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Isolation and screening of xylanase-producing microorganisms

*[1]Oyewole, O. A., [2] Binuyo, O. A., [3] Joda, J. F., [4] Nabara, H. Y., [1] Tsado, P. Y., [1] Musa, F. and [1]Akinpelu, O. J.

[1]Department of Microbiology, Federal University of Technology Minna, Nigeria.
[2]Department of Medical Microbiology, National Biotechnology Development Agency, Abuja, Nigeria
[3]Department of Genomic, Genetics and Bioinformatics, National Biotechnology Research and Development Agency, Abuja, Nigeria
[4]Department of Public Health, Texila American University, Guyana, South America

ABSTRACT

Xylanases are enzymes that break down the complex polysaccharide xylan, the major component of hemicellulose. Xylanase hydrolyzes xylan by breaking the β-1,4- glycoside linkage to produce xylose and other degradation compounds. Many degradative microorganisms such as fungi, bacteria and yeast have been found to produce xylanases. In this study, xylanase-producing microorganisms were isolated from different environmental samples - paddy field rhizosphere, earthen pond sludge, decayed wood, cow dung, and sediments from Tagwai Dam, Minna, Nigeria. The samples were serially diluted and plated on a selective agar medium, which contains xylan (1.5 g), peptone (5 g), yeast extract (3 g), sodium chloride (5 g), and agar (15 g) per 1 L of distilled water using spread plate method and incubated at 37 °C for 48 hours and 25 °C for 5 days for bacteria and fungi respectively. The plate screening method was used to screen the microbial isolates for xylanase activity. Colonies with zones of inhibition indicated xylanase activity. Positive isolates were further subcultured to obtain pure isolates and were kept in slant bottles and stored at 4°C for further analysis. The pure isolates were cultured in nutrient broth for bacteria and Sabouraud dextrose broth for fungi and from the broth, into selective broth which was used to determine xylanase activity using DNS (dinitrosalicylic acid) method. The bacterial isolates were identified based on their cultural, morphological and biochemical characteristics while the fungal isolates were stained with lactophenol cotton blue. Ten (10) bacteria were isolated from the samples, of which four (*Clostridium* sp., *Escherichia coli*, *Salmonella* sp., and *Staphylococcus* sp) were xylanase producers and four (4) xylanase-producing fungi (*Aspergillus niger, A. fumigatus, A. flavus,* and *A. sydowii* were identified for fungi) were isolated. *Escherichia coli* gave the highest activity for the bacteria (0.8260) while *Aspergillus flavus* gave the highest xylanase activity for fungi (2.1118). *Aspergillus flavus* can therefore be recommended for large-scale production of xylanase.

Keywords: Xylanase, xylan, Dinitrosalicylic acid (DNS), Enzyme, *Aspergillus flavus*, *Escherichia coli*

***Correspondence author:** oa.oyewole@futminna.edu.ng

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INTRODUCTION

Xylanases are a group of enzymes that hydrolyse xylan backbone into small oligomers, they are ubiquitous and diverse by nature (Collins *et al.* 2005). The xylanolytic enzyme system includes β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase and phenolic acid esterase (Dhiman ET AL., 2008). β-1,4-endo xylanase is responsible for the hydrolysis of the main chain xylosidic linkages and β-xylosidase releases xylosyl residues after attacking the xylo-oligosaccharides This leads to the breakdown of the backbone of xylan. Other xylanolytic enzyme helps in the cleavage of the side-chain of the molecule (Uday *et al.*, 2016). The combined action of all these enzymes converts the xylan molecule into constituent sugars. Studies have shown that xylanases are produced by a variety of sources such as bacteria, fungi, yeast and algae (Mandal, 2015), seeds, snails and crustaceans (Polizel *et al.,* 2005). However, the major producers of these enzymes are fungi and bacteria. Bacterial xylanases have different characteristics from fungal xylanases. Therefore, xylanases produced by bacteria and actinomycetes (*Bacillus* sp., *Pseudomonas* sp., *Streptomyces* sp.) are efficient in a broader pH range of 5 to 9 and temperature of 35-60ºC (Beg *et al.,* 2001; Motta *et al.*, 2013; Mandal, 2015) and are useful in different industries due to their alkali tolerance and thermostability, like the pulp and paper industries (Mandal, 2015).

