT 9 was 4.5 ± 0.02 U/ml/min at 72 h.

Production of Tannase by *P. verrucosum* using coffee pulp as substrate and obtained maximum tannase production at 96 h of fermentation period¹².

Identification of Bacterial Isolates:

Morphological Characteristics of AT 9: AT 9 formed a white, powder colony on the TAA plate. Gram's staining and microscopic view revealed a gram-positive, filamentous structure **Fig. 1B**.

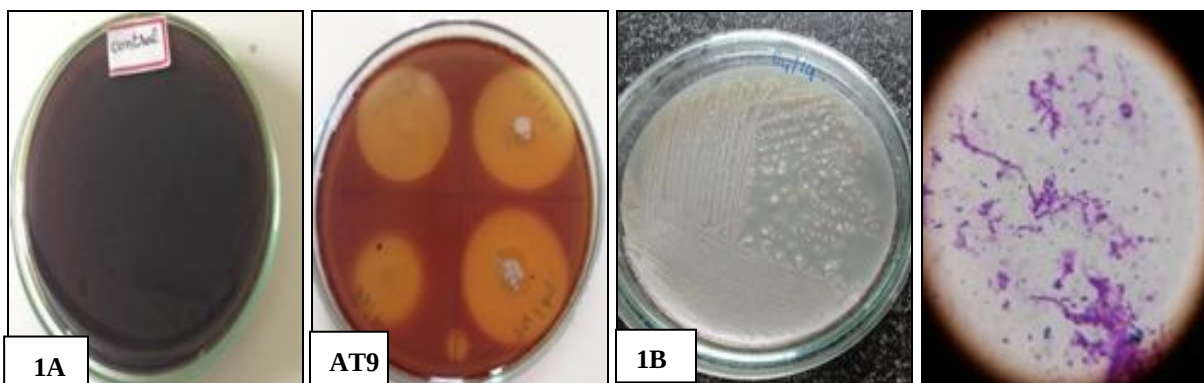


FIG. 1: (A) ZONE OF TANNIC ACID HYDROLYSIS BY VARIOUS ISOLATES ON TAA PLATE (B) GROWTH OF AT 9 ON TAA PLATE AND MICROSCOPIC VIEW OF AT 9 AFTER GRAM STAINING (1000X)

Culture Characteristics of AT 9 on Different Media: AT 9 was grown on different media (Glucose Asparagin Agar, Nutrient Agar, Czapek Dox Agar, Bennett's Agar, Potato Dextrose Agar, and Glycerol Asparagine Agar) to study the growth pattern. A comparison of the growth of isolate on different media is shown in **Fig. 2**. The color of

aerial mycelium of AT 9 was found white on Nutrient Agar, Potato Dextrose Agar, and Czapek Dox Agar. In Glucose Asparagine Agar, AT 9 gave grey mycelium color. The aerial mycelium of AT 9 in Glycerol Asparagine Agar was dark brown in color.

