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Full Length Article

Enhanced Xylanase Production by N-methyl-N-nitro-N-nitrosoguanidine Mutated *Aspergillus niger* using Agricultural Waste

Sikander Ali[1*], Saif Ullah[2], Muhammad Usman Ahmad[1], Umama Saif[1], Ayesha Saleem[1], Aimenara Shafique[1], Huma Ijaz[1] and Saba Amjad[1]

[1]*Department of Microbiology, Dr. Ikram-ul-Haq Institute of Industrial Biotechnology, Government College University Lahore, Pakistan*

[2]*Department of Mathematics, Government College University Lahore, Pakistan*

[*]For correspondence: dr.sikanderali@gcu.edu.pk

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Abstract

Xylanases (EC 3.2.1.8) are key enzymes that hydrolyze *β*-1,4-glycosidic linkages in xylan, facilitating its depolymerization. Microorganisms like bacteria, fungi, and actinomycetes are capable of producing enzymes that withstand high temperatures. This study focused on improving xylanase production by screening mutant strains of *Aspergillus niger* GCBT-35 (wild-type) that were resistant to 2-deoxy D-glucose following exposure to N-methyl N-nitro N-nitroso guanidine (MNNG) for 15–45 min. To maximize enzyme yield, various culture conditions were optimized in a solid-state fermentation system. Different agricultural by-products, including wheat straw, wheat bran, sunflower meal, soybean meal, newspaper, rice husk, rice straw, and bagasse, were tested as substrates. Wheat bran, at a 1:1 ratio (w/v), was identified as the most effective substrate for xylanase production. The highest enzyme activity (94.9 U/mg/min) was achieved 48 h after inoculation when the moistening agent had a pH of 5.0, controlled incubation temperature of 30°C and the nitrogen source was optimized to 0.2% ammonium sulfate [$(NH_4)_2SO_4$]. Xylanase production was boosted by 2.3 times compared to the wild-type strain. These findings revealed that mutagenesis significantly enhanced xylanase yield, highlighting its potential for industrial-scale enzyme production. The study highlighted the importance of strain improvement and fermentation optimization in developing cost-effective biotechnological applications for xylanase production.

Keywords: *Aspergillus niger*; Xylanase; Agricultural waste; Lignocellulosic materials; MNNG induced mutation

Introduction

A complex hemicellulose polysaccharide in plant cell walls, the xylan, is primarily found in grasses, hardwoods, and softwoods (Zhang *et al.* 2023). Its backbone is made up of D-xylose units connected by *β*-1,4- glycosidic linkages; side chains including L-arabinose, acetyl, feruloyl and glucuronopyranosyl groups are frequently added to replace them (Ghosh *et al.* 2020; Curry *et al.* 2023). Endo-1, 4-D-xylanases, *β*-xylosidases, acetylxylan esterases and ferulic acid esterases are among the xylanolytic enzymes that must cooperate in order to breakdown of xylan into fermentable sugars (Walia *et al.* 2017). One of these essential enzymes, xylanase (EC 3.2.1.8), hydrolyses glycosidic bonds and aids in depolymerisation of xylan. Natural producers of these enzymes include bacteria, actinomycetes and fungi (Liu *et al.* 2023). Heat stable xylanases classified into glycoside hydrolase families GH 10 and GH 11, which show remarkable activity at high pH and temperature, make these enzymes economical for industrial processes (Hamann and Noronha 2022). Structural changes including disulphide and hydrogen bonds and salt bridges improve the stability of enzyme in harsh conditions and make enzyme thermostable (Vandenberghe *et al.* 2020). The species of genera *Bacillus*, *Clostridum*, *Streptomyces* and *Thermotoga* are microbial source of thermostable xylanases; each of which has specific pH and temperature optimal range, which are suitable for various uses (Akpinar and Karaoğlu 2024). Depending on microbial strain employed, solid-state and submerged fermentation are the two methods for xylanase production. As bacteria requires a lot of water so submerged fermentation (SmF) is better for bacteria. On the other hand, fungi require solid surfaces for growth, so solid state fermentation (SSF) is better for them (Chadha *et al.* 2019).

