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DETERMINATION OF XYLANASE ACTIVITY USING *STREPTOMYCES*, ISOLATED FROM KALGO FADAMA LAND SOIL, KEBBI STATE, NIGERIA

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ABSTRACT

Xylanase is the most important enzymes that is currently being used in a number of industries. Xylanases hydrolyze the reprecipitated xylan on the fibers of paper pulp. The permeability of fibers increases with xylanase treatment, which allows easier removal of lignin from the fibres The aim of this research is to determine the activity of xylanase enzyme

Introduction

Xylanase is a group of enzymes consisting of endo-1, 4-β-D-xylanases, β-D-xylosidases, α-glucuronidase, acetylxylan esterase, α-L-arabinofuranosidases, p-coumaric esterase, and ferulic acid esterase, involved in the depolymerization of xylan into xylooligosaccharides to simple monosaccharide (xylose)

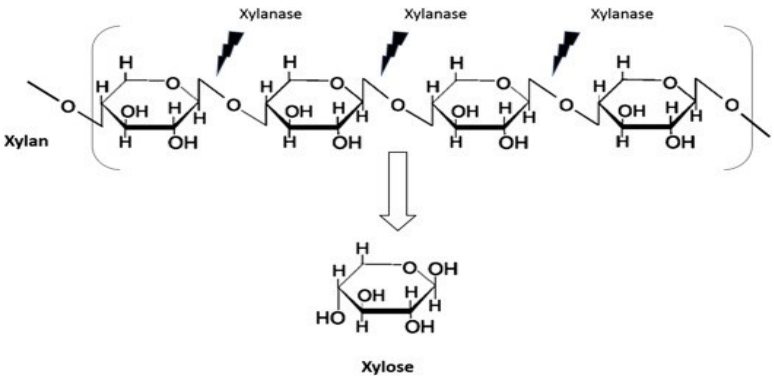

Fig 1: Hydrolysis of xylan toxylanase

Sources of Xylanase

xylanase is wide spread in nature and its presence is observed variously in a wide range of living organisms,

using soil *Streptomyces* collected from dangwarema fadama area of kalgo local government, Kebbi State, Nigeria. The soil sample was pre-treated to eliminate the commonly found microbes using physic-chemical methods. After inoculation, *Streptomyces* showing distinct morphological characteristics were selected from the isolation plate and further sub-cultured to obtain the pure form of the isolate, and then inoculated into production media for the production of the enzyme. The best Corbin sources and incubation period were determined. During the process of isolation, identification of the colonies with different morphology such as large white colonies with fluffy spores, small white sporulation powdery, colonies producing light brown pigment were determined. The xylanase was produced by isolated bacteria, and then optimization of time and carbon source were determined by measuring the enzyme activity. For time optimization, the highest activity of (0.512umol) was recorded at 32hrs followed by 28hrs (0.155umol), and then 24hrs (0.142umol) which is the lowest, and according to this research glucose was the best carbon source compared to other carbon sources. It can be concluded that the isolated bacteria (*Streptomyces*) from Kalgo Fadama land soil is capable of producing microbial xylanase enzyme. And according to this research, the best carbon source and best incubation period of xylanase enzyme were glucose and 32hrs respectively.

Keywords: Xylanase, Enzyme, Production, Activity

such as marine, terrestrial and rumen *bacteria*, thermophilic and mesophilic *fungi* [1], *protozoa*, *snails* [2], *insects* [3], *algae* [4], plants and seeds

Strategies Employed for Xylanase Production from Different Microbial Sources

The xylanase production has been carried out under submerged fermentation (SmF) and solid-state fermentation (SSF) [5]. The choice of the fermentation process usually depends on the type of microorganisms used. Bacteria require

a high amount of water during growth; therefore, SmF is preferred whereas fungi due to its mycelia nature require less moisture and can be grown under SSF [6]. Several reports suggest that submerged fermentation using bacteria and fungi is the most preferred method for xylanase production. Statistically speaking approximately 90% of total xylanase is produced globally through SmF. During SmF, the synergistic effect of different xylan degrading enzymes can be observed and even result in better biomass utilization for enhanced xylanase production [7]. Xylanase production utilizes soybean residues and rice straw as a substrate under SmF by Aspergillus oryzae LC1 and Aspergillus foetidus [8].

