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ORIGINAL ARTICLE

Isolation, screening, production optimization and identification of xylanase producing bacterial and fungal isolates

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ABSTRACT
20% of the global enzyme market is made up by xylanase enzyme. They play a significant role in the textile, paper pulp, bakery, and poultry feed sectors. The development of indigenous strains and scalable technology for the manufacture of industrial enzymes is essential given the rising demand for the enzyme. Enormous amount of agricultural waste is produced every year by food, forestry and agricultural industries. Without any proper treatment land disposal of these residues lead to harmful health effects and environmental pollution. However, this agricultural waste can be used as low-cost substrates for the production of industrial enzyme xylanase. Using readily accessible, low-cost agricultural waste residues, such as oat husk, maize cob, carrot pomace, and banana peel, isolates produced large amounts of xylanase in submerged fermentation (SmF). The best substrate among these was determined to be oats husk. The results of xylanase screening showed that 42 isolates had xylanase producing capability. Out of 42 isolates, 30 were bacterial isolates and 12 were fungal isolates. Following primary screening, 12 bacterial and 5 fungal isolates were selected for further studies. After second screening, bacterial isolate NDB4 and fungal isolate NDF5 were chosen for further production and optimization. Isolates NDB4 and NDF5 showed maximum zone of 28 mm and 32 mm respectively on screening media. NDB4 strain when cultured at 50°C temperature and 8.0 pH for 72 h showed xylanase production of 19 ± 0.5 Units/ml/min while NDF5 showed maximum activity of 21 ± 0.5 Units/ml/min at 35 and pH 5.0. Isolate NDB4 was later identified as Bacillus licheniformis using 16S rRNA gene sequencing whereas isolate NDF5 was identified as Aspergillus niger using ITS rRNA gene sequencing.
Keywords: *Xylanase activity, Submerged fermentation, 16srRNA sequencing, ITS sequencing*

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INTRODUCTION

Approximately 40 million tonnes of lignocellulose wastes, which comprise 30–40% hemicellulose, are produced by the agriculture and forestry industries [1]. The bulk of waste produced by the food sector is lignocellulosic in nature, making them ideal substrates for the production of high-value goods [2]. There are a lot of agricultural and municipal solid waste leftovers across the world, therefore using this waste as a substrate for the manufacture of enzymes and creating an effective enzymatic hydrolysis process opens up new opportunities for the productive treatment of these wastes [3,4]. Selection of a suitable microorganism is very important aspect for production of microbial enzymes. One of the main components of plant hemicellulose is xylan [5]. Xylanases are hydrolytic enzymes that break down 1,4-glycosidic bonds in lignocellulosic materials to produce xylose and xylo-oligosaccharides [6]. These enzymes have various biotechnological applications, including pre-treating pulps in the paper industry to reduce energy expenditure and improve yield and pulp characteristics [7,8]. Xylanases are also used in feed additives to enhance the nutritional and digestive qualities of agricultural silages and animal diets

[9]. Furthermore, they find applications in clarifying fruit juices, improving bread goods, extracting plant oils and coffee, and increasing the quality of leather while reducing environmental contamination [10,11,12,13]. Additionally, xylanases are utilized in saccharifying pre-treated lignocellulosic biomass to produce biofuels and other organic chemicals [14].