Xylanases have become extremely important in industry because of its wide range uses in various fields

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(Paul and Thatoi 2022). It is a sustainable bio-bleaching alternative that paper and pulp industry uses extensively to lessen the use of harmful chemicals like oxides of chlorine. Xylanases help to remove lignin from pulp by breaking down its hemicellulosic components, thus reduces the need for chemicals, lessens environmental pollution, and enhances the brightness and strength of paper (Kumar 2020). In baking, the food industry also uses xylanases to improve dough qualities, and aid in arabinoxylans in wheat flour breakdown, which improves water absorption and bread texture, produce gluten networks, and increase loaf volume (Kaur *et al.* 2019; Sun *et al.* 2022). In fabric processing, especially in bio-scouring and bio-polishing, xylanases help the textile industry to remove contaminants of hemicellulose from cotton and other materials derived from plant fibers, improving fabric smoothness, brightness, and dye absorption by lowering the use of toxic chemicals (Subash and Perumalsamy 2021; Elfaleh *et al.* 2023). It is essential for the enzymatic breakdown of lignocellulosic biomass in the producing biofuel because they release sugar that ferment and transformed it into bioethanol (Freitas *et al.* 2019). The potential of xylanases has also been utilized by pharmaceutical sector, specifically in the synthesis of xylo-oligosaccharides (XOS), which act as prebiotics and support gut health by activating *Lactobacillus* and *Bifidobacterium* (Jain *et al*. 2015). Furthermore, xylanases lower the organic load, improve biodegradability in wastewater, and break down industrial waste discharge (*e.g*., hemicellulose), have demonstrated potential in wastewater treatment (Irfan *et al.* 2016). Because of their versatility and environmental friendliness, xylanases are essential for many industrial processes that call for economic and sustainable enzymatic solutions.

The current study was aimed to create a xylanase hyper-producing strain of *Aspergillus niger* using the chemical mutagen N-methyl-N-nitro-N-nitrosoguanidine (MNNG) to enhance xylanase activity. To make this process cost effective, different agricultural wastes/by-product were used as a sustainable substrate for the enhanced xylanase production by the mutated *Aspergillus niger* strain. Optimization of fermentation conditions by using One Parameter at a Time approach (OPAT) was employed for the mutated strain to achieve maximum xylanase yield, making the process efficient and economically viable.

Materials and Methods

Organism

The fungal strain *A. niger* GCBT-35 was sourced from culture bank of Dr. Ikram ul Haq Institute of Industrial Biotechnology at GCU, Lahore, Pakistan. For culture maintenance, xylan agar slants featuring a customized blend of xylan, agar, inorganic salts and yeast extract at a pH of 5.5 were used. The culture was stored at a temperature of 4°C using a laboratory cold storage unit (Model MPR-1410, SANYO, Japan).

Inoculum preparation

A 250 mL conical flask was filled with 45.0 mL of Vogel's medium, consisting of (% w/v): trisodium citrate (0.25), KH_2PO_4 (0.5), NH_4NO_3 (0.2), $(NH_4)_2SO_4$ (0.4), $MgSO_4$ (0.02), peptone (0.1), yeast extract (0.2) and adjusted to pH 5.5. Approximately 20–25 glass beads (2.0–5.0 mm in diameter) were added to facilitate the breakdown of mycelial pellets. The medium was sterilized at 121°C, subjected to 103.4 kPa pressure for 15 min. After cooling, a 2.0 mL glucose supplement was added to enhance carbon availability. Then *A. niger* conidia were introduced into the medium, and the mixture was incubated at 30°C with agitation at 200 rpm. After incubation, cells were isolated through centrifugation at 6,000 rpm for 15 min, washed, and resuspended. This suspension was then exposed to a mutagenic agent, MNNG.