Recent trends suggest that xylanase production by SSF is also gaining popularity [9]. Bacillus sp. was used for the production of thermo-alkalophillic extracellular xylanase under SSF using wheat bran as substrate. It has several advantages such as low cultivation, operation and capital cost, a lower rate of contamination, easy enzyme recovery, and high productivity per reactor volume. The disadvantages associated with SSF are not suitable for all microorganisms (preferred for the fungal system) and require proper aeration and humidity control and up-scaling is a tedious process [10].

Components Used for Xylanase Production

Naturally, xylanolytic enzymes are induced by the different intermediate products generated by their own action. Xylan is found to be best xylanase inducer [11]. However, xylan being a high-molecular weight polymer cannot stimulate xylanase as it cannot enter the microbial cells. Therefore, a small amount of constitutive enzyme produced in the media results in the generation of low-molecular weight fragments, i.e., xylobiose, xylotriose, xylotetraose, xylose from the breakdown of xylan and further induces the xylanolytic enzymes for enhanced enzyme production [12]. Cellulose, synthetic alkyl, aryl β-D xylosides, and methyl β-D-xyloside also act as an inducer for xylanolytic enzyme production [13]. Busk and Lange (2013) observed that poor quality paper can efficiently induce the xylanase production in Thermoascus aurantiacus even in the absence of xylan and xylooligosaccharides.

Nitrogen is an important structural element required for the metabolic processes in the microbial system. Therefore, the choice of nitrogen source is important for the growth of microorganisms that subsequently affect the overall enzyme yield. Peptone, tryptone, soymeal, yeast extract, etc. have found to be suitable nitrogen source. The requirement of these nitrogen sources varies for different microorganisms; therefore, optimizing the type and level of nitrogen source in the media is an important parameter [14]. Trace elements, amino acids, and vitamins are also important parameters for the growth of different microorganisms [15]. Therefore, regulating their levels in the media is important for regulating the production of xylanase. Also, the addition of bio surfactant such as Tween 80 affected the level of xylanase production [16].

MATERIALS AND METHODS

MATERIALS

Conical flask (200ml), 100ml measuring cylinder, shaking incubator, autoclaving machine, weighing balance, filter paper, beaker, water bath, pH meter, media plates, spectrophotometer, centrifugation machine, test tubes, petri dishes, magnetic stirrer, incubator.

CHEMICAL REAGENTS

Starch casein agar (SCA), Soil sample, Banana peels, Distilled water, Trace salt solution ($FeSO_4$. $7H_2O$, $MnCl_2$. $4H_2O$, $ZnSO_4.7H_2O$, Na_2HPO_4).

METHODS

COLLECTION AND PREPARATION OF SOIL SAMPLE

The chosen site for sample collection was Dangwarema Fadama area of the Kalgo local government, Kebbi State, Nigeria. Ten cm (10cm) of soil from the surface was dug and the soil sample was collected and placed into a sterilized plastic bag and transported to the laboratory and used for isolation of *Streptomyces*. The soil sample collected was pre-treated to eliminate the commonly found microbes.

ISOLATION AND INOCULATION

One gram of soil sample was suspended in 9ml of distilled water and swirled vigorously and allowed to stand for 30 minutes. The sample was serially diluted up to 10^{-5}. An aliquot of 0.1ml of dilutions (10^{-1}, 10^{-3}, and 10^{-5}) was inoculated in fresh starch casein agar (S.C.A) plates using spread plate. The plates were incubated at 37°C for 48 hours.

GRAM STAINING

Using an inoculating loop the *Streptomyces* culture was smeared onto a microscope slide and was spread into an even thin over a circle of 15mm in diameter. The slide was allowed to air dry. Crystal violet stain was added over the dried fixed culture, after 60 seconds the stain was washed off using distilled water. Iodine solution was used to cover the smear for 60 seconds and was rinsed with water. This seep is known as fixing the dye *(Mittwer et al., 1950)*, a few drops of decolorizer was added and rinsed off with water after 5 seconds. The smear was counterstained with fuchsin solution for 60 seconds and was rinsed off with water, excess water was wiped out using cotton wool. The slide was air dried and viewed under the microscope.

BIOCHEMICAL TESTS

CATALASE

Procedure: Ensure that you have a pure bacterial culture of *Streptomyces* grown on an appropriate agar medium. Use a sterile loop or inoculating needle to obtain a small amount of bacterial growth (colonies) from the agar plate. Place the obtained bacterial growth into a sterile test tube or micro-centrifuge tube. Add a few drops of sterile distilled water to the tube to create a bacterial suspension. Mix the suspension well to ensure that the cells are evenly distributed.

