**RESEARCH PAPER**

# Screening and Isolation of Mutanase producing microorganism from different soil samples and teeth caries specimens

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**ABSTRACT**
The enzymatic degradation of microbial biofilms, particularly those formed by cariogenic bacteria, has attracted substantial interest in recent times due to its potential applications in oral health as well as industrial biotechnology. Mutanase, a specialized enzyme capable of hydrolyzing α-1,3-glucans (commonly referred to as mutans), plays a pivotal role in disrupting biofilms, especially those associated with dental caries. These biofilms are primarily composed of extracellular polysaccharides synthesized by Streptococcus mutans and related species, which contribute to tooth decay and other oral diseases. The identification and isolation of mutanase-producing microorganisms from natural environments, such as soil and dental caries specimens, is therefore a critical step in advancing biofilm control strategies.
Microbial enzymes, including mutanase, are often sourced from diverse ecological niches due to the adaptability and metabolic versatility of microorganisms. Soil, a rich reservoir of microbial diversity, harbors numerous species capable of producing extracellular enzymes to degrade complex polysaccharides. Similarly, dental caries specimens provide a unique microenvironment where microorganisms are exposed to mutans-rich biofilms, potentially driving the evolution of mutanase-producing strains. Screening these environments for mutanase producers not only aids in understanding microbial ecology but also facilitates the discovery of novel enzymes with enhanced activity and stability for therapeutic and industrial applications.
The significance of mutanase extends beyond oral health. In industrial biotechnology, mutanase is employed in processes such as the modification of glucans in food, pharmaceuticals, and bioethanol production. Furthermore, its application in biofilm disruption has implications for medical device sterilization and the prevention of biofouling in water treatment systems. These diverse applications underscore the importance of isolating and characterizing mutanase-producing microorganisms from natural sources.
Recent advancements in microbial screening techniques, including selective enrichment, high-throughput assays, and molecular characterization, have streamlined the identification of enzyme-producing strains. By leveraging these methods, researchers can efficiently isolate and evaluate microorganisms capable of producing mutanase under various environmental conditions. Studies have demonstrated that combining traditional microbiological approaches with modern molecular tools enhances the likelihood of discovering novel strains with superior enzymatic properties.
This report focuses on the systematic screening and isolation of mutanase-producing microorganisms from two distinct sources: soil samples and dental caries specimens. By exploring these environments, the study aims to identify potential microbial candidates for further enzymatic characterization and application development. Collection and Preparation of Soil and Teeth Caries Samples. Mutanases, are also called a-1,3-Glucan 3-glucanohydrolases; they demonstrate tremendous promise as dental caries-prevention medicines since mutanases hydrolyze the glucans that are water-insoluble found in dental plaque. Nevertheless, due to the lack of enzymatic preparations appropriate for oral usage, the use of mutanases has not been particularly successful economically. In the present study Mutanase producing microorganisms were isolated on mutanase producing agar medium. Qualitative determination of Mutanase was done by plate screening method using congo red as indicator. Positive cultures were further screened quantitatively in liquid medium using mutan as a substrate. Bacterial isolate (AK 27) gave maximum mutanase production that is 0.68 U/ml over 72 hours incubation period
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## INTRODUCTION

In present time, food waste and microbes combine to generate dental caries and dental plaque, the most prevalent dental illnesses worldwide. Dental caries is a common and expensive disease globally, though it is not usually fatal, it poses a significant challenge to healthcare providers (Forssten *et al*., 2010). Majorly cause of dental caries is bacterial infection caused by *Staphylococcus* species,

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*Eubacterium* species, *Neisseria* species, *Actinomyces* species, *Peptostreptococcus* species, *Micrococci* species, and so forth. Among them in the oral cavity, *streptococci* alone constitute the largest association (Daboor *et al*., 2015).

Dental plaques or bacterial biofilms are created when *Streptococcus mutans* and other bacteria are present on the surface of teeth. Dental plaque typically contains 30% water-soluble and 70% water-insoluble components, respectively. The water insoluble glucan polymer (Mutan) plays major role for developing dental plaque and dental disease (Quivey & Kriger, 1993). Mutan, a polysaccharide is basically synthesized by glucosyltransferases of oral *Streptococcal* bacteria and dietary sucrose (Wiater *et al*., 2005).

Mutanase is a family of Glucanohydrolase. It is a Polysaccharide-hydrolysing enzyme Hydrolyses α-(1→3) bonds present in mutan, which is mainly associated with water insolubility (Guggenheim *et al*., 1980).

Mutanase breaks down mutan produced by *Streptococcus.* Therefore, mutanase prevents the production of dental plaque by either inhibiting mutan or encouraging its breakdown into low-molecular-weight glucans. Because it is essential for preventing dental caries, it is capable of helping to treat dental plaque (Wiater *et al*., 2005).

**Isolation of Microorganisms Using Selective Media**

The isolation of mutanase-producing microorganisms begins with the use of selective media tailored to enrich and identify strains capable of degrading mutans. Unlike the existing content, which discusses enrichment cultures broadly, this section focuses specifically on the composition and role of selective media in isolating mutanase producers. Selective media was designed to include mutans or dextran as the sole carbon source, forcing microorganisms to utilize these polysaccharides for growth. For example, basal media supplemented with 1% mutans can be used to isolate mutanase producers. The media was set to 6.5–7.5 to mimic the natural environment of soil or oral cavities. The plates were incubated at 30°C for a duration of 48 to 72 hours under either aerobic or anaerobic conditions, contingent upon the suspected microbial group. Colonies that exhibited clear zones surrounding them, indicative of polysaccharide degradation, were selected for subsequent analysis. To enhance the specificity of isolation, antibiotics such as ampicillin or cycloheximide can be added to suppress the growth of non-target bacteria or fungi, respectively. This step ensures that only microorganisms capable of mutan degradation thrive. This approach differs from the previously described enrichment cultures by emphasizing the role of antibiotics and specific carbon sources in selective media.