Mutation technique

A sterile centrifuge tube was aseptically inoculated with a 2 mL aliquot of vegetative mycelial suspension. Four concentrations of MNNG (1.0, 2.0, 3.0 and 4.0 mg/mL) were prepared using a phosphate buffer solution. Various volumes (1.0, 2.0, 3.0 and 4.0 mL) of each MNNG concentration were added to mycelial suspension. A control experiment was conducted by replacing MNNG with sterile distilled water. After a specified time interval (15–45 min at 30°C), the tubes were centrifuged, and the supernatant containing MNNG was discarded. The mycelial cells were washed twice using phosphate buffer to eliminate any residual MNNG. Following resuspension in phosphate buffer to a final volume of 5.0 mL, the cell suspensions were vigorously agitated. Aliquots (0.1 mL) of each dilution were then transferred to individual petri plates containing xylan agar and Triton X-100 medium. The petri plates were incubated at 30°C for 72 h, after which the colony count for each plate was determined. Colonies showing a larger clear zone of xylan degradation were selected and subcultured onto xylan agar slants to promote the growth of xylanase-producing microorganisms. Sporulation was promoted by incubating the cultures at 30°C for 3–5 days. A group of 20 mutant cultures was selected and subjected to further analysis of xylanase production using a shake flask method.

Resistance and storage of culture

Selected *A. niger* cultures were gradually adapted to tolerate increasing levels of 2-deoxy D-glucose following the methods described by Suzuki *et al*. (1996) and Parvez *et al*. (1998). For future use, the cultures were preserved at 4°C.

Suspension of fungal conidia

The conidial suspension was created by introducing a surfactant solution into a mature fungal culture. A 10 mL of

sterilized Monoxal O.T. (0.005% w/v) was added to a 3–5 day old xylan agar slant culture. The mixture was then gently manipulated to release conidia from the culture surface and resulting suspension was vigorously agitated to ensure uniformity.

Fermentation technique

A 10 g sample of substrate was placed in each 250 mL conical flask, sealed with a cotton plug. Then it was moistened by the addition of 10 mL of fermentation medium that consists of (%, w/v): $NaNO_3$ 0.1, NH_4Cl 0.1, KH_2PO_4 0.1, $CaCl_2$ 0.1, $MgSO_4.7H_2O$ 0.03, meat extract 0.5, Tween-80 0.15 mL at pH 5.0. The flasks were autoclaved at 121°C and 0.1 MPa pressure for a 15 min cycle to ensure sterilization. After cooling at room temperature, the flasks were inoculated with 1 mL of the conidial suspension containing 1.2×10^6 conidia after Sharma (1989) and incubated at 30°C in an incubator for 72 h. After 72 h incubation, 100 mL of extractant and 18–20 glass beads (2–5 mm diameter) were added to each flask to facilitate comprehensive enzyme extraction. After the addition of extract and glass beads, the flasks were incubated in a rotary shaker at 200 rpm for 1 h, and the resulting filtrate was used for xylanase analysis. Triplicate experiments were performed, with all replicates running simultaneously.

Xylanase activity

The saccharifying activity of xylanase as assessed by quantifying the release of reducing sugars from xylan. This was achieved using a colorimetric assay, which involved measuring the absorbance of the reaction mixture (DNS method) in accordance with Miller (1959). The reaction mixture consisted of 1 mL of 1 % (w/v) xylan, 0.5 ml of 0.1 M acetate buffer (pH 4.5), as well as 0.5 mL of the enzyme dilution. Incubation at 50°C for 30 min was followed by measurement of the mixture's absorbance using a spectrophotometer. The xylanase activity unit (U) was defined as the amount of enzyme necessary to release 1 μmol of reducing sugar (equivalent to xylose) per min under the specified conditions, and the enzyme activity was expressed as U/mg/min.

