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Enzymatic Potential of Sewage Microbes: A Study on Protease-Producing Bacteria from Sironj, Vidisha

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Abstract: ***Microbes that like warm temperatures, like Actinomycetes and extremophiles, make the extracellular proteases that break down complex organic matter in sewage systems. Finding and studying a new strain of Streptomyces spp. (Sironj-SAM-2) that can break down proteins in sewage from Sironj, Madhya Pradesh Strain was most active at 50°C and pH 9, a prime feature for an industrial use. Widely used in wastewater treatment, detergent production, food processing, leather industries, and environmental bioremediation applications, these proteases remain stable under extreme conditions. Also, improvements in fine-tuning fermentation and genetic methods to make these enzymes more stable and increase their yield have made these products more valuable in the business world. Also, this report contains the first study from Sironj isolation of protease derived from Streptomyces species, which indicates that sewage-derived enzyme is a better alternative source for sustainable industrial and environmental aspects.***

Keywords: Industrial Enzymes, Microbial Proteases, Sironj, Sewage Microbiome

1. Introduction

Sewage systems harbor an array of organic matter, including diverse microbial precincts to serve as reservoirs of microorganisms that produce proteases. *Actinomycetes* are a type of bacteria that are very important in this situation because they release enzymes outside of cells, like proteases, that break down complex organic matter [1]. These enzymes are essential for nutrient distribution and the breakdown of proteinaceous substrates in sewage ecosystems. [2]. Extremophiles are microorganisms that do well in harsh environments like high temperature, pH, and salinity. They also make stable extremozymes that work well in conditions that are useful in industry [3]. Microbial proteases are superior to the plant and animal-based ones because they are less expensive, easier to produce in bulk, and their genes can be engineered to function in other industrial environments [4]. *Bacillus subtilis* makes alkaline proteases that are used in detergents, and *Aspergillus* spp. makes acid-stable fungal proteases that work better for food processing [5]. The pharmaceutical industry and recycling waste use these enzymes because they have substrate specificity but stereotype interaction [6]. It is possible to get the best performance and stability from enzymes by using strains of microbes that are very good at what they do, like *Bacillus subtilis, Bacillus amyloliquefaciens,* which can make serine proteases with a high yield and the best conditions (pH, temperature, and food) [7]. *Streptomyces* strains from soil and wastewater make proteases that are very stable at high temperatures and can grow in a pH range that is very broad [8]. Because of their ability to break down proteins in an efficient manner, proteases have found use in a variety of industrial applications. These applications include the formulation of detergents, the processing of food, the treatment of leather, and the production of pharmaceuticals [9]. One such place is Sironj, Madhya Pradesh, which used to have textile and farming industries. This changes the microbiomes in sewage, which might contain new proteases that have evolved to work with a wide range of niche substrates [10]. Research has shown that wastewater-derived proteases can degrade complex organic pollutants, indicating their potential for environmental bioremediation [11]. Alkaline proteases play an important role in textile wastewater treatment, owing to their contribution in using biodegradation to reduce the myriad of proteinaceous pollutants [12]. In the same way, microbial proteases are widely used in the leather processing industry to remove hair from hides and make batting instead of harsh chemicals [13]. Extremophile proteases have gotten a lot of attention for their ability to work in high temperatures and amphiprotic, viscous environments. Numerous fields, such as biopharmaceuticals, peptide synthesis, and drug delivery, can also utilize them [14]. New engineering methods, like recombinant DNA technology and site-directed mutagenesis, also help make microbial proteases more stable and useful in industry [15]. Because they have such a wide range of microbes, they can be used effectively for both industrial enzyme needs and clean, eco-friendly waste bioprocessing and environmental bioremediation. Sironj is an ancient town in India known for rich historical and biological diversity, which led to the different protease-producing microorganisms. Shankar Singh, a Sengar Rajput warrior, founded Sironj in 1103 A.D., with its agricultural history contributing to its sewage microbiome. Famous for producing muslin and calico, the town harbours unique microbes that may have adapted to the region's traditional textile processing. There are compelling reasons to investigate the microbial diversity and proteolytic potential in Sironj's rheological features.

2. Material and Methods

Chemicals, and Instruments

Chemicals and reagents used in the study came from HiMedia (Mumbai, Maharashtra, India), TCMedia (Mumbai, India), and Sunchem (Parsippany, New Jersey, USA). They were used for media formulation, screening, well diffusion assay, activity analysis (qualitative and quantitative), and phenotypic characterization. Indian Scientific, based in Bhopal, Madhya Pradesh, supplied these chemicals. All the laboratory instruments have been provided by the Department of Higher Education, Government of Madhya Pradesh.

