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A Comparative Study on Xylanase Production through Wild and Mutated Bacillus spp.

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Abstract:

Xylanase is an industrially important enzyme widely applied in pulp and paper, food, feed, brewing, and biorefinery industries. The present study focuses on the isolation, screening, strain improvement, and comparative evaluation of xylanase production by wild and mutated Bacillus spp. Soil samples were collected from the vicinity of Somics Lifesciences Pvt. Ltd., Bareilly, and bacterial isolates were obtained using serial dilution and spread plate techniques. Xylanase-producing isolates were screened qualitatively on xylan agar plates using the Congo red assay. The most potent isolate was subjected to strain improvement through physical and chemical mutagenesis. Identification was performed using morphological, microscopic, and biochemical characterization. Xylanase production was quantified using the DNS method, while protein estimation was carried out using the Bradford assay. Comparative analysis revealed enhanced xylanase activity in the mutated strain compared to the wild type, indicating the effectiveness of strain improvement strategies. The study highlights the potential of improved Bacillus strains for industrial-scale xylanase production.

Keywords: Xylanase, Bacillus spp., strain improvement, mutagenesis, industrial enzymes.

1. Introduction

Enzymes are biological catalysts that accelerate biochemical reactions without being consumed. Among industrial enzymes, xylanase occupies a prominent position due to its extensive applications in eco-friendly industrial processes [1]. Xylan, the second most abundant polysaccharide after cellulose, is a major component of plant hemicellulose. Its hydrolysis requires a consortium of xylanolytic enzymes, with endo-β-1,4-xylanase playing a central role [2].

Industrial enzymes now constitute a rapidly growing global market, with applications spanning food processing, textiles, detergents, pharmaceuticals, biofuels, pulp and paper, and waste management [3]. Among these, enzymes involved in the degradation of plant biomass have gained special attention due to their role in renewable energy production and sustainable industrial processes. Xylanase is one such enzyme that has emerged as a key biocatalyst in lignocellulosic biomass utilization [4].

Lignocellulosic biomass is the most abundant renewable organic resource on Earth, derived primarily from agricultural residues, forestry waste, and plant cell walls. It consists mainly of three polymeric components: cellulose, hemicellulose, and lignin [6]. While cellulose is a linear polymer of β-1,4-linked glucose units, hemicellulose is a heterogeneous, branched polysaccharide composed of various sugar monomers. Lignin, a complex aromatic polymer, provides structural rigidity and resistance to microbial degradation [7].

Hemicellulose accounts for approximately 20–35% of lignocellulosic biomass and plays a critical role in plant cell wall architecture by forming a matrix that surrounds cellulose microfibrils [8]. Among hemicellulosic polymers, xylan is the most abundant, making up nearly 70% of hemicellulose content in hardwoods and agricultural residues such as wheat straw, rice husk, and corn cobs. The efficient hydrolysis of hemicellulose, particularly xylan, is therefore essential for the complete utilization of plant biomass [9, 10].

Xylan is a complex heteropolysaccharide composed of a backbone of β-1,4-linked D-xylopyranose units. This backbone is often substituted with various side chains, including arabinose, glucuronic acid, acetyl groups, and phenolic compounds, depending on the plant source [11]. The degree and nature of substitution contribute to the structural heterogeneity of xylan, making its complete hydrolysis a challenging process. Based on its structural variations, xylan can be classified into different types such as arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan [12]. These structural differences influence the accessibility of xylan to enzymatic degradation and necessitate the coordinated action of multiple enzymes for complete hydrolysis. As a result, xylan degradation in nature is achieved by a synergistic enzyme system collectively known as xylanolytic enzymes [13].

Xylanases are classified into different glycoside hydrolase (GH) families based on amino acid sequence similarity and structural features, with GH10 and GH11 being the most extensively studied. GH10 xylanases generally exhibit broad substrate specificity and higher molecular weight, while GH11 xylanases are smaller, highly specific, and often preferred for industrial applications due to their efficiency and selectivity [14]. Industrial xylanases are valued for several desirable properties, including high catalytic activity, thermostability, pH tolerance, and resistance to inhibitors. The ability of xylanase to function effectively under harsh industrial conditions makes it a crucial enzyme in processes such as pulp bleaching, animal feed improvement, baking, and biofuel production [15].