Statistical analysis

Treatment effects were compared by the Fisher's least significant difference test also known as protected least significant difference method (Costat, cs6204w.exe) after Snedecor and Cochran (1980). General mathematical formula for Fisher's least significant difference test was:

$$LSD_{A,B} = t_{0.05/2\ DFW} \sqrt{MSW\left(1/n_A + 1/n_B\right)},$$

Where, t is the critical value from the table of t-distribution, *MSW* stands for *mean square within*, obtained from the results of Analysis of Variance test and n represents the number of scores used for calculation of means. Significance difference among the replicates has been presented as Duncan's multiple ranges in the form of probability (<p>) values.

Results

The results showed that mutant strain RH_{MNNG}^{13} exhibited the highest xylanolytic activity (Table 1), with 57.31 U/mg/min, which was approximately 1.4 times higher than the wild-type strain GCBT-35. The choice of substrate for xylanase production *via* solid-state fermentation is influenced by multiple factors, primarily cost and availability, necessitating the evaluation of various agricultural residues by *A. niger* RH_{MNNG}^{13} (Fig. 1). The mutant strains were isolated after the improvement of parental *A. niger* GCBT-35. Fungal mortality approached 100% at MNNG concentrations exceeding 4.0 mg/mL. The fermentation medium was incubated at 30°C for 24–168 h after conidial inoculation (Fig. 2), resulting in a gradual increase in xylanolytic activity. The maximum activity (64.31 U/mg/min) was achieved 48 h after inoculation. The impact of temperature on xylanase biosynthesis was investigated over a range of 20–45°C (Fig. 3). The mutant *A. niger* RH_{MNNG}^{13} showed promising results, producing 65.98 U/mg/min xylanase at 30°C.

A comparative study was conducted to assess the effects of different buffers, distilled water, and tap water on the extraction of xylanase, aiming to optimize enzyme yields. The results showed that distilled water (pH 7.0) yielded the highest xylanase production when used for enzyme extraction from fermented wheat bran (Fig. 4). This may be because the production of microbial enzymes is pH-dependent, and at pH values higher than neutral, the mutant strain *A.niger* RH_{MNNG}^{13} produces proteolytic enzymes that degrade xylanase activity (Fig. 5).

The effect of adding different nitrogen sources to the fermentation medium was noted to further increase the fungus xylanase production. Effects of different nitrogen sources, such as NH_4NO_3, NH_4CL, $(NH_4)_2SO_4$, $NaNO_3$ and urea were assessed to check the production of xylanase (Fig. 6). $(NH_4)_2SO_4$ produced 79.2 U/mg/min activity, out of all the nitrogen sources evaluated. After that optimization of $(NH_4)_2SO_4$ concentration, a range of 0.1–0.5% values were tested (Fig. 7). Among these, 0.2% $(NH_4)_2SO_4$ produced the most xylanase (94.9 U/mg/min) and was considered as optimal for xylanase production by *A. niger* RH_{MNNG}^{13}. Further increase in the concentration of $(NH_4)_2SO_4$ did not boost the synthesis of xylanase considerably.

Discussion

MNNG treated alkylated guanine residues, resulting in permanent lesions of DNA and clusters of similar mutations that was responsible for the mutant strains enhanced xylanase output (Tasneem *et al.* 2003). Use of wheat bran as the substrate resulted in the best enzyme production.