Fungal xylanases (*Aspergillus* sp., *Fusarium* sp., *Penicillium* sp.) are effective at a pH range of 4 to 6 and temperature below 50ºC (Mandal, 2015), thus being used in limited industrial applications. However, fungi are important producers due to their higher xylanase activity (compared with bacteria) (Motta *et al.,* 2013; Mandal, 2015) and extracellular release of the enzymes (Nair and Shashidhar, 2008). Another difference between bacterial and fungal xylanases is linked to the presence of cellulase. Few studies report fungal xylanase without cellulase activity. Xylanase enzyme plays a major important role in the industries, including the food, paper, pulp, animal feed and biofuel. The combination of the xylanase enzyme with glucanase, amylase, cellulose and pectinase have potential applications for the production of bakery foods, biscuits and wafers (Moteshafi *et al.*, 2016). There has been much mechanical enthusiasm for xylanase: as a supplement for the production of bread, and beverages, for the dying of cellulose mash and for xylitol generation. The utilization of xylanases could enormously enhance the cost-effectiveness of preparing lignocellulosic materials for the age of fluid powers and chemicals (Polizeli et al., 2005).

Despite the existing research on xylanase-producing organisms, there is still a need to explore untapped environmental sources to discover novel microorganisms with robust xylanase production potentials. Understanding the diversity of xylanase-producing microorganisms in different environments can provide valuable insights into novel microbial resources for industrial applications. Also identifying microorganisms that possess high xylanase production potential can contribute to the development of more efficient and sustainable enzymatic processes for biomass degradation and bioconversion. This study therefore aimed to isolate, screen and identify xylanase-

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producing microorganisms from different environmental samples.

MATERIALS AND METHODS

Sample Collection

Soil and sediment samples were collected from different locations, including the paddy field rhizosphere, earthen pond sludge, decayed wood, cow dung, and Tagwai Dam sediments in Minna, Nigeria. The collected samples were transferred into a clean, sterile sample collection bag to avoid contamination.

Preparation of Selective Agar Media

A selective agar medium was prepared by dissolving xylan (1.5 g), peptone (5 g), yeast extract (3 g), sodium chloride (5 g), and agar (15 g) per 1 L of distilled water. The pH was adjusted to 7.0 using phosphate buffer. The mixture was heated to dissolve, and autoclaved at 121°C and 15 psi for 15 minutes and then cooled to 45°C. The medium was then poured into sterile Petri dishes and allowed to solidify.

Isolation of microorganisms

One gram(1g) of each soil sample was mixed with 9 ml of sterile distilled water after which 1 ml was drawn and inoculated into a test tube containing 9 ml of distilled water, establishing a 1:10 dilution. The process was repeated for 5 test tubes. A 0.5 ml of diluent from test tubes 3 and 5 was directly inoculated onto a selective agar plate using the spread plate method for bacteria while test tubes 2 and 3 were used for fungi. The plates were left undisturbed briefly to allow the inoculum to be absorbed. Subsequently, the plates were inverted and incubated at 37°C for 48 hours for bacteria while for fungi the plates were incubated at 25 °C for 5 days.

Screening for Xylanase Activity

The agar plates were inspected for clear zones around colonies after incubation. These clear zones indicate xylan degradation due to the presence of xylanase activity (Burlacu *et al.*, 2016). Colonies showing such zones were considered potential xylanase producers.

Sub-culturing for Pure Isolates

Individual colonies showing xylanase activity were sub-cultured onto fresh nutrient agar plates for bacteria and on Sabouraud dextrose agar plates for fungi and slanted for further analysis at 4ºC. The media were prepared according to manufacturer's instructions, autoclaved at 121°C and 15 psi for 15 minutes and cooled to 45-50°C. The medium was poured into sterile Petri dishes and dispensed into slant bottles under aseptic conditions. The Petri dishes and test tubes were allowed to cool and solidify, creating slants in the case of the slant bottles. Incubation was at 37°C for 24 hours for bacteria and 3 days for fungi.

Preparation of Xylanase Selective Broth

After obtaining pure isolates, xylanase selective broth was prepared to assess xylanase activity. The selective broth was composed of the following components per liter of distilled water: Xylan (10 g), peptone (5 g), yeast extract (2 g), sodium chloride (5 g), and other necessary components. The pH of the broth was adjusted to 7.0 using a phosphate buffer. The mixture was heated to dissolve the components and then autoclaved at 121°C and 15 psi for 15 minutes. After autoclaving, the broth was allowed to cool

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to 45°C and then dispensed into sterile tubes (100 ml each), ensuring proper sealing with caps (Anwar *et al.*, 2023).