The major source of xylanases and related debranching enzymes utilized in industry is thermophilic bacteria [15]. The ideal temperature range for the bacteria to grow is between 60 and 80 °C. Because the temperature in the center of compost piles may reach 80°C, thermophiles can survive there [16]. Due to their traits and features, industries are always in need of microorganisms that generate large yields of thermostable enzymes. High temperatures and alkaline or acidic treatments can be applied to these enzymes without denaturing them [6]. Since xylanases generated by mesophilic organisms typically undergo denaturation at temperatures over 55°C, their utility is restricted [17]. A 10°C temperature rise in an industrial process can double the pace of the reaction. Despite the fact that less enzyme is needed [18]. The half-life of thermostable enzymes increases with temperature. Furthermore, microbial development is inhibited at temperatures above 60 °C, which lowers the danger of contamination [19]. Aerobes, anaerobes, mesophiles, thermophiles, and extremophiles are only a few of the bacteria, fungi, and actinomycetes that have generated xylanases [20,21]. Over xylanases made from plants, those from microbial sources are favoured. They are easily produced in small spaces, have stable structural characteristics, and are amenable to genetic modification [22]. Due to their rapid growth rates, bacteria are better suited than other microorganisms for producing xylanases [23]. *Arthrobacter*, *Bacillus, Cellulomonas, Micrococcus, Microbacterium, Staphylococcus,* and *Paenibacillus,* are recognized bacterial genera that produce xylanases [24,25,26,27]. In the present paper, we report the isolation, production optimization and identification of a bacteria and fungi producing xylanase. Here in this study different agricultural wastes were used for production of xylanase. Thus, this research emphasized on production of commercially important enzyme along with waste management.

MATERIAL AND METHODS

Isolation and screening of xylanase producing microorganisms

a) Soil samples were collected for isolation microorganisms producing xylanase:

Different sites were selected to collect three soil samples, namely,

1) Sample A was collected from local garden at Chandkheda, Ahmedabad
2) Sample B was collected from vegetable market at D. cabin, Sabarmati, Ahmedabad
3) Soil sample C was collected from agricultural land at Anand, Gujarat

b) Lignocellulosic wastes were used as a substrate for isolation:

Four substrates used were oat husk, carrot pomace, banana peel and corn cob

Enrichment was carried out in liquid medium. 100 ml basal mineral salt medium composed of (g/l) yeast extract 0.1, $MgSO_4$ 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, NaCl 6.0, $CaCO_3$ 0.1, $(NH_4)_2SO_4$ 1.0 containing 10% w/v substrate was sterilized by autoclaving at 121.6 °C for 20 minutes. The pH of the medium was adjusted by 0.1 NaOH/HCl to 7.0 before sterilization. 2.5 ml of 1% soil was inoculated in sterilized medium and incubated at room temperature. After 60 days of incubation degradation of substrates was observed in flasks. Maximum degradation of substrates was observed in Sample A and Sample C therefore soil sample A and C were selected for further studies.

For isolation, enrichment broth from sample A and C was streaked on solid medium containing basal mineral salt medium with 1% oats. Plates were incubated at 37°C till the development of colonies. After development of growth, plates were flooded with Gram's Iodine solution. Colonies showing zone of hydrolysis selected for further studies.

Primary screening of isolates was done on birch wood xylan agar medium. Second screening of isolates was done by performing xylanase assay and isolates showing maximum enzyme activity after 48 hours of incubation were selected for further study. After second screening NDB4 and NDF5 were selected for further studies.

Xylanase production under submerged fermentation

Xylanase production in bacterial and fungal isolates was assessed using oats husk under submerged fermentation (SmF). 10 g of substrate were mixed with 100 ml of sterile mineral salt solution (6.6 g MgCl2.6H2O, 0.5 g KHPO4, 0.5 g KH2PO4, 2.0 g (NH4)2SO4. pH 6.7). Bacterial and fungal isolates were cultured in 250 mL Erlenmeyer flasks with the moistened substrate at 50°C for 48 hours. The solid substrate was then separated and suspended in 50 mM phosphate buffer (pH 8.0). After vertexing properly to extract xylanse the sample was centrifuged for 10 minutes at 4000 rpm and 4°C to separate broth from the substrate. The supernatant was then filtered through Whatman No. 1 filter paper to obtain a clear filtrate, which was used as the crude xylanase.