Catalase Reaction: Add a small amount (usually a drop or two) of sterile 3% hydrogen peroxide directly to the bacterial suspension in the test tube. Observe the reaction immediately after adding hydrogen peroxide. If the bacteria produce catalase, you will see the formation of bubbles or

effervescence within a few seconds. This is because catalase breaks down hydrogen peroxide into oxygen and water, producing gas bubbles.

Interpretation: If bubbles or effervescence occur, it indicates a positive catalase test result, suggesting that the bacteria possess the catalase enzyme. And on the other hand if no bubbles form, it indicates a negative catalase test result, suggesting that the bacteria do not produce catalase.

Oxidase

Water was added to Oxidase reagent using a sterile pipette. Add a few drops of the solution to filter paper. Removed a colony from the blood agar plate using a sterile took pick. Then smear it on to the moistened filter paper. A purple color appears in 10seconds for Oxidase positive bacteria. No color change is seen for Oxidase negative bacteria.

Citrate

Using sterile took pick a test of an organism was strike in citrate media on straight line. The media was incubated at 37oC for 2days, the media was observed constantly for the growth of turbidity and blue color which indicates that the bacteria can digest the citrate, while no growth of turbidity shows that an organism cannot digest citrate.

Urease

100ml of urea agar was prepared, pepton 1g, glucose 1g, sodium chloride 5g, disodium phosphate 1.2g, monopotassium phosphate 0.8g, phenol red 0.012g was weighed in an electric weighing balance and was inoculated in conical flask with an 2.4ml of urea agar and 95ml of distilled water was added and was mixed very well and boiled on hot plate and was autoclaved, then was allowed to cooled at 45oC, yellow urea solution was added to urea agar it was shacked very well for proper mixing and urea agar was poured into two test tube and they were placed vertically for them to solidified, both of the was moved around the flame in order for them to dissolidified and 1ml of protease agarose and E.coli was inoculated in a separate tube which differentiated the one that is urea and incubated at 37oC for 48 hours. The color were expected to change

from yellow to pink. The yellow color is absence of urea while the pink colored shown the presence of urea positive.

Starch

Organism was strike at the Centre of the starch plate using sterile took stick and the plate was incubated for 24 hours at 37oC. After incubation, the plate was flushed with iodine solution using a dropper, it was allowed for 30second and excessed iodine was poured off. The plate was observed for the growth of zone around the bacteria. Zone was formed around the organism which indicated that the organism hydrolyzed the starch, while the blue colored indicated that the organism does not hydrolyzed the starch.

Casein

Bacteria isolated was picked with sterile took stick and was strike straight on the milk agar plate and was the media was incubated at 37oC for 24 hours. The milk agar plate for observed for presence or absence of growth of proteolysis around the bacteria. The proteolysis grow around the bacteria which confirmed that it's gram positive.

MEDIA PREPARATION

The media (Starch Casein Agar) was prepared by dissolving 63 gram in 1000ml distilled water. The media was gently heated to dissolve completely. Then the media was sterilized by autoclaving at 15 psi (121^{o}C) for 15 minutes and allowed to cool to 40 ^{O}C. the media was mixed well and poured into fresh petri dishes and allowed to soilidify.

ENZYME PRODUCTION

The xylanase enzyme production was carried out in the basal medium containing in g\L: banana peels (10), NH_4NO_3 (1), NACL (2), NaH_2PO_4 (2.6), $FeSo_4$ (1.0), $MgSo_4.7H_2O$ (0.5) with pH 7.5. The media was autoclaved at 121^{o}C for 30 minutes and allowed to cool. A 50ml of the production media was transferred into a 100ml conical flask and using a sterile wire loop the pure isolate from the sub-cultured plate was collected and agitated into production media. The

media was incubated for 24hrs at 40°C with shaking incubation period. The next day, the fermented broth was centrifuged at 5000rpm for 20 minutes. The cell free supernatant was collected and used as crude enzyme.

XYLANASE ACTIVITY ASSAY

Assays for crude xylanase were performed using 0.1% of banana peels in 50 mM sodium phosphate buffer, pH 7.0. The reaction mixture was composed of 1.8 ml substrate and 0.2 ml crude enzyme. The mixture was incubated in a water bath at 60°C for 15 min. The released reducing sugar was measured after stopping the reaction by heating the reaction mixture at 100°C in a water bath. After cooling the reaction tubes to room temperature, the absorbance was measured at 575 nm.