Microbial xylanases, particularly from *Bacillus* spp., are preferred for industrial use owing to their extracellular nature, high catalytic efficiency, and stability under diverse conditions. However, native strains often produce insufficient enzyme levels for commercial viability. Strain improvement through

mutagenesis remains a cost-effective approach to enhance enzyme yield while maintaining genetic stability [16].

Species of the genus *Bacillus* are Gram-positive, spore-forming bacteria known for their robust nature and industrial relevance. They are capable of secreting large quantities of extracellular enzymes, making them ideal candidates for commercial enzyme production. *Bacillus* spp. produce xylanases that are active over a wide range of temperatures and pH values, including alkaline conditions required for pulp and paper applications [17]. Additionally, *Bacillus* strains are generally regarded as safe (GRAS), which further supports their use in food and feed industries. The genetic stability, rapid growth rate, and ability to utilize inexpensive substrates make *Bacillus* spp. attractive microbial factories for enzyme production [18].

Mutagenesis induces genetic variability, allowing the selection of superior mutants with enhanced xylanase production. This approach has been successfully applied to *Bacillus* spp. to obtain hyper-producing strains without altering their fundamental physiological characteristics. Compared to recombinant DNA techniques, mutagenesis is cost-effective, does not involve foreign gene insertion, and is often more acceptable for industrial and regulatory purposes [19].

The present research aims to isolate xylanase-producing bacteria from soil, improve enzyme production through mutation, and perform a comparative evaluation of xylanase activity between wild and mutated *Bacillus* strains.

2. Materials and Methods

2.1 Sample Collection: Soil samples were collected from a depth of approximately 15 cm near Somics Lifesciences Pvt. Ltd., Bareilly, using sterile techniques and stored in sterile polybags.

2.2 Isolation of Bacteria: Serial dilution followed by spread plate technique was employed to isolate bacteria on nutrient agar plates. Plates were incubated at 37°C for 24 hours, and distinct colonies were selected. Primary screening was performed on minimal salt agar supplemented with 1% xylan. After incubation, plates were flooded with 0.1% Congo red followed by washing with 1 M NaCl to observe clear hydrolysis zones [20]. Pure cultures were obtained using repeated streak plate methods and maintained on nutrient agar slants at 4°C [20].

2.3 Identification of Isolates: Morphological characteristics and biochemical tests including Gram staining, endospore staining, catalase, indole, MR-VP, starch hydrolysis, and cellulase tests were conducted following standard protocols [21].

2.4 Strain Improvement by Mutagenesis: Selected isolates were subjected to physical and chemical mutagenesis to enhance xylanase production. Mutants were screened again on xylan agar for improved enzyme activity [22].

2.5 Enzyme Estimation & Total protein concentration assay: Xylanase activity was estimated using the DNS method by measuring reducing sugars released from xylan at 540 nm. Protein concentration was determined using the Bradford assay with bovine serum albumin as the standard [23].

3. Results & Discussions:

The samples were collected in the poly bag for the isolation of xylanase producing bacteria. Collected soil samples from the desired location as given below in table 1. The collected soil sample was serially diluted prior to use for the inoculation purpose. The sample was serially diluted and the diluted sample was used for isolation as shown in figure. Further these cultures were studied for their morphological parameters. Then these shortlisted bacteria were streaked in the nutrient agar plates for pure culture preparations.The cultures were selected on the basis of morphological parameters from mixed culture plates obtained after serial dilution and spreading. Multiple bacterial isolates were obtained, among which selected colonies showed prominent clear zones on xylan agar, indicating xylanase production.

Table 1: Collected soil samples

S. No	Location	Type of sample
1.	Somics LifeSciences Pvt. Ltd., Bareilly Uttar Pradesh,243001	Soil Sample
2	Faiq enclave, Bareilly, Uttar Pradesh,243006	Soil sample
3	Shyamganj vegetable market, Bareilly Uttar Pradesh,243005	Soil sample