**High-Throughput Screening for Mutanase Activity**

Once isolated, microorganisms were subjected to high-throughput screening to identify those with significant mutanase activity. This section introduces a novel methodology not covered in existing content: the use of microplate assays for rapid screening.

Microplate assays involve culturing isolated strains in 96-well plates containing liquid media supplemented with mutans. After incubation, the supernatant is collected and assayed for mutanase activity using a colorimetric method. For instance, the 3,5-Dinitrosalicylic acid (DNS) assay can be employed to quantify reducing sugars released during mutan degradation. The intensity of the color change, determined at 540 nm using a multimode microplate reader, correlates with enzymatic activity. Strains exhibiting the highest activity were selected for further characterization. This method is advantageous because it allows for the simultaneous screening of hundreds of isolates, significantly accelerating the identification process. Additionally, the use of microplate readers ensures high sensitivity and reproducibility, making it a valuable tool for large-scale studies.

**Molecular Characterization of Mutanase-Producing Strains**

After identifying potential mutanase producers, molecular techniques were employed to characterize the strains and confirm their enzymatic capabilities. This section focuses on genetic analyses addressed in existing content.

**16S rRNA Sequencing**

The 16S rRNA gene is amplified using polymerase chain reaction (PCR) and sequenced to identify bacterial isolates at the species level. Specific primers, such as 27F and 1492R, were used for amplification. The resulting sequences were compared against databases like the National Center for Biotechnology Information (NCBI) using BLAST to determine phylogenetic relationships. For fungal isolates, internal transcribed spacer (ITS) sequencing is performed using primers such as ITS1 and ITS4.

The current study's objective is to isolate mutanase-producing microorganisms that aid in the elimination of insoluble glucans from biofilms in the oral cavity made by dieses causing microbes.

**Material and Method**

**Collecting soil samples**

The sampling of soil was done from waste dumped at paper mills located at different areas of Gujarat (Table 1), using clean sterile spatula in a sterile tube from the depth of 1 cm from soil surface.

**Drying and Sieving**: Soil samples were air-dried at room temperature to reduce moisture content. Large debris, such as stones and plant material, was removed by sieving through a 2 mm mesh.

**Serial Dilution**: A 1 g aliquot of soil was taken in 9 mL of sterile phosphate-buffered saline (PBS) or saline, and vortexed to dislodge microorganisms. Serial dilutions (e.g., 10^-1 to 10^-6) were prepared to reduce microbial density for plating.

**Enrichment Cultures**: To enhance the isolation of mutanase-producing microorganisms, enrichment cultures were established by inoculating soil suspensions into media containing dextran or mutans as the sole carbon source. These cultures were incubated at 30°C for 48–72 hours under aerobic or anaerobic conditions, depending on the target microbial group.

Table 1: Soil samples collected from waste dumped at paper mills in various locations of Gujarat.

| Sr. no. | Sample ID | Location |
|---|---|---|
| 1 | Soil sample - 1 | Fortune taxcone Paper Mill, Trasad, Dholka, Gujarat. |
| 2 | Soil sample - 2 | |
| 3 | Soil sample - 3 | Laxmi PaperFactory, Ahmedabad, Gujarat. |
| 4 | Soil sample - 4 | |
| 5 | Soil sample - 5 | Karan Paper Mills, Chhatral, Gujarat. |
| 6 | Soil sample - 6 | |

**Collecting dental samples infected with caries:**

Teeth with various levels of caries were gathered from different dental clinics located at Dholka. A total of eleven caries-ridden teeth were collected from Dholka's dental clinics (Fig. 1). The sample was chosen at random, with no consideration given to the patients' age, gender, race, or socioeconomic situation.

Teeth Caries Sample Collection: Clinical Sampling Protocols

Teeth caries samples were collected from individuals with active dental caries. The sampling process involves the following steps:

Patient Selection: Individuals with visible caries lesions were selected. Ethical approval and informed consent were obtained before sample collection.

Sterile Sampling: A sterile dental excavator or probe was used to scrape carious material from the lesion. Care was taken to avoid contamination with saliva or other oral tissues.

Sample Storage: The collected material was transferred to sterile containers that contained sterile phosphate buffered saline pH 7.0 (Fig. 3). The samples were stored at 4°C and processed within 24 hours to maintain microbial viability.

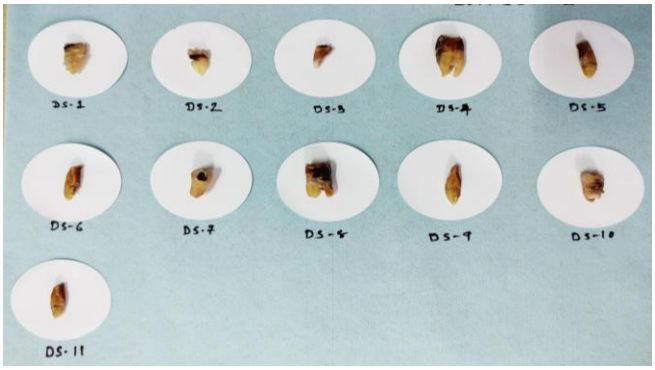



**Fig. 1: Caries infected teeth sample**

**Isolation of culture from Soil samples:**

The isolation of microbial cultures from soil samples was a fundamental technique in environmental microbiology and soil ecology. This process begins with the collection of a soil specimen, which was mixed with purified water to create a suspension. The suspension undergoes a series of serial dilutions, which was starting from 10^-1 to 10^-6, to lower the microbial concentration and allow for the growth of individual colonies. These diluted samples were then spread onto specific growth media: potato dextrose agar (PDA) for fungi and nutrient agar was used for bacteria.