OPTIMIZATION OF TIME

Optimization of time was carried out by subjecting the fermented broth to incubation. After the first twenty four hours of incubation, an interval of four hours was given and then the reaction was stopped by heating method and the enzyme activity was measured agains
a control. The same procedure was repeated after another interval of four hours two times(28-32hrs).

OPTIMIZATION OF CARBON SOURCE

To detect the effect of various carbon-sources on xylanase production 100 ml Erlenmeyer flasks were prepared containing 10 ml of prepared production medium supplemented with 0.2% (w/v) of one of the following carbon sources: glucose, Cellulose, and Sucrose giving a total of 0.4% carbon source. Xylanase assays were performed accordingly.

RESULTS

ISOLATION OF *STREPTOMYCES*

In the process of isolation and identification, colonies showing different morphology such as large white colonies with fluffy spores, milky color colonies producing light brown pigment were observed from the plate.

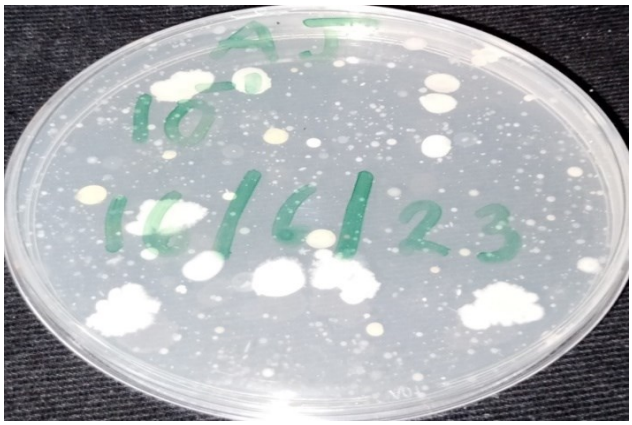

Fig 1: *Streptomyces* colonies with different morphological characteristics.

IDENTIFICATION OF STREPTOMYCES

In the process of physical identification, microbial characteristics and biochemical tests, the colonies with different morphology and microbial characteristics such as large white colonies with fluffy spores, small white sporulation powdery colonies producing light brown pigment was viewed from the base of the plate, yellow colonies pigment diffused through the media and large white colonies with greyish centre and fluffy spores were observed on starch casein agar isolation agar plate. The biochemical tests (casein, catalase, citrate, urease, oxidase and starch) were all positive.

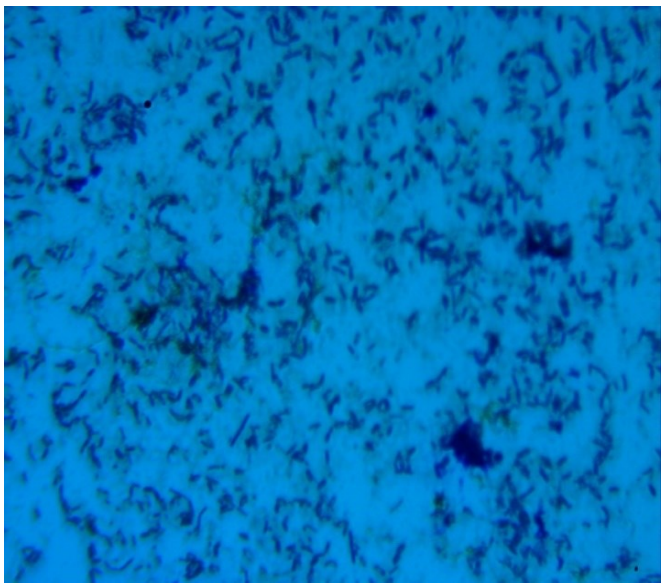

Fig 2: Microscopic view of stained *streptomyces* (Gram+ve rod-shaped bacteria)

Table I: Physical microbial and biochemical characteristics of bacterial isolates for identification.

Characteristics	Observation
Shape	Irregular
Spore surface	Smooth
Color	Milky
Gram staining	+ve(rod shaped)
Catalase	+ve
Oxidase	+ve
Urease	+ve
Citrate	+ve
Starch	+ve
Casein	+ve

PRODUCTION OF XYLANASE

The bacteria (*streptomyces*) has successfully induced and the xylanase enzyme has been produced. After incubating it into production media at 30°C for 24hrs using banana peels as substrate.

Fig 3: Submerged fermentation culture for enzyme production.

XYLANASE ACTIVITY ASSAY

After production of the enzyme, the supernatant after centrifugation was used as crude free enzyme to measure the enzyme activity.