Table 1: Screening chemically mutated *A. niger* strains for enhanced xylanase production

MNNG-treated mutant strains	MNNG conc. (mg/mL)	Time of exposure (min)	Xylanase saccharifying activity (U/mg/min)
Parental	-	-	40.67 ± 0.75^{ef}
RH_{MNNG}^{1}	1.0	15	40.67 ± 0.75^{ef}
RH_{MNNG}^{2}			35.44 ± 1.33^{g}
RH_{MNNG}^{3}			35.97 ± 1.64^{g}
RH_{MNNG}^{4}			19.99 ± 1.1^{ij}
RH_{MNNG}^{5}			35.08 ± 0.94^{g}
RH_{MNNG}^{6}			34.40 ± 0.54^{g}
RH_{MNNG}^{7}			28.16 ± 1.62^{h}
RH_{MNNG}^{8}	2.0	30	43.99 ± 1.73^{de}
RH_{MNNG}^{9}			19.07 ± 1.15^{j}
RH_{MNNG}^{10}			42.67 ± 2.33^{ef}
RH_{MNNG}^{11}			53.70 ± 1.29^{bc}
RH_{MNNG}^{12}	3.0	45	40.38 ± 1.93^{f}
RH_{MNNG}^{13}			57.31 ± 1.23^{a}
RH_{MNNG}^{14}			46.79 ± 1.76^{d}
RH_{MNNG}^{15}			54.19 ± 3.4^{abc}
RH_{MNNG}^{16}			43.96 ± 2.8^{de}
RH_{MNNG}^{17}	4.0	30	57.39 ± 2.06^{a}
RH_{MNNG}^{18}			53.04 ± 2.8^{bc}
RH_{MNNG}^{19}			51.57 ± 0.73^{c}
RH_{MNNG}^{20}			55.52 ± 2.3^{ab}

The ± represents standard deviation calculated from three independent replicate experiments. The values designated by letters differ significantly at $P \leq 0.05$. Fermentation period 72 h, initial pH 5.0, incubation temperature 30°C

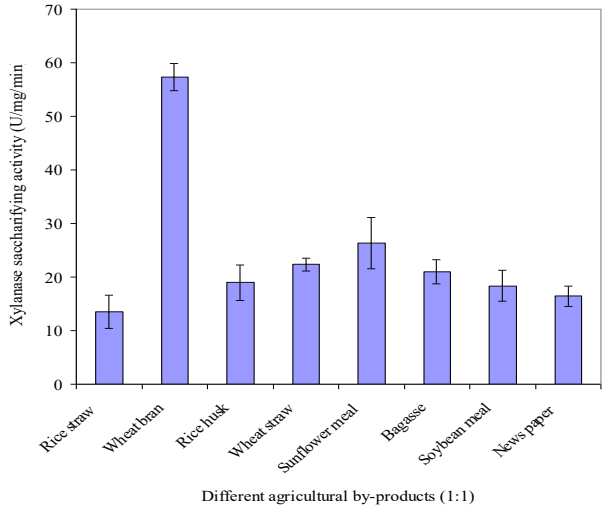

Fig. 1: Assessment of agricultural residues for xylanase production by *A. niger* RH_{MNNG}^{13}. Optimized fermentation conditions (72 h, pH 5.0, 30°C). Error bars represent variability among triplicate experiments

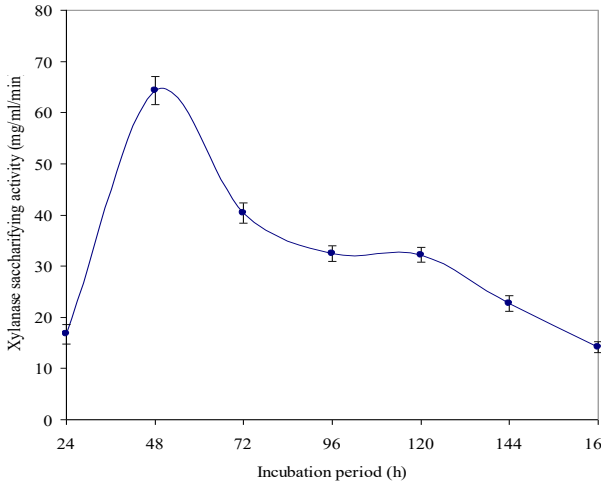

Fig. 2: Effect of time duration for xylanase production by *A. niger* RH_{MNNG}^{13}. Optimized conditions (pH 5.0, 30°C). Standard deviation values are indicated by error bars. A significant correlation was observed between the initial pH of the medium and xylanase production by mutant *A. niger* RH_{MNNG}^{13} (Fig. 3). Maximum xylanolytic activity was obtained at pH 5.0