Optimization of Xylanase production

Cultural conditions were optimized for maximum xylanase production by testing various factors including substrate type, incubation period, temperature, and pH. Different substrates such as oat husk, corn cobs, banana peel, and carrot pomace were used. Fermentation was conducted for 96 hours to identify the best substrate for xylanase production. Sampling was done at 24, 48, 72, and 96 hours to determine the optimal time for xylanase production with NDB4 and NDF5 isolates. Enzyme biosynthesis at pH range of 2-12 (2, 5, 7, 8, and 12) was analyzed to identify the optimum pH for xylanase production. Xylanase production at temperatures ranging from 4 to 70 ℃ (4, 28, 37, 50 and 70 ℃) was tested to optimize temperature conditions. All experiments were performed in triplicate.

Determination of crude xylanase activity

Xylanase activity was measured by monitoring the release of reducing sugars from xylan using the DNSA technique [28]. The substrate assay comprised of 1% corn cob xylan (1.0 mL) in phosphate buffer (pH 6.5). Crude xylanase enzyme (1.0 mL) was added to the substrate and incubated at 50°C for 10 minutes, then adding DNS solution, boiling for 10 minutes, and cooling. A negative control without enzyme was prepared using the same process. Absorbance was measured at 540 nm using a T60-UVvisible Spectrophotometer against blank (water). One unit of enzyme activity was defined as the release of 1 micromole of reducing sugar equivalent to xylose per minute, under the standard assay condition [29]. Enzyme activity was expressed as Unit/ml/min. Enzyme activity was determined in triplicates.

The following formula was used to calculate the activity of the crude xylanase:

$$Unit/ml/min\ enzyme = \frac{\mu mol\ of\ xylose\ liberated\ x\ dilution\ factor}{Time\ of\ assay\ (minutes)\ x\ Volume\ of\ enzyme\ (ml)}$$

Here, time of assay (minutes) is 10 and the volume of enzyme (ml) is 1.

DNA extraction and PCR amplification

The sample of bacteria and fungi was taken out and placed in a mortar. 1 ml of extraction buffer was added, and the homogenate was then transferred to microfuge tube of 2 ml. The tubes were filled with equal volumes of phenol, chloroform, and isoamyl alcohol in a ratio of 25:24:1 and mixed by gently shaking. The tubes were centrifuged at 14,000 rpm for 15 minutes at room temperature. A fresh tube was used to capture the top aqueous phase, and it was then combined with an equal amount of chloroform: isoamyl alcohol (24:1). After centrifugation at 14,000 rpm for 10 minutes at ambient temperature, the aqueous phase at the top was collected and placed in a fresh microcentrifuge tube. DNA precipitation from the solution was done by adding 0.1 ml of 3 M Sodium acetate (pH 7.0) and 0.7 ml of isopropanol. The tubes were centrifuged at 4 °C for 15 min at 14,000 rpm after being incubated at room temperature for 15 min. The DNA pellet was rinsed twice with 70% ethanol and then with 100% ethanol before being air-dried. Finally, the DNA was dissolved in TE buffer (Tris-Cl 10 mM, pH 8.0, EDTA 1 mM). The DNA was mixed with 5 µl of DNAse free RNAse A (10 mg/ml) to eliminate the RNA. For amplification, 130 ng of extracted DNA and 10 pM of each primer were utilized.

Statistical analysis

Statistical analysis was based on triplicates. The values of different parameters were expressed as the mean ± standard deviation. On analyzing the data and performing t - test the difference was found to be statistically significant ($P < 0.05$).

RESULTS AND DISCUSSION

Isolation and screening of bacteria

Total 42 isolates were selected from different soil samples. Out of 42 isolates, 30 were bacterial isolates and 12 were fungal isolates. Following primary screening, 12 bacterial and 5 fungal isolates were selected for further studies. After secondary screening, bacterial isolate NDB4 and fungal isolate NDF5 were chosen for further production and optimization. Isolates NDB4 and NDF5 showed maximum zone of 28 mm and 32mm respectively on screening media as shown in figure 1. Growth of NDB4 and NDF5 in production medium is shown in figure 2.