Sampling Procedure

Samples of sewage were taken from three well-known spots in Sironj, Vidisha, Madhya Pradesh (India) such as Bhawani Nagar Sewage Site, Pachkuiya Ward and Sewage Dumping Site Sironj for isolating and characterizing protease producing bacteria. To reduce contamination, 2 cm deep samples were aseptically collected in sterile 15 ml Falcon tubes. These places were chosen because they might be able to support groups of microbes that can survive in alkaline conditions and make proteases. Samples were collected under alkaline pH conditions, which enhance the growth of proteolytic bacteria. The collected samples were then sealed in sterile bags with proper labelling and conditions for the isolation of microbes and biochemical characterization.

Medium

A non-selective medium was prepared using gently heated, 1% casein agar, 2.5 g of casein, 2.5 g of beef extract, 1.2 g of sodium chloride, and 3.7 g of agar in 250 ml double-distilled water. The pH was carefully adjusted to 7.0, followed by the sterilization of the medium through autoclaving at temperature of 121°C for 21 minutes. The mixture then transferred into pre-sterilized petri plates and allowed the medium to solidify.

Screening for Protease-Producing Bacteria

The serial dilution method, applying sterile double-distilled water as the diluent. In order to find out what kinds of microbes were in the sewage; samples were serially diluted from 10^{-4} to 10^{-7} in 10 ml test tubes. Then 100 µl of diluted sewage samples were inoculated on casein agar plates. Plates were incubated for a duration of 24-48 hours at 35°C temperature in a BOD incubator. Casein agar plates were replicated in triplicate to confirm the colony's purity. Pure isolates were maintained on nutrient agar slants for subsequent biochemical analysis.

Biochemical Tests

Bacterial isolates with protease activity were characterized using a comprehensive set of biochemical and morphological tests. For structural and growth-related consistency, Gram staining and colony morphology were examined after 24 and 48 hours of incubation. Oxidase and catalase tests were conducted, along with methyl red and Voges-Proskauer tests, to assess mixed-acid fermentation or acetoin production. In addition, the indole test was performed. Triple Sugar Iron (TSI) agar was used to test substrate utilization. Citrate agar was used to see how well the substrate absorbed citrate, and urea tests were used to see how well the substrate broke down urea. The proteolytic activity was confirmed by breaking down casein, and cellulose degradation tests gave us more information about the enzyme profile. Nitrate reduction and carbohydrate fermentation (glucose, lactose, and sucrose) tests were also conducted to assess acid and gas production. The morphological observations were made using a Magnus MLXi Plus binocular microscope.

Well Diffusion Assay

Isolated bacterial colonies that are producing clear zones on casein agar were inoculated into liquid medium. LB medium was prepared using 2.5 g of LB broth powder and 1.0 g of casein in 100 ml double-distilled water. Each isolate was inoculated in 100 ml of the Luria-Bertani medium. The culture media were incubated at 37°C for 24 hours. The supernatant was collected by centrifuging the culture at 10000 rpm for 10 minutes. Radial diffusion assays on casein agar plates were used to detect protease activity. Casein agar wells (7 mm diameter) were made, and 100 µl of the cell extract was added to the wells. Relative enzyme activity was calculated by measuring the diameter of the zone of hydrolysis surrounding the wells after incubation at 37°C for 24 hours. A reaction-stopping solution of 10% trichloroacetic acid was added to calculate the REA.

REA = Diameter of Zone of Clearance (in mm) / Well Diameter (in mm)

UV-Visible Photometry

To test protease activity, the Folin-Ciocalteu method was used to measure the amount of tyrosine that was released when casein was broken down. To do the test, 0.5 ml of enzyme solution and 1 ml of 1% casein solution were mixed in 50 mM sodium phosphate buffer (pH 7.5). The mixture was incubated at 37°C for 30 minutes. One milliliter of 10% trichloroacetic acid (TCA) was added to stop the reaction. The mixture was then centrifuged at 10,000 rpm for ten minutes. The clear supernatant that was left over (0.5 ml) was mixed with 0.5 ml of Folin-Ciocalteu reagent and 2.5 ml of 0.5 M sodium carbonate. After a further 30-minute incubation at 37°C, the absorbance at 660 nm was recorded.

Evaluation of Protease Production in Isolates at Different pH Conditions

The protease-producing ability of bacterial isolates was characterized by immersing them in a 1% casein solution within Luria Bertani (LB) broth. Since enzyme synthesis is influenced by pH, the pH of the medium was adjusted between 4.0 and 12.0 using 0.5% HCl and 1 % NaOH. The inoculum was added to the medium and incubated at 37°C for 24 hours with shaking at 150 rpm. After incubation, the cells were collected by centrifugation at 10,000 rpm for 10 minutes, and the supernatant was obtained to evaluate protease activity at different pH levels.