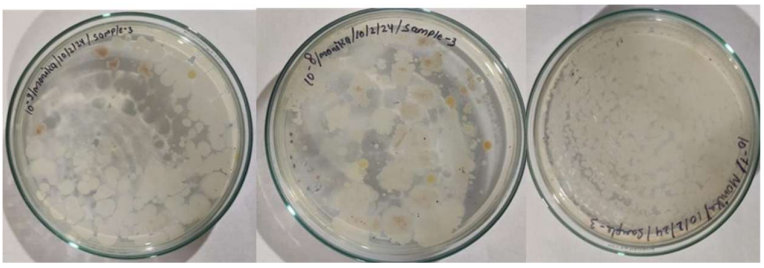

Figure 1: spreading of microbial culture of 10^{-7} 10^{-8} and 10^{-9}dilution factors respectively of soil samples

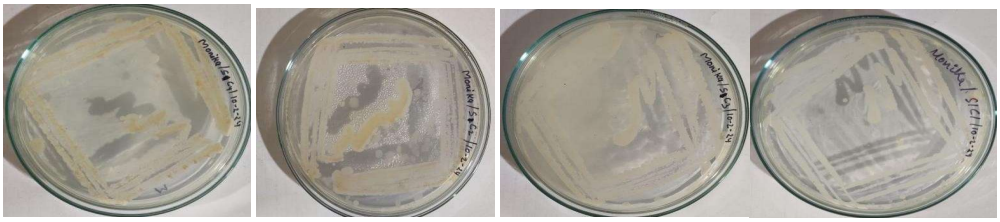

Figure 2: Pure culture of bacterial isolates from mixed culture plates by quadrant streak plate method

Primary screening on minimal salt agar supplemented with 1% xylan revealed that only a few isolates were capable of hydrolyzing xylan efficiently. The appearance of clear zones around colonies after Congo red staining confirmed xylanase activity. Among all isolates, the strain designated **S1C4** showed the largest and most distinct zone of clearance, indicating strong extracellular xylanase secretion. The formation of a clear halo zone is a direct consequence of xylan hydrolysis, where enzymatic degradation of xylan prevents Congo red binding, resulting in zone formation.

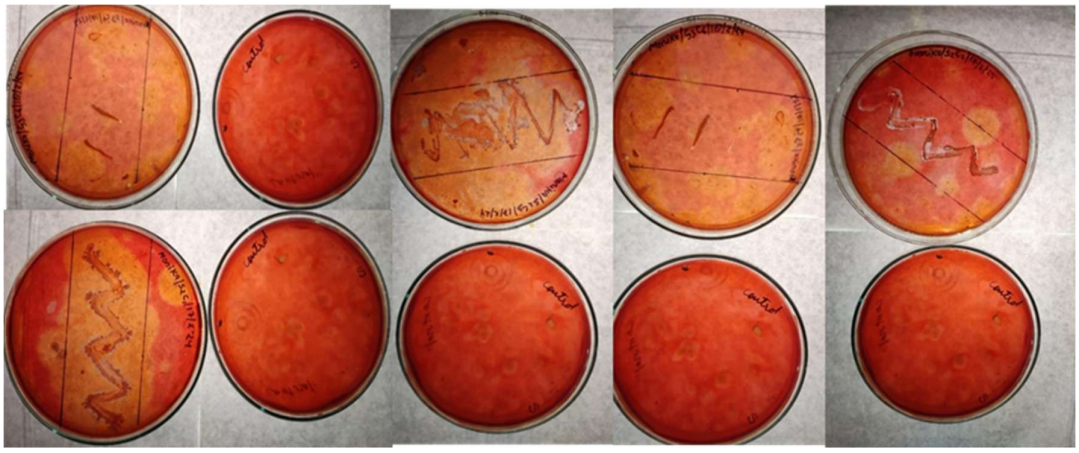

Figure 3: S1C4 shows positive result in primary and secondary screening.

Quantitative screening of xylanase production was carried out using the DNS method, which measures the release of reducing sugars from xylan substrate. A standard calibration curve for xylose was constructed to ensure accurate enzyme activity estimation. The enzyme activity values obtained confirmed that S1C4 produced appreciable levels of xylanase under submerged fermentation conditions.

Graphical representation of enzyme activity showed a clear distinction between different isolates, with S1C4 exhibiting the highest xylanase activity. This validates the qualitative screening results obtained through the Congo red assay. The correlation between zone diameter and enzyme activity highlights the reliability of plate assays as a preliminary screening tool.