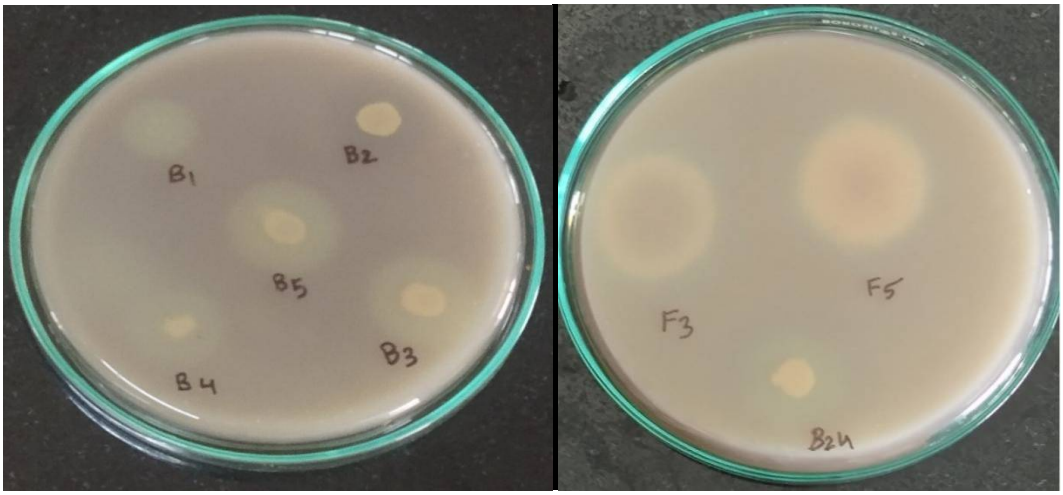

Figure 1: Clear zone of xylan hydrolysis observed on Birchwood Xylan agar medium

Figure 2: Growth of NDB4 and NDF5 isolates in production medium

Several agricultural wastes consisting of Oats husk, Corn cob, Banana peel and Carrot pomace were used as substrates for production of xylanase by *Bacillus licheniformis* (NDB4) and *Aspergillus niger* (NDF5). It is quite evident from the results that both *Aspergillus niger* and *Bacillus licheniformis* produced small amount of enzyme during the 24 hours of incubation. With the increase in incubation time, both bacterial (NDB4) and fungal isolate (NDF5) produced the maximum xylanase of 15 ± 0.5 Unit/ml/min and 17 ± 0.5 Unit/ml/min ($P < 0.05$) after 72 hours of incubation when oats was used as a substrate. NDB4 produced 5 ± 0.5 Unit/ml/min, 5 ± 0.5 Unit/ml/min and 11± 0.5 Unit/ml/min of xylanase using banana peel, carrot pomace and corn cob as a substrate respectively. NDF5 produced 7 ± 0.5 Unit/ml/min, 8 ± 0.5 Unit/ml/min and 12 ± 1 Unit/ml/min of xylanase using banana peel, carrot pomace and corn cob as a substrate respectively. With a 96-hour incubation, enzyme decreased due to depletion of nutrient by the action of the organism in the growth medium. This is most likely due to production of extracellular protease by bacteria [30]. The results after 4 days incubation at ambient temperature showed that Oats remained the best substrate for xylanase production by both isolates as depicted in figure 3a and 3b. In a study shown by [31] it was observed that *Bacillus licheniformis* produced xylanase (3.24 U/ml) using banana peel as substrate. In a pervious study by [32], *Aspergillus niger* produced 28.69 U/ml of xylanase using corn cob as a substrate. Bacteria are the main, most appealing extracellular xylanase producers [33,34]. *Bacillus licheniformis* generated the most xylanase during the study (37.91±1.04 U/ml) when the circumstances were ideal. When compared to un-optimized conditions, this enzyme output was ten times greater in optimized conditions. The ability of *Bacillus* species to release vital extracellular enzymes makes them useful industrial bacteria for the breakdown of xylan [35,36].