The efficacy of wheat bran was probably influenced by its balanced macronutrient and micronutrient composition, which included proteins (1.32%), fats (1.9%), ash (1.8%), carbohydrates (69.0%), fibre (2.6%), and important minerals like sulphur (0.12%), magnesium (0.17%), potassium (0.35%), calcium (0.05%), and phosphorus (0.35%). Moreover, wheat bran provided the cells with an appropriate anchor, promoting the microorganism's growth and subsequent synthesis of xylanase (Park *et al.* 2002). The highest enzyme synthesis was also noted by Roy (2004) when growing on wheat bran. Unlike wheat bran, perhaps the whole spectrum of nutrients needed for the best possible enzyme production and fungal growth was not supplied by other substrates. Sharma *et al.* (2021) claimed that the mutant *A. niger* strains notably increased the extracellular xylanase synthesis and thereby demonstrating that how well mutagenesis works to increase enzyme yields. In addition to supporting sustainable and affordable enzyme production, using agricultural residue as carbon source encourages waste valorization. Use of UV and chemical mutagenesis to select stable, high-yielding mutants, which offered an effective method for producing xylanases in an industrial setting.

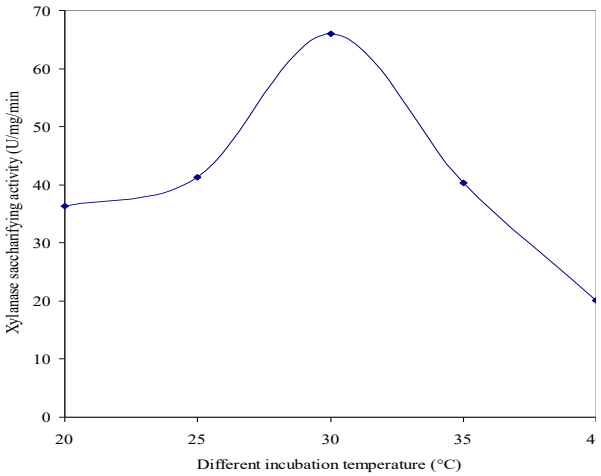

Fig. 3: Effect of incubation temperature for xylanase production by *A. niger* RH_{MNNG}^{13}. Optimized conditions (48 h incubation; pH 5.0)

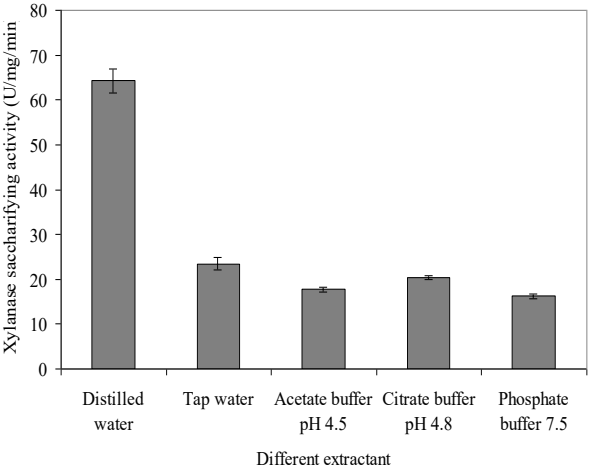

Fig. 4: Effect of various extractant for xylanase production by *A. niger* RH_{MNNG}^{13}. Optimized conditions (48 h incubation; pH 5.0; 30°C). Standard deviation values are indicated by error bars