Fig 4: Cell free supernatant for enzyme assay.

TABLE II: Enzyme assay

Enzyme and control	Absorbance (u/ml)
Control	0.118
Test	0.512

OPTIMIZATION OF TIME

The time optimization was done by subjecting the fermented broth to incubation on a rotary shaker for 24hrs. Two mls (2mls) of the mixture was centrifuged and the reaction was stopped by heating at 100°C for three mins, and then allowed to cool at room temperature and the first absorbance was taken at 575nm with control as 0.118. After an interval of exactly four hours (28hrs), the same procedure was done and absorbance was taken which appears to be higher than the first one. Lastly after another interval of 4hrs (making 32hrs), the same procedure was performed and absorbance taken,

this last absorbance seems to be the highest among all, suggesting that the isolate (*streptomyces*) produces the desired enzyme with longer incubations as shown by the graph below

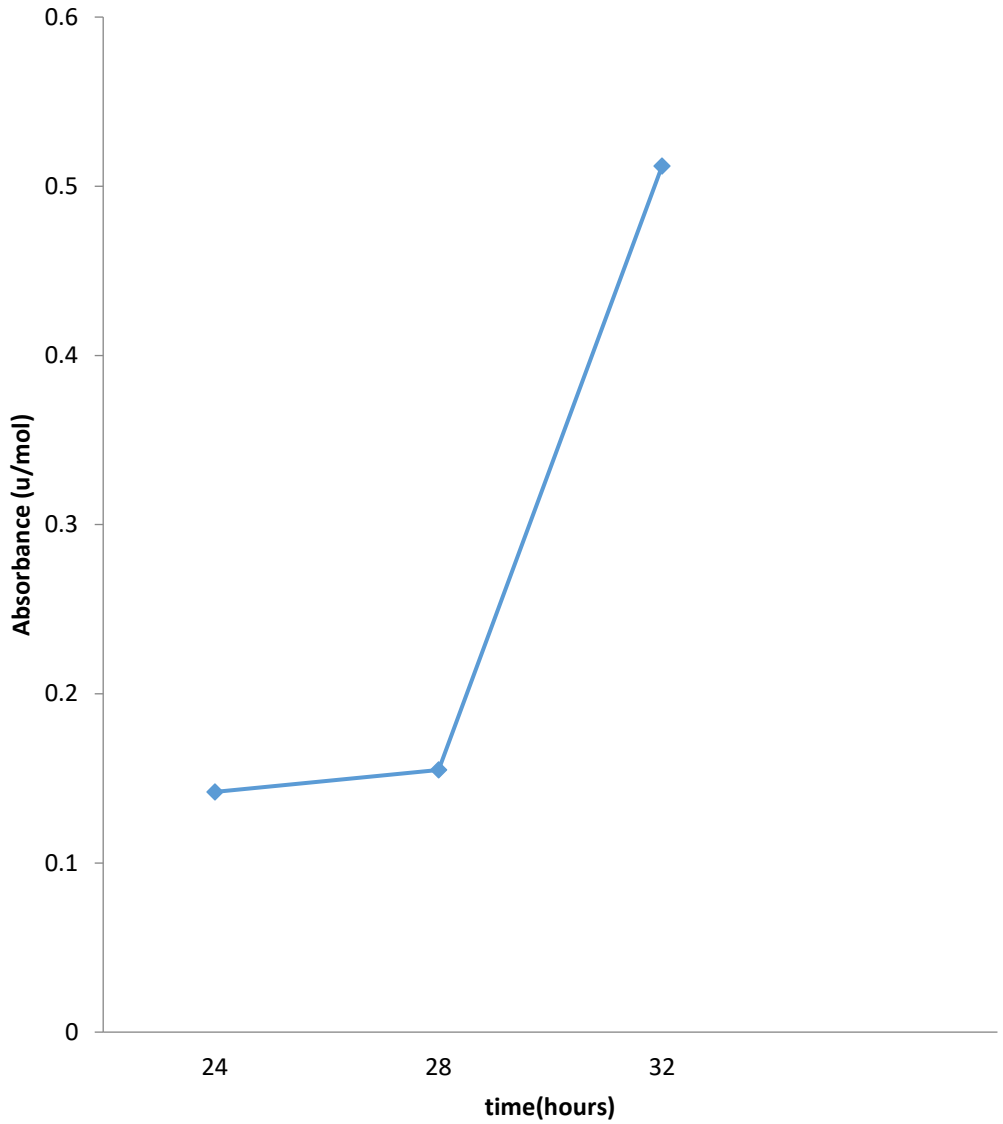

Fig 5: Optimization of time for the production of *Streptomyces*

OPTIMIZATION OF CARBON SOURCES

Three test tubes were set containing 10ml with distilled water and supplemented with 0.02(w/v)/g with different carbon sources (glucose, sucrose, and cellulose) accordingly. The reaction mixture was incubated at 40C