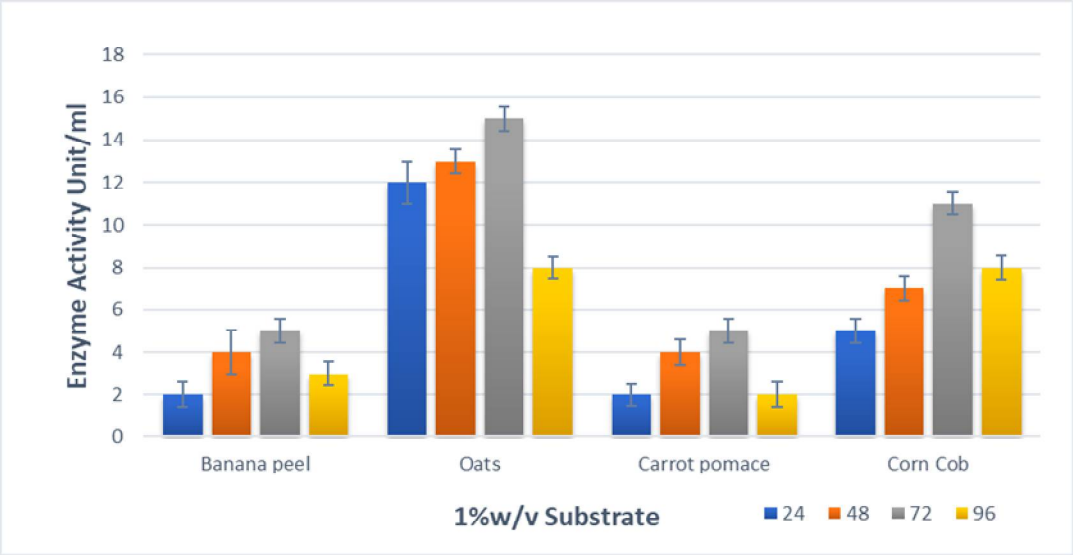

Figure 3a: Effect of incubation time and substrate on production of Xylanase in NDB4