The inoculated plates were incubated under conditions that favor the growth of the target microorganisms. Bacterial cultures were typically incubated at 37°C on nutrient agar, while fungal cultures were grown at a lower temperature of 30°C on PDA. After incubating for 3-5 days, resulting microbial colonies were sub cultured onto fresh, specific media to obtain pure isolates. For bacterial isolates, additional confirmation techniques such as Gram staining was employed to further characterize the microorganisms. This methodical approach allows researchers to isolate and identify a diverse range of soil microbes, providing valuable insights into soil microbial communities and their potential applications in various fields, including agriculture, bioremediation, and biotechnology. 1 gm of soil specimen was taken and mixed well. The soil was allowed to settle dawn and the resultant suspension was subjected to serial dilutions from $10^{-1}$ to $10^{-6}$. From each dilution, 1ml of sample was spread on potato dextrose agar plate (PDA) and nutrient agar plate. Bacterial cultures were obtained using nutrient agar plates incubated at 37°C, while fungal cultures were isolated using PDA at 30°C for 3-5 days. After incubation the micro-organisms were sub-cultured on specific culture medium (Fig. 4). For further confirmation of bacterial isolates, gram staining was performed (Fig. 5).

**Isolation of culture from dental samples infected with caries:**

The process of isolating and culturing microorganisms from samples involves several steps to ensure proper growth and identification. Initially, a small volume (0.1 mL) of each sample suspension was spread onto two different types of culture media: nutrient agar for bacterial growth and potato dextrose agar (PDA) for fungal growth. These media provide the necessary nutrients and environmental conditions for the respective microorganisms to thrive. The plates were then incubated at specific temperatures to promote optimal growth: 37°C for bacterial cultures on nutrient agar and 30°C for fungal cultures on PDA. The incubation period typically lasts 3-5 days, allowing sufficient time for visible colonies to form.

Following the initial incubation, the isolated microorganisms were sub-cultured onto specific culture media for further studies. This step allows for the separation and purification of individual microbial strains, enabling more detailed analysis and characterization. For bacterial isolates, an additional confirmation step was performed using Gram staining. This technique helps differentiate bacteria based on their cell wall composition, providing valuable information for classification and identification. The entire process, from initial plating to sub-culturing and staining, was crucial for accurately isolating and identifying microorganisms from environmental or clinical samples, forming the foundation for subsequent microbiological investigations. A 0.1 mL of each sample suspension (see 2.2) of was spread onto plates of nutrient agar and PDA. The incubation of nutrient agar plate was done at 37ºC for

bacterial culture isolation and PDA for fungal isolation at 30ºC for 3-5 days. The micro-organisms were then sub-cultured on specific culture medium for further studies (Fig. 4). Gram staining was performed for further confirmation for bacterial isolates (Fig. 5).

**Primary screening of isolates for mutanase production**

The isolation of mutanase-producing microorganisms begins with the use of selective media tailored to enrich and identify strains capable of degrading mutans. Unlike the existing content, which discusses enrichment cultures broadly, this section focuses specifically on the composition and role of selective media in isolating mutanase producers. Selective media were designed to include mutans or dextran as the sole carbon source, forcing microorganisms to utilize these polysaccharides for growth.

The isolated organisms were grown on 1% mutan containing nutrient agar medium. Incubation of plates was done at 37ºC for 7 days. Following incubation, plates were treated with 0.1% of Congo red dye allowing to sit for 15 min. at the room temperature. All plates were then washed using 10 ml of a 1M NaCl solution. Mutanase activity was seen by a clear zone of congo red decolorization surrounding the microbial colony. The best isolates were selected for further studies (Sudheer *et al*., 2016).

**Secondary Screening of mutanase producing micro-organisms:**

The secondary screening of mutanase-producing microorganisms involves a more detailed evaluation of the best isolates identified during primary screening. This process begins with the cultivation of selected isolates in a nutrient-rich seed medium, typically 50 ml of nutrient broth. After initial growth, 1 ml of this seed culture was transferred to a larger volume (150 ml) of specialized mutanase-producing medium. This medium was carefully formulated to promote mutanase production and contains essential components such as mutan, yeast extract, peptone, various salts (KH2PO4, KNO3, MgSO4.7H2O, NH4Cl), and inositol, with the pH set to 7.0 ± 0.2.

The inoculated flasks were then incubated under controlled conditions, typically at 30ºC with continuous shaking at 150 rpm to ensure proper aeration and mixing. To monitor mutanase production, samples were collected at 24-hour intervals. These samples undergo centrifugation to separate the cells from the culture broth, yielding a clear supernatant. The supernatant was then used to conduct enzyme assays, which quantify the mutanase activity produced by the microorganisms. This systematic approach allows researchers to identify the most promising mutanase-producing strains and optimize cultivation conditions for enhanced enzyme production (Buddana SK., 2016).