Inoculation and Incubation in Xylanase Selective Broth

A loopful of each pure isolate was inoculated into each prepared xylanase selective broth using aseptic techniques. The tubes were securely closed and incubated at 37°C for 24 hours for bacteria and 3 days for fungi (Anwar *et al.*, 2023).

Determination of Xylanase Activity using DNS Method

The DNS method (dinitrosalicylic acid method) was employed to quantitatively assess xylanase activity. A 1 ml of the cultured xylanase selective broth was mixed with 1 ml of 1% xylan solution in a test tube. The mixture was incubated at 50°C for 10 minutes to allow enzyme-substrate interaction. After incubation, 1 ml of DNS reagent was added to stop the reaction and develop color. The test tube was placed in boiling water for 5 minutes and then cooled. The appearance of a color change from blue to orange indicated positive xylanase activity (Senthil *et al.*, 2014).

Optical density measurement

Measurement of optical density was taken for each isolate with the use of a spectrophotometer, using the wavelength of 600nm for 3 consecutive days for bacteria and 5 days for fungi.

Identification of isolates

Morphological characteristics, Gram staining and biochemical tests were used to identify bacteria isolated. Colony characteristics, spore structures and hyphal morphology were used to identify each fungus isolated (Suleman *et al.*, 2016)

RESULTS

Cultural, Morphological and Biochemical Identities of the Bacterial Isolates

Ten (10) bacteria were isolated from fresh soil samples. They exhibited wide morphological variation (Table 1). Bacterial morphotypes were selected based on their macroscopic properties such as colony colour, shape, elevation, texture and appearance of the zone of inhibition around them, which indicated the production of xylanase to utilize xylan as a carbon source on the xylanase selective media plate.

Table 1: Colony morphology of bacterial isolates

Isolate code	Form	Size	Colour	Elevation	Texture	Opacity
PF3	Circular	Small	Grey-yellow	Raised	Smooth	Translucent
PF5	Circular	Small	Grey	Raised	Smooth	Translucent
TD3	Circular	Small	Grey-yellow	Raised	Smooth	Semi-opaque
TD5	Circular	Small	Grey-yellow	Raised	Smooth	Semi-opaque
WR3	Circular	Small	Grey-yellow	Raised	Smooth	Translucent
WR5	Circular	Small	Grey-yellow	Raised	Smooth	Semiopaque
EP3	Circular	Small	Off-white	Raised	Smooth	Opaque
EP5	Circular	Small	Off-white	Raised	Smooth	Opaque
CD3	Circular	Small	Whitish	Raised	Smooth	Opaque
CD5	Circular	Small	Cream	Raised	Smooth	Opaque

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The Gram's reaction and biochemical characteristics of bacterial species isolated from the soil samples are shown in Table 2. The isolates obtained were Gram-positive rods, Gram-positive cocci and Gram-negative rods in shape. The biochemical test used for the identification of the isolates include catalase, indole, citrate, urease, and methyl-red tests. Catalase test for the isolates were positive except for PF3 and PF5. Methyl-red test for all isolates was positive.

Table 2: Biochemical identification of bacterial isolates

Isolate code	Gram reaction	catalase	indole	Citrate utilization	Urease	Methyl red	Suspected organism
PF3	+ rods	-	-	+	-	+	*Clostridium* sp.
PF5	+ rods	-	-	+	-	+	*Clostridium* sp.
TD3	+ rods	+	+	-	-	+	*Escherichia coli*
TD5	+ rods	+	-	+	-	+	*Salmonella* sp
WR3	+ rods	+	+	-	-	+	*Escherichia coli*
WR5	+ rods	+	+	-	-	+	*Escherichia coli*
EP3	+ cocci	+	-	+	+	+	*Staphylococcus* sp.
EP5	+ cocci	+	-	+	+	+	*Staphylococcus* sp.
CD3	+ cocci	+	-	+	+	+	*Staphylococcus* sp.
CD5	+ cocci	+	-	+	+	+	*Staphylococcus* sp.

Keys: +: Positive; -: Negative

Xylanase activity

All the obtained isolates showed positive results to the test for xylanase activity using DNS method. The result of the test was confirmed by the colour change from blue to orange.

Optical activity

The PF3 showed the highest activity as shown in Table 3, with the highest daily progressive increase and had the highest activity on day 3 of the spectrophotometric reading (0.8260).