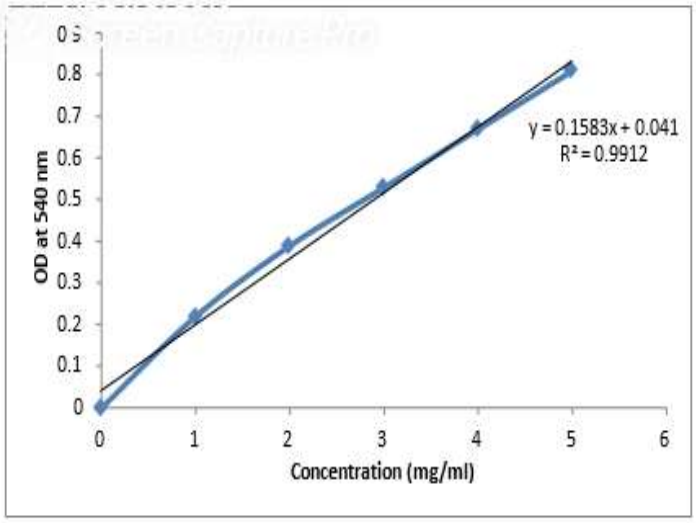

Figure 4: standard calibration curve for enzyme activity through DNS Assay

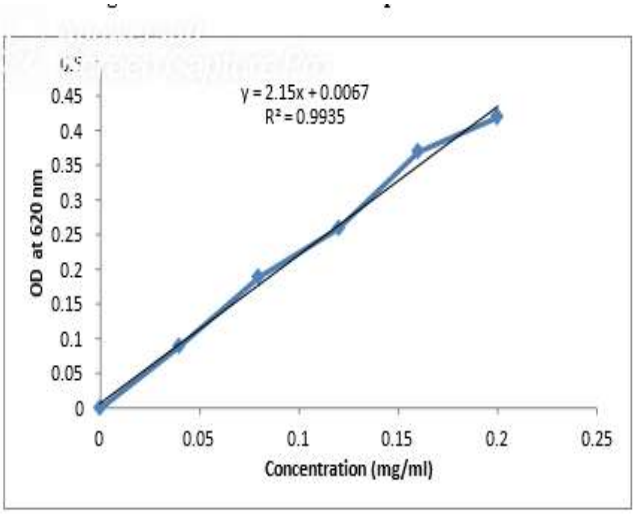

Figure 5: standard calibration curve for total protein concentration through Bradford assay

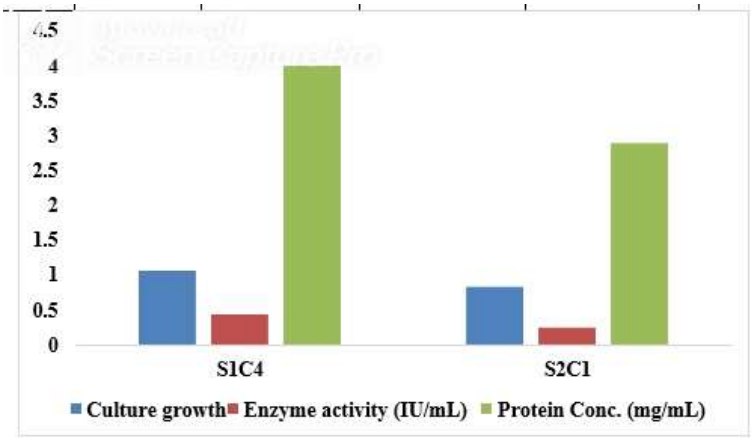

Figure 6: Graphical illustration of the Quantitative screening of xylanase production.

The selected isolate S1C4 was subjected to morphological, microscopic, and biochemical characterization to determine its taxonomic identity. Gram staining revealed **Gram-positive cocci-shaped cells**, indicating bacterial characteristics consistent with members of the genus *Bacillus*. Although endospore staining showed negative results, other biochemical properties supported its classification as a *Bacillus* species. Biochemical tests demonstrated positive results for glucose and sucrose fermentation, catalase activity, starch hydrolysis, and xylan hydrolysis, confirming the organism's ability to metabolize complex polysaccharides. The positive catalase test suggests aerobic metabolism, a characteristic feature of *Bacillus* spp. The negative indole and methyl red tests, along with a positive Voges–Proskauer reaction, further supported its biochemical profile.