**Mutanase enzyme assay:**

The mutanase enzyme assay described involves a time-course study to measure enzyme activity over an extended period, from 12 to 120 hours. This approach allows for the observation of enzyme kinetics and potential changes in activity over time. The assay utilizes cell-free broth, likely obtained from a microbial culture, as the source of the mutanase enzyme. The substrate, mutan, was suspended in a sodium acetate buffer at pH 5.5, providing optimal conditions for enzyme activity. The reaction was conducted at 50°C temperature for 30 minutes, allowing sufficient time for the enzyme to catalyze the breakdown of mutan into reducing sugars.

The Nelson-Somogyi method, a colorimetric assay, was employed to quantify the reducing sugars produced as a result of mutanase activity. This method was widely used for its sensitivity and reliability in detecting small amounts of reducing sugars. The enzyme activity was quantified in units (U). The standardized definition for enzyme activity quantification allows for comparison of mutanase activity across different experiments and studies, facilitating reproducibility and consistency in enzyme characterization (Nelson, N., 1944., Buddana *et al*., 2019).

One unit of enzyme activity = Under the specified conditions, the amount of enzyme that catalysed for the release of reducing sugar is equivalent to 1µmol/min. (Nelson, N., 1944., Buddana *et al*., 2019).

**Identification of mutanase producer AK 27:**

**Morphological examination of isolate**

Morphological characteristics of the pure isolate AK 27 were studied by phenotypic identification on nutrient agar. Microscopic analysis was done by gram's staining and observed in a light microscope under high power (100X).

**Molecular examination of isolate**

The cetyl trimethylammonium bromide (CTAB) method, a widely used technique for DNA extraction, was employed to isolate the genomic DNA from the AK 27 culture. This method, as described by Wilson (2001), is particularly effective for isolating high-quality DNA from various organisms, including bacteria. Following DNA extraction, the 16S rRNA gene, a highly conserved region in bacterial genomes, was amplified using polymerase chain reaction (PCR). This amplification step was crucial for obtaining sufficient quantities of the target gene for subsequent analysis.

The amplified 16S rRNA gene sequence was then submitted to the Gene Bank Database, a comprehensive repository of genetic sequences. To determine the taxonomic identity of the AK 27 strain, the sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI). BLAST compares the submitted sequence against a vast database of known sequences, allowing for the identification of closely related species based on nucleotide similarities. Finally, a phylogenetic tree was constructed using the maximum likelihood method, as described by Kimura (1980), utilizing MEGA 5.05 software. This tree visually represents the evolutionary relationships between the AK 27 strain and other related species, providing insights into its taxonomic position and potential functional characteristics.

## RESULT & DISCUSSION

**Isolation of microorganisms from soil sample:**

Six soil specimens were taken from waste that was disposed of at various paper mills (Fig. 2). From soil samples about 24 different bacterial and 3 fungal isolates were obtained.

**Fig. 2: Processing of soil samples collected from waste dumped at different paper mills.**

**Isolation of microorganisms from dental samples infected with caries:**

11 distinct caries-infected teeth samples were acquired from various dental clinics shown in Fig. 1. Eight bacterial strains were isolated on Nutrient agar media after 24 Hrs of incubation.