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Table 3: Xylanase activity (optical density)

Isolate code	Form	Size	Colour
Clostridium sp.	0.4076	0.5690	0.6410
Clostridium sp. *Escherichia coli*	0.1946 0.6394	0.2054 0.6976	0.6598 0.8260
Salmonella sp	0.3795	0.3937	0.7619
Escherichia coli	0.2087	0.2193	0.6179
Escherichia coli	0.3038	0.3754	0.5899
Staphylococcus sp.	0.2913	0.5609	0.5918
Staphylococcus sp.	0.1898	0.2411	0.4466
Staphylococcus sp.	0.2630	0.4401	0.5551
Staphylococcus sp.	0.2566	0.5822	0.7646

Control: 0.1778, Wavelength used: 600 nm

Fungi

After the screening for xylanase activity, the fungal isolates obtained from the study were *Aspergillus niger, A. sydowii, A. flavus* and *A. fumigatus* as presented in Table 4 while their growth in xylanase medium is presented in Table 5.

Table 4. Fungi isolated from various samples

Samples	Location and coordinate		Suspected isolate
Dam Sample	Tagwai Dam	90 31' 51.6" N|60 28' 46.08"E90 31' 55.16"E	*A. niger*
Earthen Pond	Lapai Gwari	9039'13.76"N|6031'31.39"E	*A. sydowii*
Paddy field	Bosso	9035'34.68"N 16034'30.76"E	*A.flavus*
Cow dung	Garatu	9031'20.66"N 160 30' 6.78"E	*A. fumigatus*

Table 5. Showing Fungal Growth in Xylanase Medium

Days	*A. sydowii*	*A. niger*	*A. fumigatus*	*A. flavus*
0	0.5321	0.6189	0.2120	0.6189
1	0.7634	0.9406	1.0941	1.1612
2	0.6585	0.5962	0.1758	0.1140
3	1.2750	1.4292	0.4909	2.1118
4	0.6496	1.1289	1.0941	0.6682
5	0.7628	0.6613	1.3716	1.2769

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DISCUSSION

Based on the biochemical reactions, the suspected organisms were identified as *Clostridium* sp. for PF3 and PF5, *Escherichia coli* for TD3, WR3, and WR5, *Salmonella* sp. for TD5, and *Staphylococcus* sp. for EP3, EP5, CD3, and CD5. The results of xylanase production among the different isolates showed that *Clostridium* sp., *E. coli*, and *Staphylococcus* sp. had an increase in xylanase activity from Day 1 to Day 3. Notably, *E. coli* exhibited the highest activity by Day 3, suggesting a robust xylanase production.

Different microbial strains possess unique capabilities for enzyme production. *Escherichia coli* might have exhibited rapid growth and sustained viability, leading to a continuous increase in xylanase activity over the fermentation period. *Escherichia coli* may have possessed efficient regulatory mechanisms governing xylanase gene expression. These regulatory elements could respond dynamically to environmental cues, resulting in a significant upregulation of xylanase production. Belorkar and Kausar (2018) recognized the diverse capacities of microbial strains for xylanase production, with *Bacillus* sp. being commonly acknowledged. In this case, the prominence of *E. coli* aligns with Belorkar and Kausar (2018) emphasis on the variability of microbial strains in enzyme production. Evolutionary pressure or adaptation to the specific conditions of the fermentation process could contribute to its superior performance. Kallel *et al.* (2016) underscored the role of inducer and repressor molecules in gene expression for xylanase production, with xylan often acting as inducers. Xylanase activity in *Clostridium* sp., *Salmonella* sp., *E. coli*, and *Staphylococcus* sp. fluctuated over the three days. *Salmonella* sp. and *Staphylococcus* sp. however, showed a consistent increase in activity, indicating potential sustained enzyme production.

Each microbial species may have a unique metabolic rate, affecting the production and utilization of enzymes. Variations in metabolic activities among *Clostridium* sp., *Salmonella* sp., *E. coli*, and *Staphylococcus* sp. could lead to fluctuations in xylanase production. Belorkar and Kausar (2018) and Suleman *et al.* (2016) emphasized that different microbial strains exhibit varying capacities for xylanase production. *Clostridium* sp., *Salmonella* sp., *E. coli*, and *Staphylococcus* sp. likely possessed distinct genetic backgrounds influencing their ability to produce xylanase. This inherent variability could result in different patterns of enzyme activity over time.