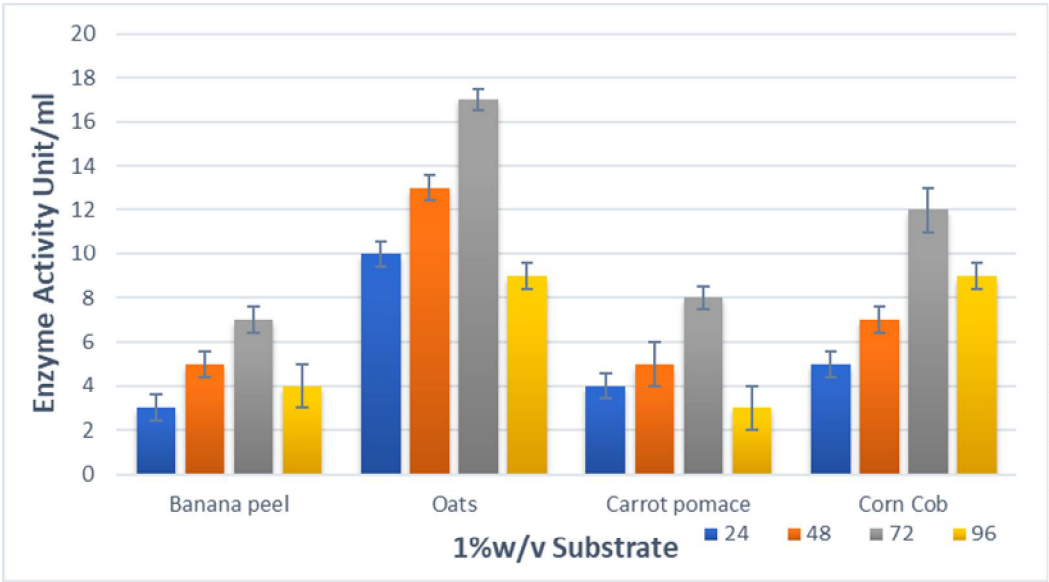

Figure 3b: Effect of substrate on production of Xylanase in NDF5

By adding 0.1N HCl and 0.1N NaOH, the starting pH of the medium was changed to a variable pH range. In the pH range (2-12) enzyme production was evaluated. The maximum production of xylanase 17 ± 0.8 Unit/ml/min and 15 ± 1 Unit/ml/min was found to be the best at pH 8 and pH 5 respectively using oat husk as substrate by *Bacillus licheniformis* and *Aspergillus niger* respectively as shown in figure 4. On analyzing the data, the difference was found to be statistically significant ($P < 0.05$). At pH 8, the xylanase enzyme from *B. licheniformis* showed the highest level of activity, with a maximum activity of 38.6 U/ml [31]. Maximum enzyme production was observed for a different species known as *B. pumilus* GRE7 at pH 8[37]. The maximum quantity of xylanase was produced at pH 8 by *B. subtilis* and *B. megaterium* [38]. *A. niger* produced highest xylanase at pH 5.0 of 4.72 ± 0.04 Unit/ml[39].

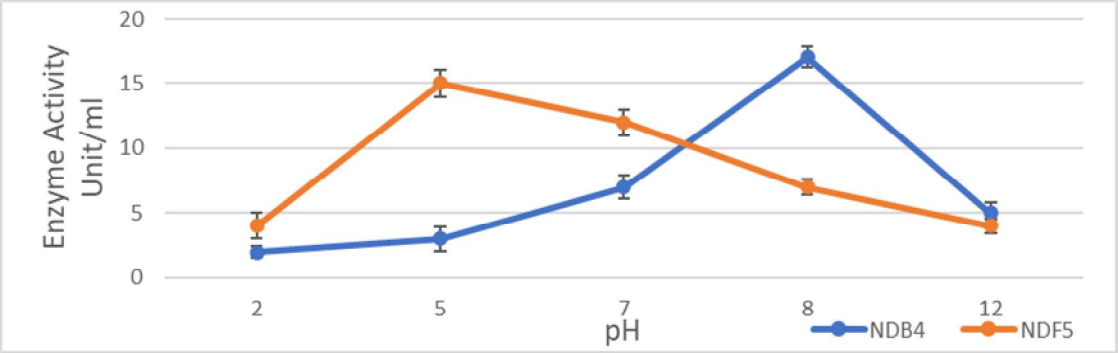

Figure 4: Effect of pH on production of Xylanase in NDB4 and NDF5

Different incubation temperatures, such as 4, 28, 37, 50, and 70 °C, were used to produce xylanase. It was shown that maximum xylanase production was observed at 50°C of 19 ± 0.5 Unit/ml/min by *B. licheniformis* and 21 ± 0.5 Unit/ml/min ($P < 0.05$) at 37°C by *A. niger* as shown in Figure 5. Xylanase production declined at 70°C in both bacteria and fungi. Xylanase from *B. licheniformis* in a study done by [31] showed highest activity at 55°C. *Bacillus pumilus* AJK during submerged fermentation at 55°C discovered similar results [40]. The impact of temperature on the activity of UV-mutated A and wild-type A *A. niger* xylanase enzyme after partial purification was observed. The temperature range used was 25 °C to 60 °C. The outcome showed that 45 °C was the temperature at which enzyme activity was at its peak while the enzyme activity significantly decreased at a temperature of 60 °C [41].