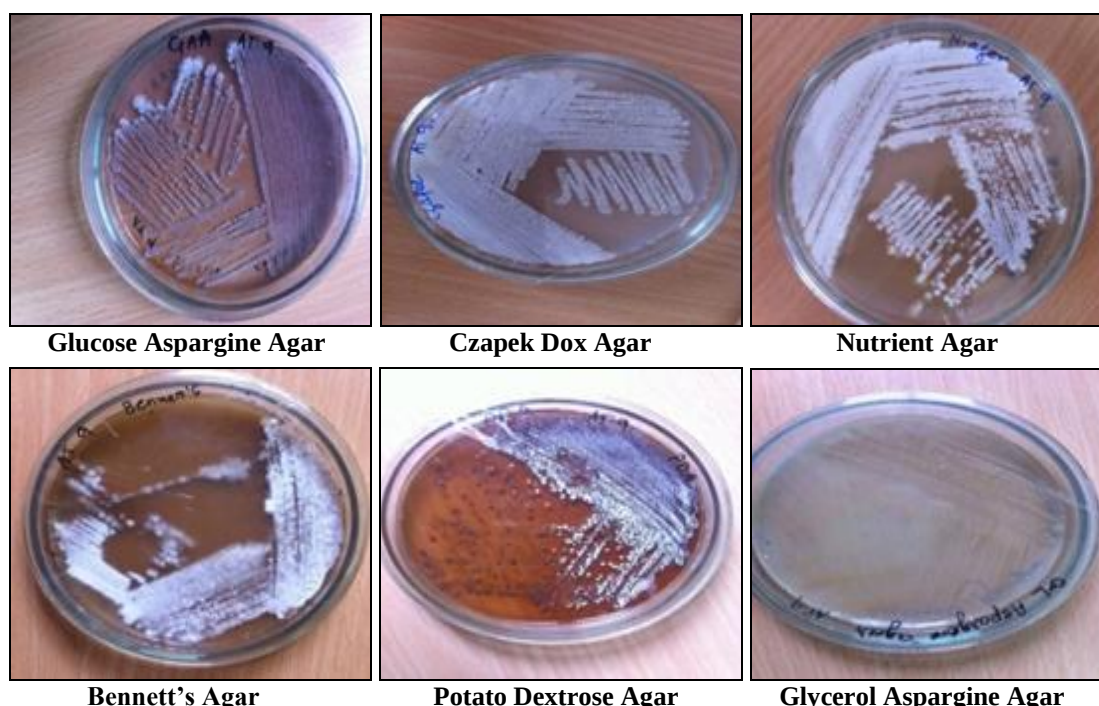


FIG. 2: GROWTH OF ISOLATE AT 9 ON DIFFERENT MEDIA

Biochemical Characteristics of Isolate AT 9: The biochemical characterization of AT 9 was carried out for identification purposes. AT 9 cultures utilized arabinose and gave good growth in the medium. No growth was observed with sucrose, mannitol, and Trehalose, glucose. Cellulase, Lipase and xylanase production were observed by the culture. Nitrate reduction and arginine utilization were positive, while Citrate utilization and ONPG utilization, malonate utilization were negative.

Characterization of Bacterial isolate by 16s rRNA Sequencing: The isolate AT 9 showing tannase production was identified at GSBTM by sequencing, the obtained sequences of tannase producing isolate were compared with deposited

sequences in NCBI database, using BLAST as a search tool.

In most cases, the high identity values obtained allowed us to assure that AT 9 isolate belongs to *Streptomyces* spp. with identity values 100 %. An evolutionary study done by using the 16S rRNA sequence for actinomycetes reveals that in the phylogenetic tree the position of AT 9 is nearby the *Streptomyces gancidicus*.

The sequence data of isolate AT 9 was deposited in the Gene bank Database under the accession number MF464014. The phylogenetic tree was constructed via the neighbor-joining algorithm using the Blast package **Fig. 3**.

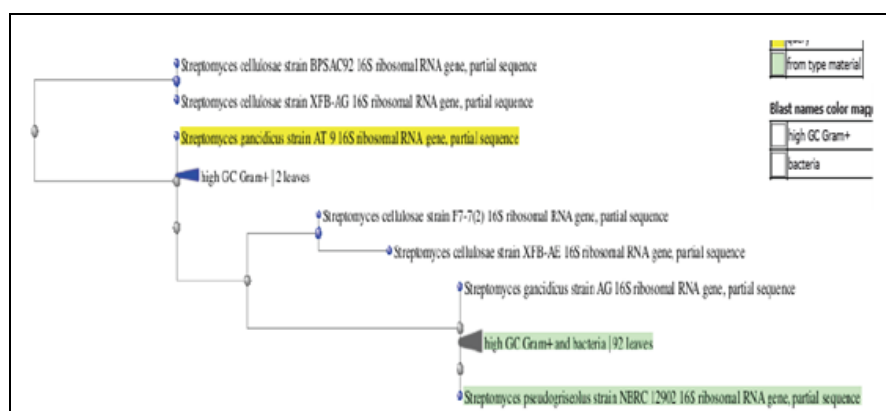


FIG. 3: UNROOTED TREE SHOWING THE PHYLOGENETIC RELATIONSHIPS OF MEMBERS OF THE GENUS *STREPTOMYCES* AND SOME RELATED SPECIES. THE TREE, CONSTRUCTED BY USING THE NEIGHBOR-JOINING METHOD

Optimization Studies:

Optimization of Physicochemical Parameters to Maximize the Production of Tannase by *Streptomyces ganncidicus*:

Effect of Inoculum Size on Tannase Activity:

Tannase activity of *Streptomyces ganncidicus* was maximum when 4 plugs of culture were added to inoculation medium (TA Broth).

The enzyme activity of the bacterium at 24 h was 3.9 U/ml/min. The activity decreased after 48 h and again increased after 72 h. The results are represented in **Fig. 4**.