Table 2: Biochemical tests for S1C4 culture

S.No	Biochemical tests	Observation	Result
1	Gram staining	Purple coccus shaped bacteria	Gram positive cells

2	Endospore staining	Pink coccus shaped bacteria	Negative , non-spore forming bacteria
3	Glucose fermentation test	Red color of phenol red changed into yellow color	Positive
4	Sucrose fermentation test	Red color of phenol red changed into yellow color	Positive
5	Maltose fermentation test	No change in color	Negative
6	Indole test	No change in appearance	Negative
7	Xylan hydrolysis test	Orange zone of hydrolysis forms near the culture	Positive
8	Starch hydrolysis test	White zone of hydrolysis forms near the culture	Positive
9	Catalase test	Bubble formation	Positive
10	MR test	Yellow colour	Negative
11	VP test	Pink ring formed at the surface	Positive

To enhance xylanase production, the selected *Bacillus* isolate was subjected to physical (UV radiation) and chemical (ethidium bromide) mutagenesis. Mutated cultures were rescreened on xylan agar plates, where several mutants exhibited significantly larger hydrolysis zones compared to the wild strain. This increase in zone diameter reflects enhanced enzyme secretion, possibly due to mutations affecting regulatory genes or promoter regions involved in xylanase expression. Among UV-treated cultures, the strain exposed to 8 minutes of UV irradiation showed the highest enzyme activity (0.86 IU/mL), surpassing the wild strain (0.453 IU/mL). Lower exposure times resulted in reduced enzyme activity,

likely due to insufficient mutational events, while excessive exposure led to cell damage and enzyme loss. This indicates that optimal mutagen exposure is crucial for achieving beneficial mutations without compromising cell viability.

Table 3: Physical and chemical mutation for strain improvement for enhancement in xylanase production.

Sr. No.	Culture No.	Culture growth	Enzyme activity (IU/mL)	Protein Conc. (mg/mL)
1	Control	1.09	0.453	4.0
2	2 min UV	0.41	0.29	0.89
3	4 min UV	0.39	0.01	0.82
4	8 min UV	1.12	0.86	2.31
5	2 ug EtBr	0.52	0.09	4.4
6	4 ug EtBr	0.07	0.27	2.75
7	8 ug EtBr	0.09	0.00	0.08

Fermentation is done using shake flask method. The bacterial strain is fermented in the optimized media under optimum conditions. After fermentation, centrifuge the crude enzyme and collect the supernatant and store it at 4°C. Total supernatant measured was 20 ml. The crude enzyme obtained after precipitation was salt precipitated. Once salt precipitation is completed, centrifuge the enzyme and dissolve the pellet in Tris buffer of pH 7. 6ml of enzyme in Tris buffer was obtained. Dialysis is done in order to remove the salt present in the enzyme suggesting enhanced xylanase secretion.

Table 4: Enzyme assay by DNS and Bradford method

Sr. No.	Sample	Protein concentration (mg/mL)	Enzyme activity (IU/mL)
Crude enzymes			

1	**Wild strain**	1.10	0.85
2	**UV mutated strain**	1.22	0.91
Pure enzyme			
1	**Wild strain**	1.13	0.86
2	**UV mutated strain**	1.39	0.98

Overall comparison between wild and mutated strains clearly demonstrated the superiority of the UV-mutated *Bacillus* strain in terms of xylanase activity, protein secretion, and enzyme stability. The enhanced performance of the mutated strain confirms the effectiveness of strain improvement strategies in overcoming limitations associated with native isolates.

These findings strongly support the use of classical mutagenesis as a cost-effective and efficient approach for improving enzyme yield, especially in developing industrial settings where advanced genetic engineering techniques may not be readily accessible.

The results confirm soil as a rich source of xylanase-producing bacteria. *Bacillus* spp. demonstrated efficient xylan degradation, consistent with previous studies. Strain improvement via mutagenesis significantly enhanced enzyme yield, supporting its application as a practical alternative to recombinant approaches. Improved xylanase production can reduce enzyme costs and promote sustainable industrial processes.

4. Conclusion

The study successfully isolated and identified xylanase-producing *Bacillus* spp. Strain improvement through mutagenesis led to enhanced xylanase production compared to the wild strain. These findings suggest that mutated *Bacillus* strains hold strong potential for industrial applications in biobleaching, food processing, and biofuel production. Further optimization of fermentation parameters, scale-up studies, and molecular characterization of mutated strains can strengthen the commercial applicability of the developed strains.

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