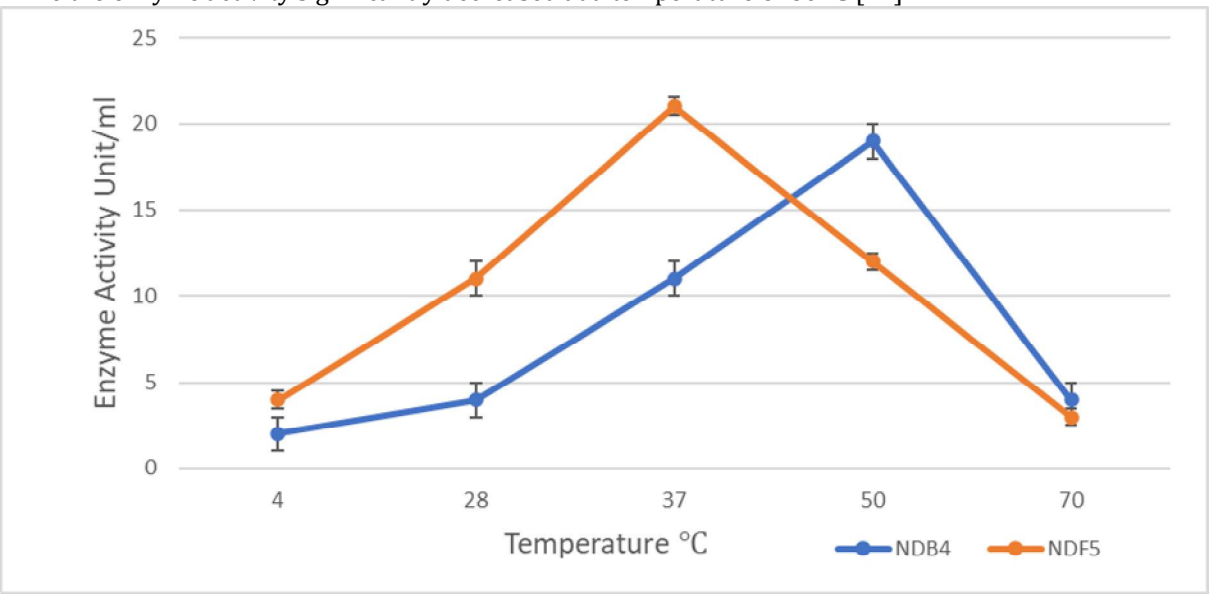

Figure 5: Effect of temperature on production of Xylanase in NDB4 and NDF5

Identification of isolates

DNA from bacteria and fungi was isolated, and its purity was determined by electrophoresis on an agarose gel. After purification, the PCR product was quantified and thereafter prepared for sequencing. The acquired sequences were examined and aligned. Sequences that have been aligned and tested using the NCBI Genbank database. Figures 6 and 7 display images of 16S rRNA and ITS rRNA amplified by PCR and genomic DNA electrophoresis. The isolates of bacterial and fungal organisms were identified based on maximum identity score. Figures 8 and 9 display genomic aligned sequence of both isolates NDB4 and NDF5. NDB4 was found to be *Bacillus licheniformis* strain 3724 and its accession no is MT604981.1 while NDF5 was identified as *Aspergillus niger* strain YCB1240 and its accession no is OK091639.1.