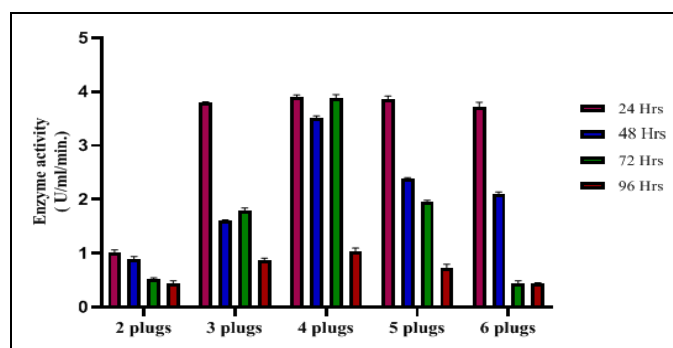


FIG. 4: EFFECT OF INOCULUMS SIZE ON TANNASE ACTIVITY BY *STREPTOMYCES GANNCIDICUS* EFFECT OF TEMPERATURE, pH, TANNIC ACID CONCENTRATION, METAL IONS ON TANNASE ACTIVITY

Temperature is also another critical factor that needs to be controlled and optimized. The significance of the optimum temperature in the development of the fermentation process is such that it could determine the inhibition of growth, cell

viability, and death. In general, an increase in the temperature of the fermenting medium due to respiration. The highest Tannase activity was observed at 30 °C (3.55 U/ml) at 72 h of incubation, it indicating maximum production of tannase enzyme by *Streptomyces ganncidicus* cultures at 30 °C **Fig. 5A** The activity decreased as the temperature increased.¹³

Tannase production by microbes and its enzyme activity is affected by the pH. Maximum enzyme production was observed at pH 6.5 after 72 h, and the enzyme activity was 2.58 U/ml/min. The activity decreased by 96 h. It was also observed that though acidic pH is more favorable for tannase production. The optimum initial pH reported for tannase production was in the acidic pH range of 4.5 to 6.5.^{14, 15, 16, 17, 18}. The results are represented in **Fig. 5B**. The experiments were conducted using different concentrations of tannic acid (1%, 2%, 3 %, and 4 %) in the TA broth.

Tannase production increased by 2% tannic acid concentration, it was 3.39 U/ml/min after 72 h of incubation, and it was further decreased, or the concentration of tannic acid measured. The results are presented in **Fig. 5C**. Metals play a major role as cofactors in enzymes. **Fig. 5D** showed the tannase activity of AT 9 (*Streptomyces ganncidicus*) when different metals at a concentration of 1 mM was added to TA broth.

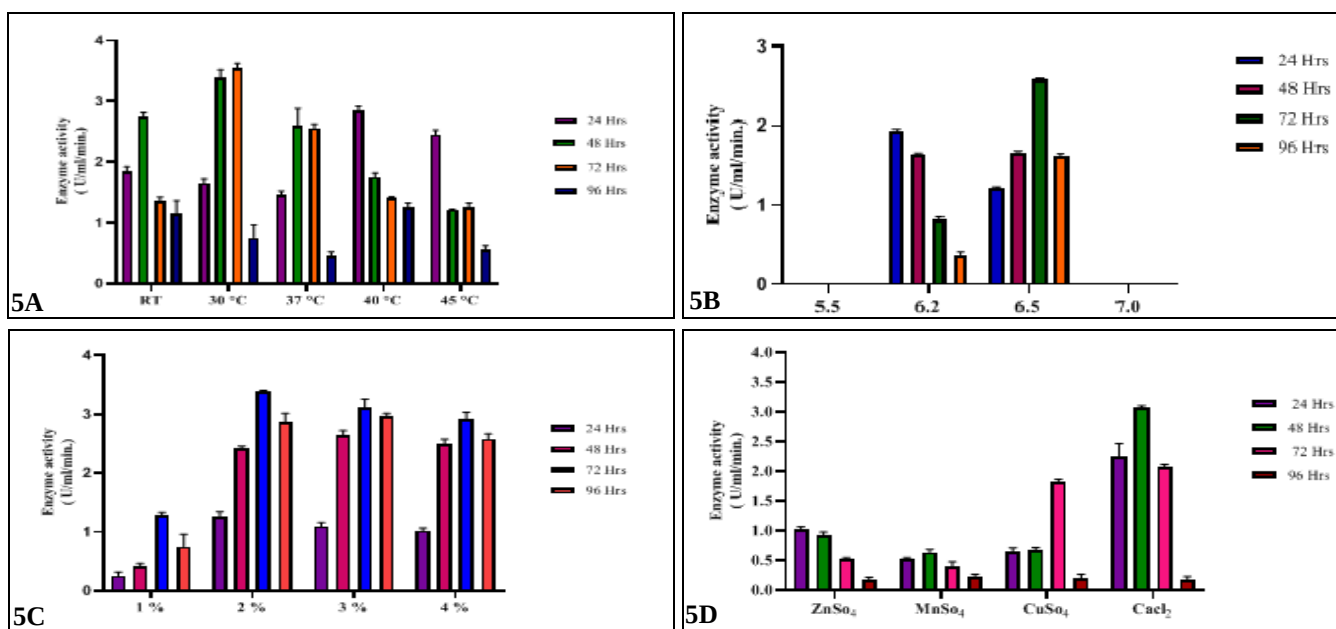


FIG. 5: EFFECT OF TEMPERATURE (5A), pH (5B) TANNIC ACID CONCENTRATION (5C) AND METAL IONS (5D) ON TANNASE ACTIVITY

From all the metal ions, the addition of CaCl_2 in TA broth induced maximum tannase production, and the production was maximum after 72 h of incubation (3.060 U/ml/min).