The availability and composition of the substrate play a crucial role in enzyme production. Fluctuations in xylanase activity may be attributed to the adaptation of microbial isolates to the changing substrate conditions and the efficiency of nutrient utilization by different isolates over the three-day period. Microbial isolates respond differently to the composition of substrates, and *Salmonella* sp and *Staphylococcus* sp might efficiently utilize the provided substrate for sustained xylanase production (Dhaver *et al.*, 2022).

Variations in environmental factors such as pH, temperature, and aeration can impact enzyme production. Each microbial species may respond differently to these conditions, leading to the observed fluctuations in xylanase activity.

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Variations in pH and temperature over the three days may influence enzyme activity differently for each isolate (Yadav *et al.*, 2019; Favaron *et al.*, 2023). Genetic factors play a significant role in enzyme production. Differences in the genetic regulation of xylanase genes among the microbial isolates can result in varying expression patterns over time, contributing to the observed fluctuations.

Salmonella sp. and *Staphylococcus* sp. showing a consistent increase in xylanase activity might indicate their ability to sustain enzyme production under certain conditions. These isolates may have stress response mechanisms that allow them to adapt and maintain higher enzyme levels. Differences in the genetic regulation of xylanase genes among microbial isolates can result in varying expression patterns over time (Kallel *et al.*, 2016).

The accumulation of end-products during the fermentation process can impact enzyme production. Differences in how each isolate interacts with and responds to the accumulation of end-products may contribute to the observed variations. The growth and viability of microbial cells can influence enzyme production. Variations in growth rates and cell viability among the isolates may lead to fluctuations in xylanase activity. The inherent variability could result in different patterns of enzyme activity over time (Suleman *et al.*, 2016; Belorkar & Kausar, 2018). Microbial physiology is inherently dynamic, and enzyme production is influenced by the dynamic nature of microbial processes. Changes in cellular processes and interactions over time can result in fluctuations in xylanase activity.

The findings align with existing research efforts focused on screening, isolating, and characterizing xylanase-producing bacteria. Various studies have explored different sources, including soil, to identify bacteria capable of xylanase production. Such as the work of Ammoneh *et al.* (2014) and Belorkar and Kausar (2018) involved screening *Bacillus* isolates from soil samples, showcasing the relevance of soil as a habitat for xylanase-producing bacteria.

The optical density of the control remains relatively constant and low, indicating that the observed changes in xylanase activity in the isolates are likely due to bacterial enzymatic activity rather than spontaneous degradation of xylan. This finding aligns with research efforts focused on xylanase production optimization and screening for high-producing strains. The work of Anwar *et al.* (2023) and Favaron *et al.* (2023) emphasized the importance of optimizing xylanase production for industrial applications. The observed variations in xylanase activity among isolates underscore the significance of strain selection for optimal enzyme production. The combined results from colony morphology, biochemical identification, and xylanase activity provide a comprehensive understanding of the isolated bacterial strains. The diverse characteristics and enzymatic capabilities of these strains offer potential avenues for further exploration.

The xylanase-producing fungi isolate displayed clear zones colour representing xylanase activity. The low enzyme activity displayed by some isolates may be due to the presence of contaminants or enzyme activities being too low for complete hydrolysis of the substrate for visualisation on the substrate agar. Isolate identified as *Aspergillus flavus* exhibited

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the highest activity at pH 10 with optical density of 2.118 indicating the organism is an alkaliphile that also produced isoforms with different pH and temperature optima. Isolate identified as *Aspergillus niger* exhibited the activity at pH 10 with the value 1.4292. Also, the isolate identified as *Aspergillus fumigatus* exhibited xylanase activity with the value of 1.3716. Isolate identified as *Aspergillus sydowii* exhibited xylanase activity with the value of 1.2750 exhibiting the lowest xylanase activity. The variations in xylanase activity among isolates may have implications for potential industrial applications, such as biofuel production or food processing, where xylanase is used to break down plant cell wall components.

CONCLUSION

Ten (10) bacterial isolates were obtained from samples in this research, of which four (4) were xylanase producers. Similarly, 4 fungi were identified as xylanase producers. The bacterial isolates identified were *Clostridium* sp., *Escherichia coli, Salmonella* sp., and *Staphylococcus* sp while *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Aspergillus sydowii* were identified for fungi. The best bacterial isolate for xylanase production was *E. coli,* showing the most daily progressive increase in turbidity (0.8260) and for the fungi *Aspergillus flavus* had the highest tendency of producing xylanase after 3 days (2.118). The optimal conditions for xylanase production can be determined, which may allow efficient and cost-effective xylanase production.

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