Characterization of the Isolates for the Production of Protease at Different Temperatures

Protease production was evaluated by cultivating bacterial isolates in LB broth supplemented with 1% casein at an optimized pH. The bacterial isolates were cultured at temperatures ranging from 20°C to 80°C for 24 hours. The cells were collected by centrifuging at 10,000 rpm for 15 minutes after incubation. The protease activity was then measured in the supernatants that were free of cells. This range of temperatures helped figure out the best conditions for

enzyme activity and showed what kinds of thermophilic conditions help make proteases. The Folin-Ciocalteu method was used to measure proteolytic activity. Tyrosine release from casein hydrolysis was measured at 660 nm using a standard curve for tyrosine.

3. Results

Protease activity on casein agar plates was screened for various isolates. Protease activity was detected from the zone of hydrolysis seen on the surface of the agar (Figure-1). The strain displaying a larger zone of hydrolysis was selected for further evaluation as Sironj-SAM-2 and preserved by virtue of consecutive subculturing (Figure 2). The Gram staining of *Streptomyces* spp. revealed Gram-positive, filamentous structures. After 48 hours of incubation, extensive filamentous growth was observed (Figure-3). The biochemical test results, showing the isolate's characteristics, including positive reactions for Gram staining, catalase, urease, casein hydrolysis, nitrate reduction, and cellulose degradation, while it was negative for citrate utilization, oxidase test, indole test, MR test, VP test, and H_2S test (Table-2).

The bacterial isolates from sewage samples were assessed for proteolytic activity using the zone of hydrolysis (Table-1) and relative enzyme activity (REA). Among the five isolates, SAM-2 showed the highest protease activity with a 20 mm zone of hydrolysis and an REA of 2.85, followed by SAM-1 and SAM-3 (18 mm, REA 2.57), SAM-5 (16 mm, REA 2.28), and SAM-4 (15 mm, REA 2.14). These results highlight SAM-2 as the most efficient protease producer, making it a promising isolate for further study and industrial applications (Table-1).

Figure 1: The sample collection site for Sironj-SAM-2 in Sironj Panchkuiya Ward

Figure 2: Zone of hydrolysis of Sironj-SAM-1, Sironj-SAM-2 and Sironj-SAM-3 after 24 hours

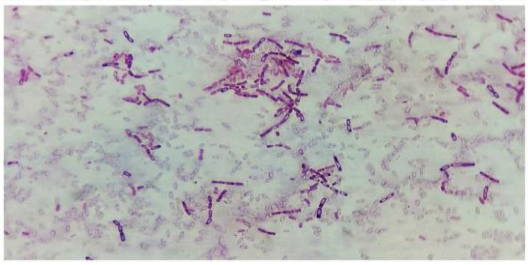

Figure 3: Microscopic image of Sironj-SAM-2 isolate after 48 hours of incubation and Gram staining.

Table 1: Zone of hydrolysis (in mm) and the relative enzyme activity (REA) for each isolate

S. No.	Isolates of Sewage Sample (Sironj-SAM)	Zone of Hydrolysis in mm	Relative Enzyme Activity (REA)
1.	SAM-1	18	2.57
2.	SAM-2	20	2.85
3.	SAM-3	18	2.57
4.	SAM-4	15	2.14
5.	SAM-5	16	2.28

Table 2: Results of Biochemical Tests for Sewage Isolate Sironj-SAM-2

S. No.	Biochemical Test	Result
1.	Gram Staining	Positive
2.	Catalase	Positive
3.	Oxidase	Negative
4.	Indole	Negative
5.	MR	Negative
6.	VP	Negative
7.	Citrate Utilization	Negative
8.	H_2S Production	Negative
9.	Urease	Positive
10.	Casein Hydrolysis	Positive
11.	Nitrate Reduction	Positive
12.	Cellulose Degradation	Positive

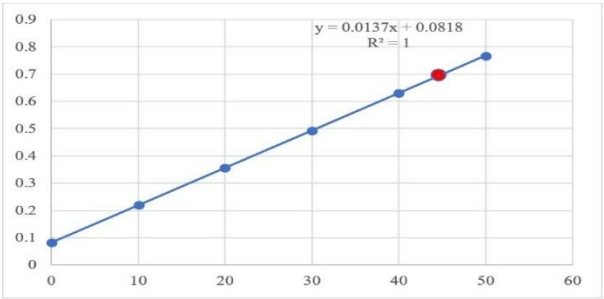

Figure 4: Standard tyrosine curve Sironj-SAM-2 showing an absorbance 0.695 at 660nm