Because of the efficient breakdown of susceptible xylan substrates, fungal aging, self-produced inhibitors, and decreased available nitrogen, incubating longer than 48 h did not increase the synthesis of xylanase (Reese *et al.* 1969). Gutierrez-Correa and Tengerdy (1998) found that the highest xylanolytic activity was reached after 72 h of incubation, which corroborates with the current results. A steady rise in xylanolytic activity throughout the course of incubation period indicated that *A. niger* GCBCX-20 effectively acclaimed to the fermentation conditions, resulting in increased enzyme synthesis (Haq *et al*. 2004). A 48 h incubation period and an ideal temperature of 30°C promoted maximum xylanase synthesis, suggesting that an exact control of culture conditions is essential for higher yields. Time-efficient fermentation techniques are essential, as prolonging the incubation period past the ideal point could not yield a significant increase in enzyme production

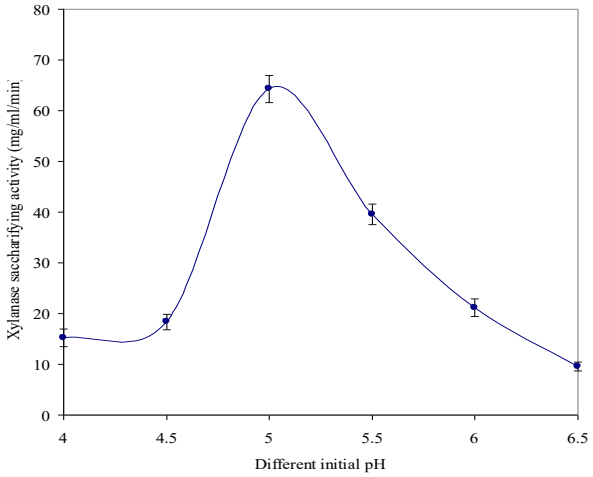

Fig. 5: Effect of pH for xylanase production by *A. niger* RH_{MNNG}^{13}. Optimized conditions (48 h incubation; 30°C). Standard deviation values are indicated by error bars

(Suleman *et al.* 2016). The production of xylanase was negatively impacted by temperature increase above 30°C. According to Murthy *et al.* (2009), fungal growth and enzyme synthesis are inhibited by the denaturation of enzymes at high temperature and the lower moisture content under solid-state fermentation conditions. This procedure was made simple and economical by percolating the bioreactor with an appropriate extractant after cultivation to extract enzyme from its substrate (Shokrkar *et al.* 2018).

A. niger RH_{MNNG}^{13} xylanase saccharifying activity was affected by several extractants. The results indicated that parameters including temperature, pH and the quantity of surfactants were required for the best enzyme production (Guimaraes *et al.* 2013). Sodium dodecyl sulphate treatment significantly improved xylanase activity in our study, suggesting its potential role for enzyme efficiency, which is consistent with findings reported by Fasiku *et al*. (2023) with *A. niger* and *B. megaterium*. The production of xylanase reduced when pH was altered because the organism requires a slightly acidic pH for growth and synthesis (Gomes *et al.* 1994). Gaspar *et al*. (1997) and Haq *et al*. (2004) also emphasize the importance of medium pH in mould metabolism.

According to Cui *et al.* (2016), the impact of various nitrogen sources on *A. niger* RH_{MNNG}^{13}'s xylanase saccharifying activity showed that nitrogen availability had a major impact on enzyme production, comparable to the patterns of expression seen in *A. niger* A09. Results revealed that ammonium-based nitrogen sources, including $(NH_4)_2C_2O_4$, increased xylanase activity. This was probably due to regulated gene expression and optimized metabolic pathways for effective xylan breakdown. The significance of careful nutrient selection to attain the highest productivity of xylanase for commercial application was underscored by the varying effects of available nitrogen on enzyme activity (Shahryari *et al.* 2019). As noted by Ameen (2023) in *A. fumigatus* KSA-2, the impact of varying ammonium sulphate concentrations on the xylanase saccharifying activity by *A.*