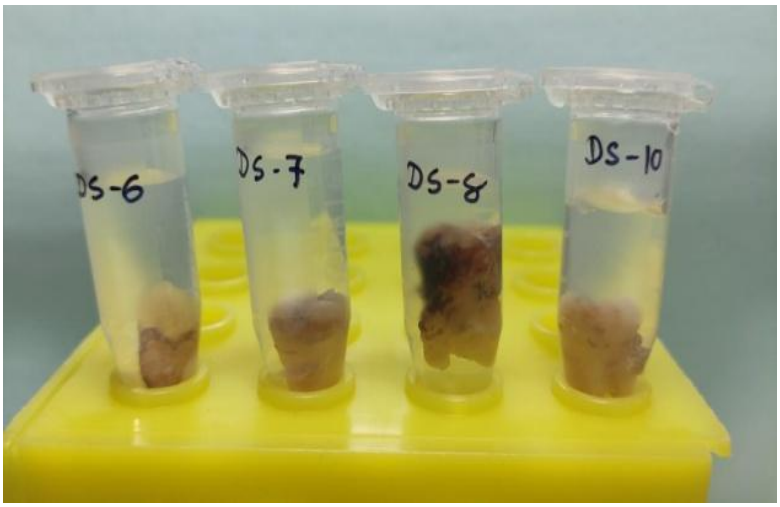



**Fig. 3: Processing of dental samples**

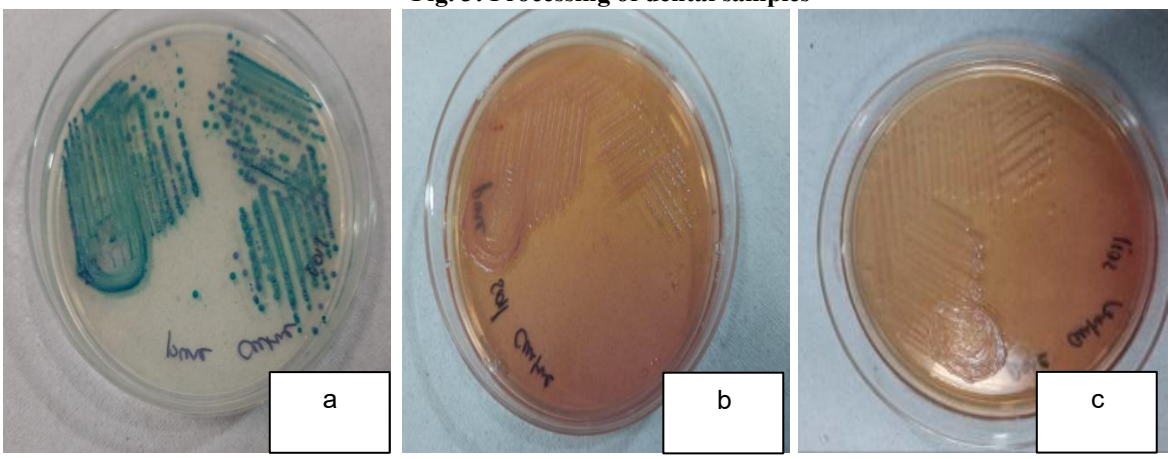



**Fig. 4: Growth of micro-organisms on specific media like Hychrome agar (a) and MacConkey agar plates (b and c).**

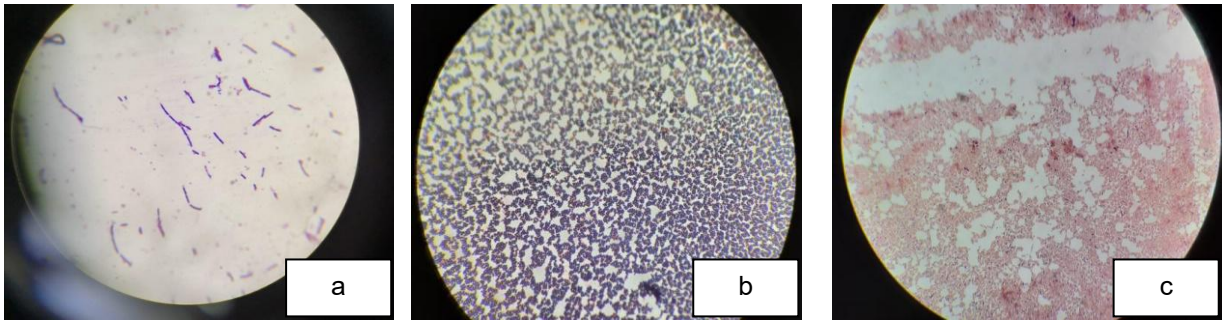



**Fig. 5: Microscopic observation of gram staining for different type of bacterial isolates including Gram positive bacilli (a), Gram positive cocci (b) and Gram negative cocci (c).**

**Primary screening of Mutanase producing micro-organisms**

Mutanase production was assessed quantitatively using method of selective plate. To screen the 32 chosen isolates, mutan-enriched medium was used. On addition of congo red dye, 8 strains of bacteria showed good result. Among 8 bacterial strains, 3 bacterial cultures (AK 04, AK 10 and AK 27) were showing higher area of hydrolysis compared to the others (Table 2, Fig. 6).

**Table 2: Amount of congo red decolorization by 32 bacterial isolates**

| Sr.no. | Amount of Mutan hydrolysis | Sr.no. | Amount of Mutan hydrolysis |
|---|---|---|---|
| AK 01 | - | AK 17 | - |
| AK 02 | ++ | AK 18 | - |
| AK 03 | - | AK 19 | - |
| AK 04 | +++ | AK 20 | ++ |
| AK 05 | - | AK 21 | - |
| AK 06 | - | AK 22 | - |
| AK 07 | ++ | AK 23 | - |
| AK 08 | - | AK 24 | - |
| AK 09 | - | AK 25 | - |
| AK 10 | +++ | AK 26 | ++ |
| AK 11 | - | AK 27 | +++ |
| AK 12 | - | AK 28 | - |
| AK 13 | - | AK 29 | - |
| AK 14 | - | AK 30 | - |
| AK 15 | - | AK 31 | - |
| AK 16 | ++ | AK 32 | - |
| '-' Negative, '++' Moderate hydrolysis, '+++' substantial hydrolysis | | | |

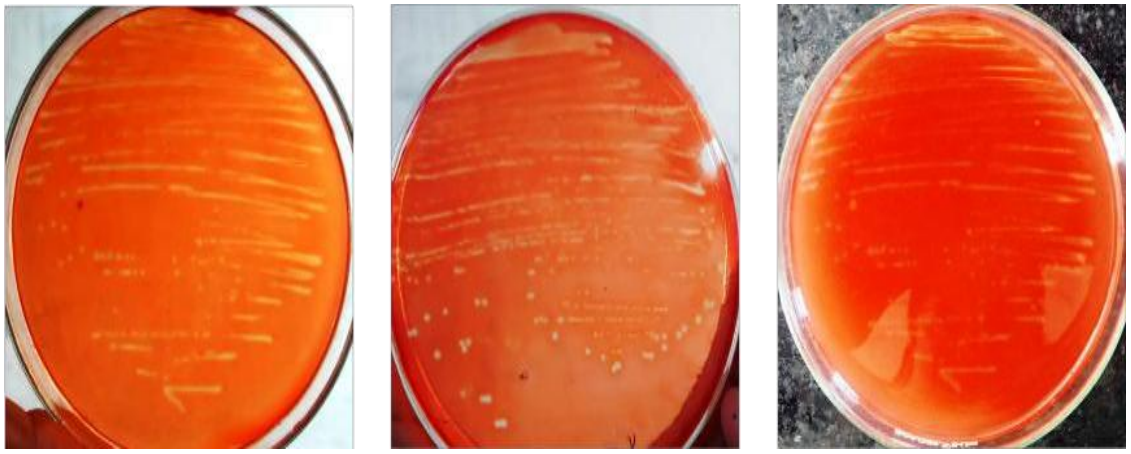


**Fig. 6: Enzymatic hydrolysis on Mutan containing agar plates stained with Congo red dye.**

As stated by Sudheer *et al*. 2019, the clear zone on plate treated with congo red indicates the presence of mutanase producing micro-organism. Congo red interacts with α-(1→3)-glycosidic bonds, resulting in red color, whereas the mutanase hydrolysed component of mutan remains colorless (Lamb & Toy 2005). Thus, the clear zone indicates the presence of enzyme hydrolysis by mutanase producing microorganism (Florencio C *et al*., 2012).