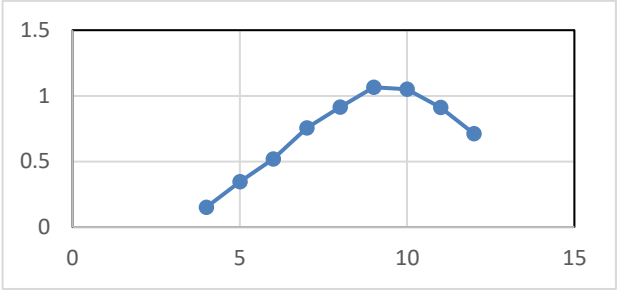

Figure 5: Effect of pH on Protease Activity Measured by Absorbance at 660 nm

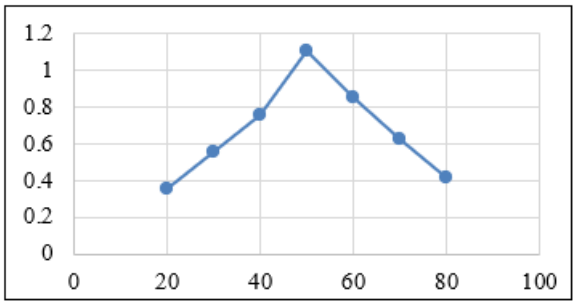

Figure 6: Temperature-Dependent Protease Activity of the Sironj-SAM-2 Isolate

A standard curve for tyrosine concentration was established based on the results obtained through the Folin-Ciocalteu method, with a linear relationship observed between the concentration of tyrosine (10–50 µg/ml) and 660 nm absorbance. The absorbance of the sample (0.695) corresponded to an interpolated tyrosine concentration of approximately 44.7 µg/ml. The standard curve showed a strong Pearson correlation (R^2=0.9862), indicating that this assay accurately quantified protease activity. Since the absorbance values for Sironj-SAM-2 were in the linear range of the standard curve, it indicated that significant proteolytic activity was present in this sample (Figure -4).

The results showed an increasing activity from 0.151 absorbance at pH 4 to a maximum of 1.065 at pH 9, indicating the enzyme working efficiently under alkaline conditions. At pH 9 and lower, activities were significantly higher compared to higher pH levels, and absorbance values reduced to 1.05 at pH 10, 0.911 at pH 11, and 0.712 at pH 12. The results indicate maximal activity of the protease at pH 9, although decreased activity is observed in both extremes of acidity and basicity (Figure-5).

The results demonstrated slow activity at 20°C (0.351 OD) up to an activity maximum of 1.100 OD at 50°C, showing the optimal temperature for enzymatic activity. However, a drop-in activity at temperatures above 50°C was noted, with OD values at 60°C, 70°C, and 80°C being 0.851, 0.623, and 0.412, respectively, showing a 67.52% and 86.80% decrease in activity compared to the activity at 50°C (Figure-6).

4. Discussion

Streptomyces species are widely recognized for their ability to produce industrially significant proteases, which are stable under extreme conditions such as high alkalinity and temperature. These proteases play a crucial role in various industries, including textiles, detergents, pharmaceuticals, and bioremediation. Studies have demonstrated that *Streptomyces griseus* and *Streptomyces* spp. exhibit optimal protease activity in alkaline conditions (pH 9–11) and temperatures around 50°C, making them suitable for industrial applications [16] [17]. Furthermore, advancements in fermentation optimization and genetic engineering have enhanced protease yield in *Streptomyces clavuligerus* and *Streptomyces albidoflavus*, with techniques like Response Surface Methodology (RSM) leading to significant improvements [18] [19]. Additionally, *Streptomyces* spp. derived proteases have been effectively used in wastewater treatment by breaking down proteinaceous pollutants from the textile and leather industries, supporting sustainable waste management [20]. The increasing use of recombinant DNA technology and site-directed mutagenesis has further improved enzyme stability and specificity, expanding their industrial applications in food processing, pharmaceuticals, and environmental bioremediation [21] [22].

5. Conclusion

The study from Sironj is the first to isolate and describe a *Streptomyces* strain that produces protease from sewage in Sironj, Madhya Pradesh. The study found that Sironj-SAM-2 is a very good protease in both hot and acidic conditions, working best at 50°C and pH 9. This implies that industry could potentially utilize it. To find out how well *Streptomyces* spp. work, researchers test their ability to make antimicrobial metabolites that kill pathogenic microorganisms like bacteria and fungi. As a result of their unique ability to work well in harsh growth conditions, proteases are very useful for treating wastewater, making detergents, processing food, and working with phospholipids. Also, techniques for improving fermentation or genetic modification can make enzymes more stable and increase their yield, which means they can be used in more commercial situations.

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