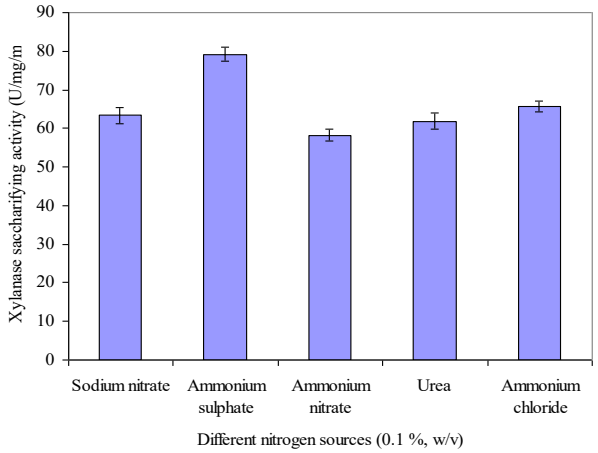

Fig. 6: Effect of different nitrogen sources for xylanase production by *A. niger* RH_{MNNG}^{13}. Optimized conditions (48 h incubation; pH 5.0; 30°C). Standard deviation values are indicated by error bars

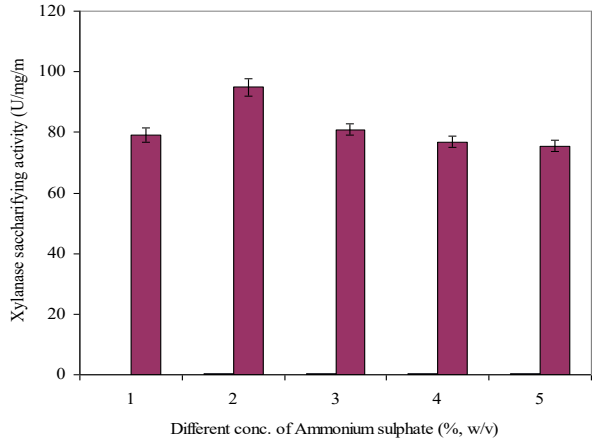

Fig. 7: Effect of different concentrations of ammonium sulphate for xylanase production by *A. niger* RH_{MNNG}^{13}. Optimized conditions (48 h incubation; pH 5.0; 30°C). Standard deviation values are indicated by error bars

niger RH_{MNNG}^{13} demonstrated that salt precipitation greatly contributed to the yield and purity of enzyme. As in wheat bran fermentation, 70% ammonium sulphate concentration resulted in improved xylanase activity and stability, as well as increased enzyme recovery (Dhaver *et al.* 2022). In industrial applications, the findings of this study emphasize how crucial it is to maximize xylanase saccharifying effectiveness by optimizing the precipitation conditions.

Conclusion

By using traditional mutagenesis, this study demonstrated that microbial strains might be altered to produce more effective enzymes. By using MNNG in chemical mutagenesis, the highly productive *A. niger* mutated strain RH_{MNNG}^{13} was created with 2.3 times more xylanolytic activity as compared to *A. niger* GCBT-35. Additionally, solid-state fermentation conditions must be optimized to maximize enzyme yield. For industrial applications these findings demonstrated a significant ($P \leq 0.05$) increase in xylanase production by process optimization and targeted strain improvement.

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Author Contributions

SA and MUA conceptualized the research, supervised and designed the experiments as well as reviewed the manuscript. SU helped with statistical analysis and authored the main text of the manuscript. US, AS, AM, HI and SA conducted experimental work. MUA provided a review of the manuscript. All authors participated in reviewing and approving the final manuscript, as well as in the preparation of graphs and tables.

Conflict of Interest

The authors declare that there are no conflicts of interest associated with this study.

Consent to Publish

All authors have given their consent for the publication of this research paper and its accompanying materials.

Data Availability

All data generated or analyzed during this study are included in this manuscript.

Ethical Approval

Not applicable.

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