SLUDGE-DERIVED ENZYMES AS GREEN CATALYSTS FOR ENVIRONMENTAL REMEDIATION

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Introduction:

1. Activated sewage sludge contains diverse microbial communities that naturally produce enzymes such as amylase, protease, lipase, cellulase, peroxidase, and laccase. These enzymes have the ability to degrade synthetic dyes, break down organic pollutants, and contribute to microplastic degradation, making them valuable for environmental remediation.
2. Recovering enzymes from sludge transforms waste into low-cost, sustainable biocatalysts, supporting circular bioeconomy principles. Additionally, 16S rRNA microbial profiling helps identify key enzyme-producing microorganisms, improving extraction efficiency and application potential.
3. In India, nearly 75,000 MLD of sewage is generated, while treatment capacity is only about 26,000 MLD, leaving a large portion untreated. This wastewater contains high organic load (COD and BOD), nutrients, and pathogens, leading to severe environmental pollution. Existing treatment systems such as activated sludge processes and SBRs face limitations in efficiency and sustainability.
4. This study focuses on extracting enzymes from sewage sludge and evaluating their potential as eco-friendly catalysts for wastewater treatment and environmental remediation.

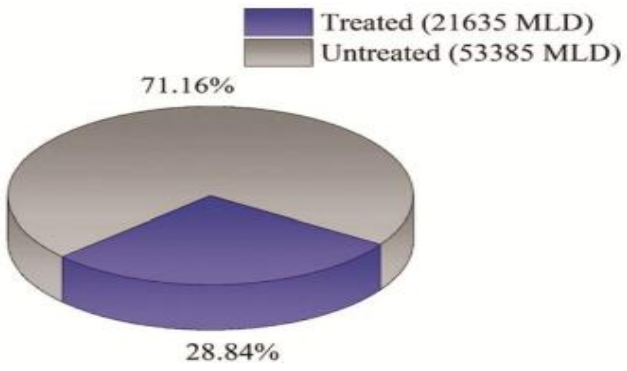

Figure 1: Sewage generation and treatment capacity in class I and class II cities

Objectives:

1. To extract crude enzymes from raw and activated sewage sludge using cell disruption techniques.
2. To identify the presence of industrially important enzymes such as amylase, protease, lipase, cellulase, peroxidase and laccase.
3. To characterize the microorganisms responsible for enzyme production.
4. To evaluate the potential applications of the extracted enzymes in environmental remediation.

Methodology:

1. Sample Collection:

Raw and activated sewage sludge samples were collected from a local SBR-based sewage treatment plant. The samples were collected in sterile containers and transported under controlled conditions to preserve microbial activity.

2. Pre-processing of Sludge:

The collected sludge samples were centrifuged at 7000 rpm for 10 minutes to separate the biomass. The supernatant was discarded, and the pellet was resuspended in Tris-Cl buffer (pH ~7) to maintain enzyme stability.

3. Crude Enzyme Extraction (Cell Disruption):

The resuspended samples were subjected to ultrasonication (5 kHz for 10 minutes) to disrupt microbial cells and release intracellular enzymes. The mixture was then centrifuged at 10,000 rpm for 15 minutes at 4°C to remove cell debris. The supernatant obtained was collected as crude enzyme extract and stored at 4°C.

4. Protein Estimation:

The total protein content of the crude enzyme extract was determined using the Lowry method. A standard calibration curve was prepared using BSA, and absorbance was measured at 660 nm. Activated sludge showed higher protein concentration compared to raw sludge, indicating greater enzyme availability.

5. Enzyme Activity Assays:

Enzyme assays were performed to confirm the presence and activity of key enzymes:

i. Amylase activity: Determined using the DNS method, where the enzyme was incubated with starch substrate, and the reducing sugars released were measured at 540 nm after colour development.
ii. Protease activity: Determined using casein as substrate, where the hydrolysis of protein was quantified by measuring absorbance at 280 nm after stopping the reaction.
iii. Lipase activity: Assessed using p-nitrophenyl butyrate (PNPB) as substrate. The hydrolysis of PNPB resulted in the release of p-nitrophenol, and the absorbance was measured at 400 nm.
iv. Cellulase activity: Estimated using the DNS method with carboxymethyl cellulose (CMC) as substrate. The reducing sugars released were measured at 540 nm.

v. Laccase activity: Measured using guaiacol as substrate. The oxidation of guaiacol resulted in a brown-coloured product, and the increase in absorbance was recorded at 470 nm.

vi. Peroxidase activity: Determined using guaiacol in the presence of hydrogen peroxide. The formation of tetra guaiacol was monitored at 470 nm.