Similar to the present work, Mutanase producing microorganism *Paenibacillu*s was also isolated form soil sample (Sumitomo *et al*., 2007). Similar technique was used for detection of mutanase produced by *Paracoccus mutanolyticus* (Buddana *et al*., 2016).

**Secondary screening of Mutanase producing micro-organisms**

Three of the top bacterial strains (AK 04, AK 10 and AK 27) were chosen for secondary screening applying the submerged cultivation technique following the first primary screening. The amount of enzyme activity was checked by Nelson-Somogyi's method after every 24 Hrs up to 120 Hrs by collecting 2ml of cell-free broth for each time point. Mutanse activity by different cultures at various intervals is shown in Table 3. The maximum enzyme activity was observed at 72 Hrs for mutanase production. The bacterial isolate AK 27 produced the highest amount of mutanase (0.68 U/ml) while AK 04 and AK 10 produced 0.51, and 0.43 U/ml activity of mutanase respectively (Table 3 and fig. 7).

Similarly, *Trichoderma harzianum* was used in the study of mutanase production under submerged conditions by A. Wiater and J. Szczodrak in 2005. The researchers achieved an optimal production of 0.33 U/ml after 72 Hrs. Pleszczyńska *et al*. in 2010 also obtained 0.35 U/ml mutanase activity from a bacterial strain *Paenibacillus sp*. MP-1.

In the present study, it is noticeable in Fig. 7, that following 24 Hrs incubation period, mutanase production rose and peaked at 72 Hrs, while concurrently declined after 72 Hrs.

**Table 3: Production of mutanase at different time intervals by different isolates.**

| Sr. no. | Enzyme activity (U/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Sampling time point (Hrs.)** | **12** | **24** | **48** | **72** | **96** | **120** |
| 1 | Sample 1 (AK04) | 0.04 | 0.07 | 0.24 | 0.51 | 0.38 | 0.22 |
| 2 | Sample 2 (AK10) | 0.02 | 0.05 | 0.16 | 0.43 | 0.27 | 0.19 |
| 3 | Sample 3 (AK 27) | 0.09 | 0.11 | 0.37 | 0.68 | 0.55 | 0.42 |

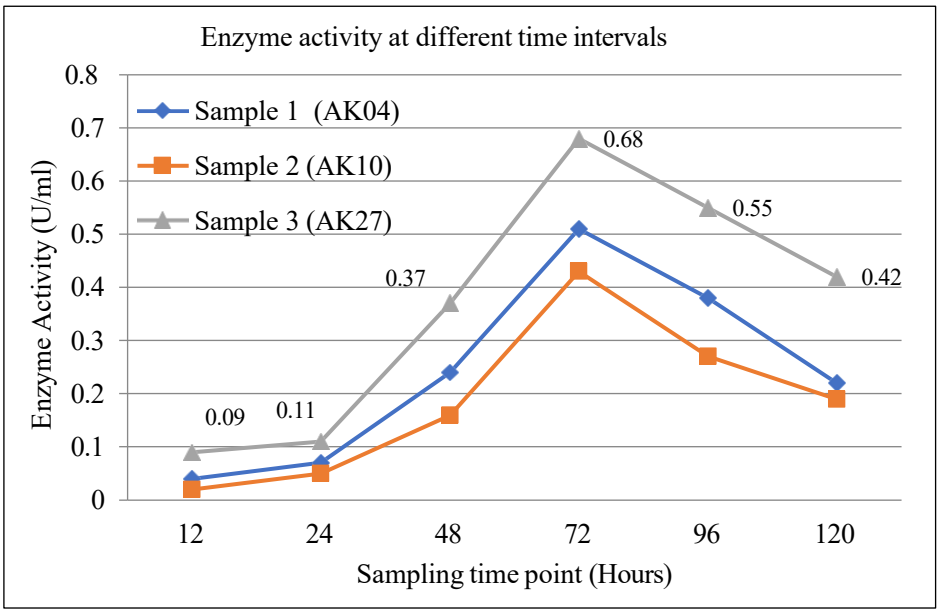



**Fig. 7: Time course study for mutanase production.**

**Identification of mutanase producer AK 27:**

**Morphological identification of AK 27**

Morphological evaluation was performed phenotypically by cultivating pure culture (AK 27) on nutrient agar plates revealed that the color of colonies was creamy white, smooth texture with entire margin, convex round shaped and non-motile. Microscopic slide analysis was done by gram staining revealed that the isolate was gram negative cocci shaped (Fig. 8).