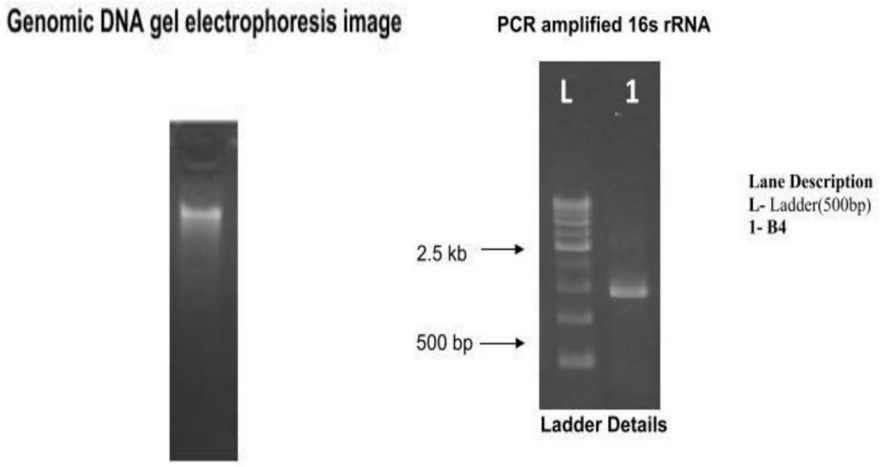

Figure6. Genomic DNA electrophoresis and PCR amplified 16srRNA

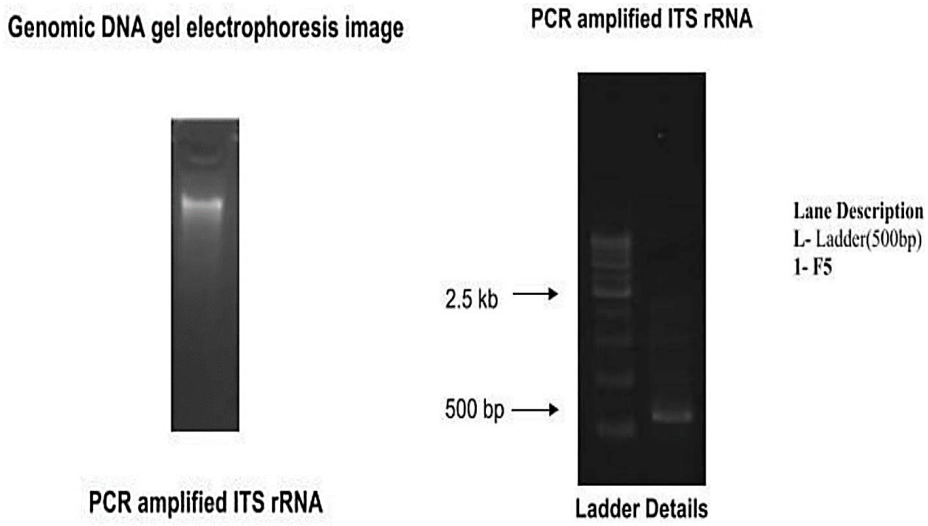

Figure 7. Genomic DNA electrophoresis and PCR amplified ITS rRNA

ACAATGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAG
TTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCT
GCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTT
TGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAAGCCCT
ATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCA
CCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGC
GTTTGCTGCAGCACTAAAGGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGT
ATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGT
GTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGT
TTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACGCCC
AATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTT
AGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGTTTTACGATCCGAAAAC
CTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGG
AGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCC
GTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCTAAAAGCCACCTTTTATAATTGAA
CCATGCGGTTCAATCAAGCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCA
CGTG

Figure 8. Aligned Sequence Data of Sample – NDB4 (1287bp)

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TAATAGCGGGAAGGGCGATGAGCCATCCTCCCATCCGTGTCTATTATACCCTGTTGCTTCGGCGGGCCCGCCGCTT
GTCGGCCGCCGGGGGGGCGCCTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAG
CGTGCAGTCTGAGTTGATTGAATGCAATCAGCTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAA
CAACGCAACGAAATGCGATAACTAATGAGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCG
CCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCC
GTCCCCCTCTCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGT
CACATGCTCTGTAGGATTGGCCGGCGCCTGCCGAGGTTTTCCAGCCGATGATATCTCAAAAAAAAAAAAGGGG

Figure 9. Aligned Sequence Data of Sample – NDF5 (527bp)

CONCLUSION

Agricultural residues/wastes can be used as a substrate for Xylanase biosynthesis. Local agricultural wastes like Oats husk, Corn cob, Banana peel and Carrot pomace can give better results. However, maximum results were obtained using oats husk and corn cob. Results of the fermentation showed that 50°C, pH 8 and incubation time of 72 hours with 1% w/v oat husk were the best conditions for the biosynthesis of Xylanase by isolate NDB4 while 37°C, pH 5, and incubation time of 72 hours with 1%w/v oats husk were the best conditions for the production of Xylanase by isolate NDF5**.** Isolate NDB4 was identified as *Bacillus licheniformis* strain 3724 while NDF5 was identified as *Aspergillus niger* strain YCB1240*.*

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