6. Microbial Characterization:

- Microbial characterization of sewage sludge was carried out using both culture-based and molecular methods. The sludge samples were subjected to serial dilution and plated on nutrient agar (for bacteria) and potato dextrose agar (for fungi) using the spread plate technique. The plates were incubated under suitable conditions to obtain distinct colonies.
- Based on colony morphology, representative isolates were selected and purified through repeated subculturing. Pure cultures were then subjected to molecular identification using 16S rRNA gene sequencing for bacteria and ITS region sequencing for fungi.

7. Environmental Application:

Dye degradation studies were carried out using a quantitative broth-based method to evaluate the bioremediation potential of the system. Nutrient broth was prepared and inoculated with the isolates. Synthetic dyes- crystal violet (CV), methylene blue (MB), and malachite green (MG), were added at known initial concentrations.

The experimental flasks were incubated under controlled conditions, and a control setup without bacterial inoculation was maintained. After 48 hours, samples were collected and centrifuged to remove biomass.

The degradation of dyes was quantitatively monitored by measuring the absorbance of the supernatant at their respective maximum wavelengths using a spectrophotometer:

i. Crystal violet (CV): 590 nm
ii. Methylene blue (MB): 664 nm
iii. Malachite green (MG): 617 nm

The percentage degradation of each dye was calculated.

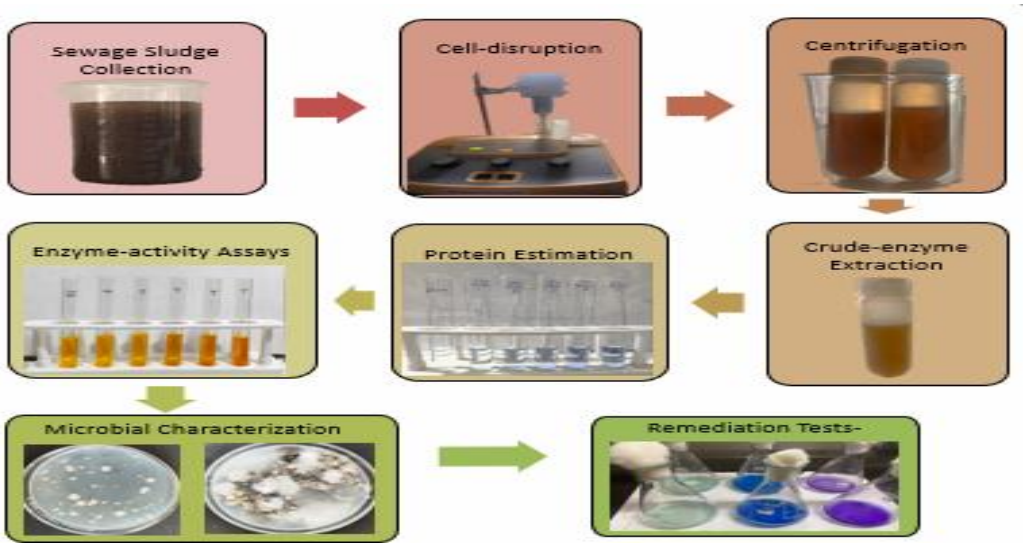

Figure 2: Methodology flowchart

Results and Conclusion:

- Activated sludge showed higher protein content (1.97 mg/mL) than raw sludge (1.067 mg/mL).
- Amylase activity increased significantly from 0.099 U/mL (raw) to 1.50 U/mL (activated).
  Specific amylase activity improved from 0.050 U/mg to 0.720 U/mg after activation.
- Protease activity increased from 0.289 (raw) to 0.402 U/ml (activated). Specific activity increased from 0.139 U/mg to 0.204 U/mg upon activation.
- Lipase activity showed the greatest increase, from 3.44 U/mL (raw) to 7.09 U/mL (activated).
  Specific lipase activity increased from 1.746 U/mg to 3.598 U/mg.
- Cellulase activity rose from 0.116 U/ml to 0.139 U/mL after activation.
  Specific activity improved from 0.058 U/mg to 0.067 U/mg.
- Laccase absorbance increased from 0.611 (raw) to 0.773 (activated) at 470 nm.
  Laccase activity rose from 0.578 U/mL to 1.780 U/mL after activation.
  Specific laccase activity improved from 0.279 U/mg to 0.903 U/mg.
- Peroxidase activity increased from 11.94 U/mL to 19.906 U/mL after activation.
  Specific activity improved from 5.77 U/mg to 10.104 U/mg, indicating enhanced efficiency.
- Results confirm activated sludge supports higher enzyme production due to aeration and enriched microbial metabolism.

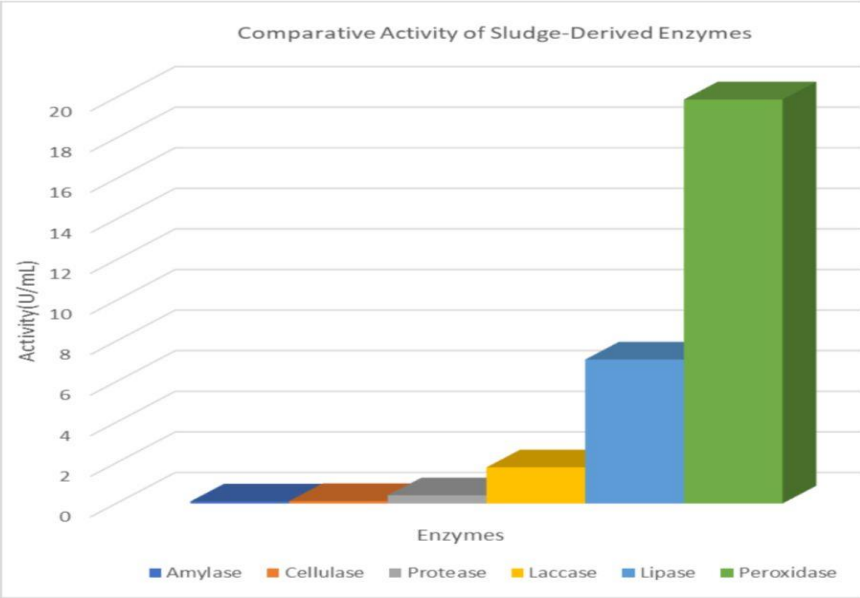

Figure 3: Comparative activity of extracted enzymes

- Molecular identification revealed enzyme-producing bacteria *Bacillus subtilis* and *Enterobacter cloacae.*
  Fungal isolates were identified as *Fusarium oxysporum* and *Aspergillus fumigatus*, known for hydrolytic and oxidative enzymes.
- Activated sludge demonstrated higher microbial biomass and catalytic efficiency than raw sludge.
- Dye degradation studies showed a continuous decrease in absorbance for crystal violet, methylene blue, and malachite green, indicating effective biodegradation. After

48 hours, degradation was highest for crystal violet (~52.48%), followed by methylene blue (~28.22%) and malachite green (~28.15%) with *Bacillus subtilis*.
- Multiple enzyme activities confirm degradation potential for carbohydrates, lipids, and aromatic pollutants.
- Activated sludge is validated as a cost-effective and sustainable enzyme source for environmental applications.
- The study supports applications in wastewater treatment, bioremediation, pollution control, and green biotechnology.

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Future Scope:

1. Optimization of enzyme extraction and development of immobilization techniques to improve stability and reusability.
2. Scale-up and integration of sludge-derived enzymes into real wastewater treatment systems and bioreactors.
3. Detailed characterization and enhancement of enzyme production using genetic and metabolic engineering approaches.
4. Expansion of applications to treat a wider range of pollutants, including industrial dyes and mixed effluents.
5. Investigation of degradation of emerging contaminants such as pharmaceuticals and endocrine-disrupting compounds.
6. Study of microplastic degradation (PE, PP, PET, PS) using oxidative and hydrolytic enzymes for sustainable waste management.