The results thus show that Ca^{+2} is an inducer for tannase production and Mn^{2+} is acting as an inhibitor for tannase production. CaCl_2 to be the most suitable for tannase production by *Aspergillus japonicus* as compared to the other used mineral salts (NaCl , KCl , CuSO_4 , MnSO_4 , FeSO_4 , ZnSO_4 and CdSO_4)¹⁹. The inhibitory effect of heavy metal ions is well documented in the literature¹⁸. Further, the decreased activity in the presence of divalent cations could be due to the nonspecific binding or aggregation of the enzyme. Tannase from *A. oryzae* and *P. chrysogenum* were heavily inhibited by Zn^{2+} , Cu^{2+} , and Fe^{2+} .²⁰

Effect of Carbon and Nitrogen Sources on Tannase Production: In submerged fermentation, the concentration of tannic acid was found to be a

crucial factor influencing the levels of enzymes it is evident from **Fig. 6A** that only tannic acid supported the significantly higher value of tannase activity (6.27 U/ml/min). Other carbon sources like lactose, glucose, sucrose and fructose did not show any significant effect on tannase production. Thus, from the results, it was evident that only tannic acid serves as a better carbon source for tannase production.

To determine the best nitrogen source for tannase production, Sodium nitrate (0.6% w/v) was replaced with different inorganic and organic nitrogen sources in the production medium. All the nitrogen sources used % equivalent nitrogen similar to that of 0.6% (w/v) sodium nitrate, and the results are presented in **Fig. 6B**. In the present study, the maximum tannase activity was exhibited by ammonium chloride at 48 h of incubation with 9.64 U/ml/min tannase activities. The report is similar to *S. marcescens*, which gave 0.75 U/mL of enzyme activity²¹.

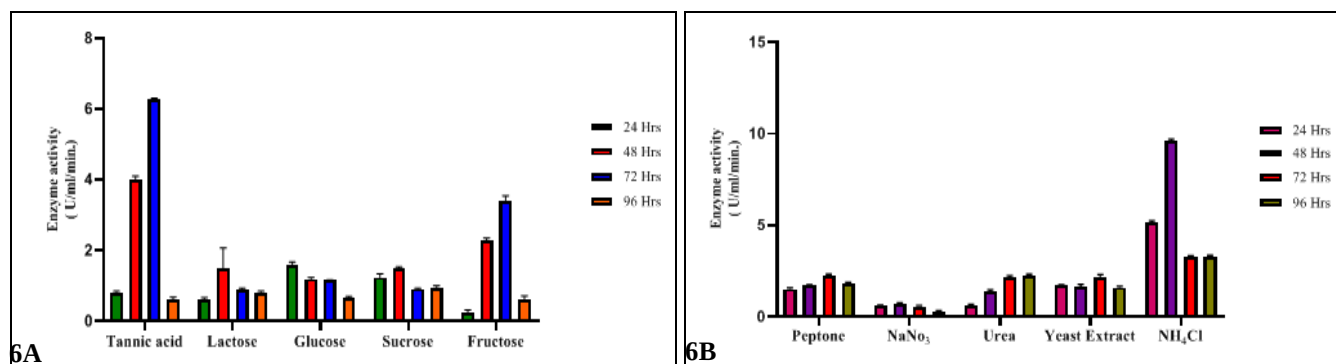


FIG. 6: EFFECT OF DIFFERENT CARBON (6A) AND NITROGEN SOURCES (6B) ON TANNASE ACTIVITY BY *STREPTOMYCES GANNCIDICUS*

CONCLUSION: A tannase producing actinomycetes AT 9 was selected from the isolates obtained from soil samples collected from CLRI located in Naroda, Ahmedabad, Gujarat, on the basis of the zone of hydrolysis formed on TAA agar plates. The AT 9 was identified as *Streptomyces gancidicus*, Gen Bank Accession Number MF 464014.

Cellulase, lipase, and Xylanase were also produced by *Streptomyces gancidicus*. Optimization of various nutritional and physical parameters for maximum tannase production under submerged fermentation by *Streptomyces gancidicus*. During optimization, the maximum tannase activity was obtained in 4 plugs of culture as inoculum size, 2%

tannic acid as carbon source, and peptone (0.6%) as a nitrogen source. The optimum temperature for maximum enzyme production was at 30 °C and 6.5 pH, respectively. Ca^{+2} was significant for tannase production by *Streptomyces gancidicus*.

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CONFLICTS OF INTEREST: There are no conflicts of interest.

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