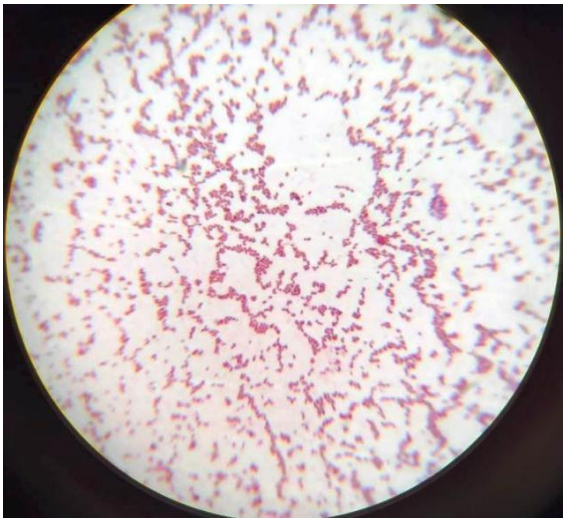
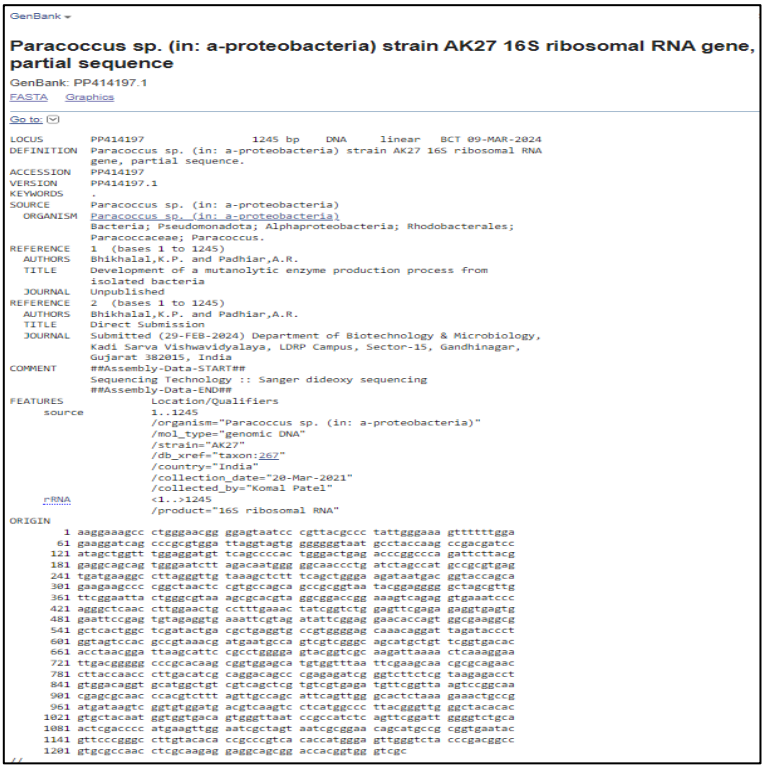


**Fig. 8: Microscopic view of gram staining for bacterial isolates AK 27.**

**Molecular Identification of bacterial strains**

For 16S rRNA sequencing, the culture exhibiting maximum mutanase activity was selected because 16s is most widely utilized housekeeping genetic marker for examining the bacterial taxonomy and phylogeny (Janda and Abbott, 2007). 16S rRNA gene sequence was done to identify bacteria or assign close relationships at both species and genus scales (Clarridge, 2004). Sequencing was done using universal primers, yielding FASTA sequences with a query length of 1245 bases that was sent to NCBI, PP414197 was the accession number of AK 27 (Fig. 9).



GenBank

**Paracoccus sp. (in: a-proteobacteria) strain AK27 16S ribosomal RNA gene, partial sequence**

GenBank: PP414197.1

FASTA Graphics

Go to:

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  AUTHORS   Bhikhalal,K.P. and Padhiar,A.R.
  TITLE     Development of a mutanolytic enzyme production process from
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  JOURNAL   Unpublished
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  TITLE     Direct Submission
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      181 gaggcagcag tgggaatctt agacaatggg ggcaaccctg atctagccat gccgcgtgag
      241 tgatgaaggc cttagggttg taaagctctt tcagctggga agataatgac ggtaccagca
      301 gaagaagccc cggctaactc cgtgccagca gccgcggtaa tacggagggg gctagcgttg
      361 ttcggaatta ctgggcgtaa agcgcacgta ggcggaccgg aaagtcagag gtgaaatccc
      421 agggctcaac ctggaactg cctttgaaac tatccggtctg gagttcgaga gaggtgagtg
      481 gaattccgag tgtagaggtg aaattcgtag atattcggag gaacaccagt ggcgaaggcg
      541 gctcactggc tcgatactga cgctgaggtg ccgtgggggag caaacaggat tagataccct
      601 ggtagtccac gccgtaaacg atgaatgcca gtcgtcgggc agcatgctgt tcggtgacac
      661 acctaacgga ttaagcattc cgcctgggga gtacggtcgc aagattaaaa ctcaaaggaa
      721 ttgacgggggg cccgcacaag cggtggagca tgtggtttaa ttcgaagcaa cgcgcagaac
      781 cttaccaacc cttgacatcg caggacagcc cgagagatcg ggtctttctcg taagagacct
      841 gtgggacaggt gcatggctgt cgtcagctcg tgtcgtgaga tgttcggtta agtccggcaa
      901 cgagcgcaac ccacgtcttt agttgccagc attcagttgg gcactctaaa gaaactgccg
      961 atgataagtc ggtgtggatg acgtcaagtc ctcatggccc ttacgggttg ggctacacac
     1021 gtgctacaat ggtggtgaca gtgggttaat ccgccatctc agttcggatt gggtctgca
     1081 actcgacccc atgaagttgg aatcgctagt aatcgcggaa cagcatgccg cggtgaatac
     1141 gttcccgggc cttgtacaca ccgcccgtca caccatggga gttgggtcta cccgaaggcc
     1201 gtgcgccaac ctcgcaagag gaggcagcgg accacggtgg gtcgc
//
```

**Fig. 9: AK 27 strain NCBI submission result (Accession number PP414197)**

The requested sequence was matched with highly comparable sequences from the NCBI database by the BLAST (https://blast.ncbi.nlm.nih.gov/Blast) software in order to investigate sequence similarity. BLAST analysis revealed that the AK 27 strain had 99.8% sequence similarity showing with the *Paracoccus sp*.; Fig. 10 shows the phylogenetic tree constructed for strain AK 27. Form this result *Paracoccus sp.* AK 27 strain showing highest similarity with *Paracoccus sp.* RSP02. These two strains were showing most similarity to the *Paracoccus yeei* strain. Similar to this result, Buddana *et al*., 2019 have produced mutanase using *Paracoccus mutanolyticus* RSP02.

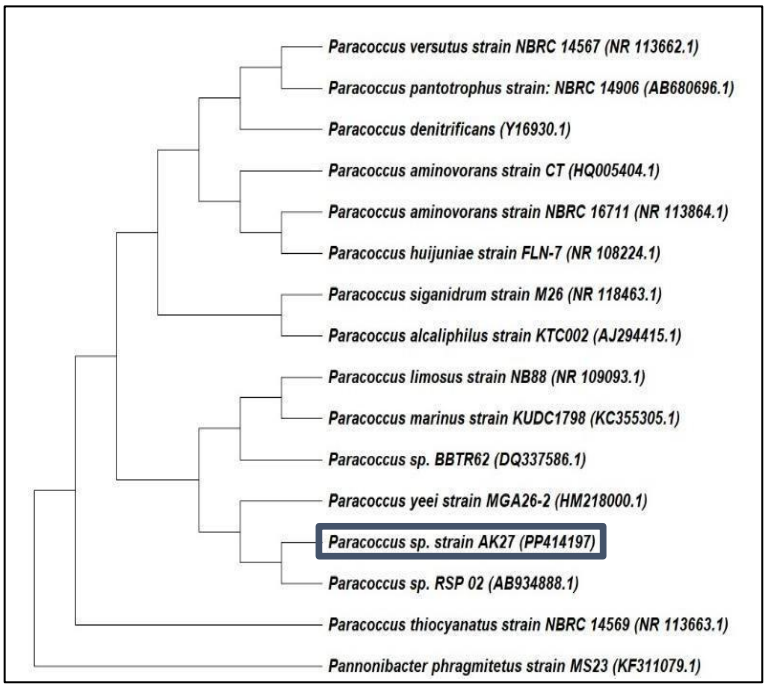


**Fig. 10: Phylogenetic tree showing the phylogenetic relationship of the distribution of mutanase producing strain (AK 27) with other species.**

## CONCLUSION

The research findings indicated that after 72 Hrs, AK 27 (*Paracoccus sp*.), which was isolated from soil samples, produced the highest amount of mutanase activity, 0.68 U/ml. By optimizing the growth conditions and cultivation media, it is possible to increase the production and activity of mutanase, which will facilitate further research towards the development of industrially relevant applications. This study successfully screened and isolated mutanase-producing microorganisms from diverse soil samples and teeth caries specimens, employing a combination of targeted sampling, selective media, and advanced molecular and biochemical characterization techniques. Soil samples from agricultural fields, industrial waste sites, and forest floors, as well as carious material from dental lesions, were processed using stringent quality control measures to ensure the viability and specificity of microbial isolates. The use of enrichment cultures and selective media with mutans or dextran as the sole carbon source enabled the isolation of microorganisms with mutan-degrading capabilities, as evidenced by clear zones of polysaccharide degradation around colonies. High-throughput screening methods, such as microplate assays, further facilitated the rapid identification of strains with significant mutanase activity. Key findings include the identification of rare microbial strains from unique environments, such as saline soils and sugarcane processing waste, which exhibited enhanced mutanase activity compared to isolates from conventional sources. Molecular techniques, including 16S rRNA and ITS sequencing, confirmed the phylogenetic diversity of the isolates, while zymogram analysis and enzyme activity assays quantified their mutanase production. Optimization of production conditions using response surface methodology (RSM) demonstrated that enzyme yield could be significantly increased by fine-tuning parameters such as pH, temperature, and nutrient sources. Additionally, the study highlighted the industrial relevance of mutanase enzymes by evaluating their thermostability, pH stability, substrate specificity, and compatibility with detergents and additives, identifying strains with robust properties suitable for commercial applications.

The implications of these findings are substantial, particularly for industries such as oral healthcare, where mutanase enzymes could be utilized to degrade dental plaque biofilms, and in biotechnology, where they may play a role in polysaccharide processing. Future research should focus on exploring underutilized environments, such as extreme habitats, to uncover novel mutanase-producing strains with unique enzymatic properties. Advanced methodologies, such as microfluidic droplet-based isolation and co-culture systems, should be further developed to enhance the efficiency of strain isolation and enzyme production., including and, could also provide insights into enzyme optimization for specific industrial applications. Also, Structural studies, such as computational modelling and X-ray crystallography, could offer valuable insights into the optimization of enzymes for particular industrial applications. These advancements will pave the way for the large-scale production and commercialization of mutanase enzymes, offering significant benefits across multiple sectors.

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