on a rotary shaker for 24hrs. The absorbance of each carbon source was taken at 575nm with control as 0.118 and glucose with an absorbance of (0.198umol) appears to be the best carbon source with the highest activity followed by sucrose (0.190umol) and then cellulose (0.156umol) as shown by the chart below.

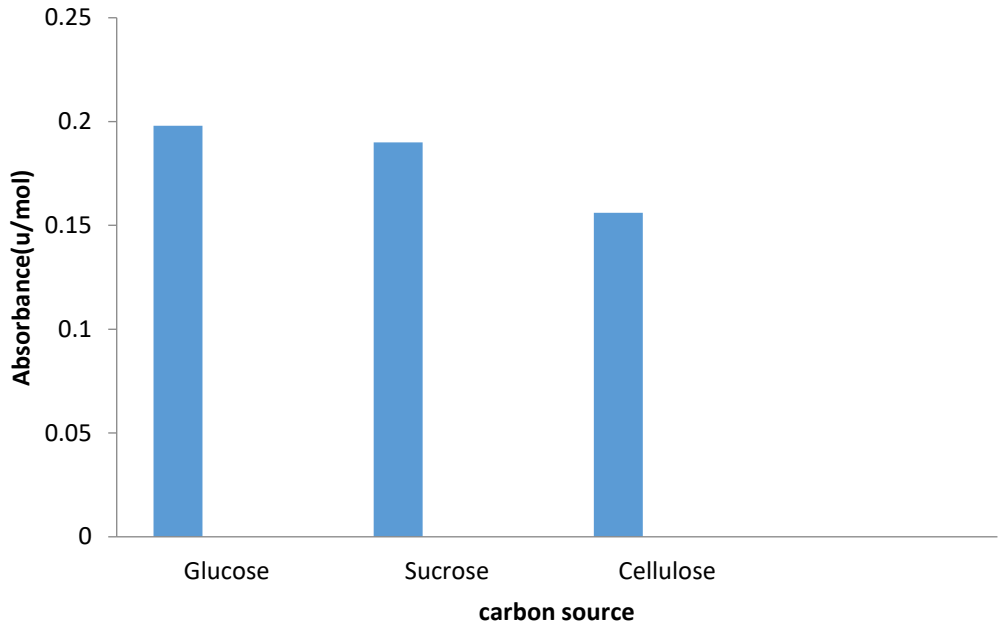

Fig 6: Optimization of carbon source.

DISCUSSION

The experiment was designed to assess the effect of varying growth conditions such as incubation time and carbon sources on xylanase yield. *Actinomycetes* produce a great variety of extracellular enzymes, of which xylanases are of significant industrial importance [17]. Cellulase-free xylanases are useful in the development of eco-friendly pulp bleaching process. They have been reported from *actinomycetes* such as *Streptomyces sp*, *Saccharomonospor*, *S. roseiscleroticus*, *Streptomyces sp*. Ab106, *S. albus, S. chromofuscus,* and S. *violaceoruber* [17]. The isolate (*streptomyces*) was identified based on morphological, biochemical and microbial characteristics which correlates to the findings of [18]. For production of high titers of any enzyme, optimizing the growth parameters is of prime importance in industrial enzymology. One of

these parameters, the initial pH of the medium, has been reported to strongly influence many enzymatic systems by affecting the transport of a number of chemical products and enzymes across the cell membrane [19]. The initial pH of this current study was 7.5 pH before incubation and this seem to have affect the enzyme production by the *streptomyces* after 24hrs of incubation with and absorbance of 0.512 umol. In this present research, during optimization of carbon source with incubation of 24hrs at 40°C, glucose was the best carbon source with an absorbance of (0.198 umol) utilized by *streptomyces* in xylanase production which correlates with the findings of Rodriguez *et al.,* (2005) who also reported a higher production of xylanase enzyme when glucose is used as the carbon source (14umol) which can be attributed to Carbon catabolite repression by glucose. Although this regulatory phenomenon has not been well characterized in *streptomycetes*, the expression of genes encoding extracellular hydrolytic enzymes such as xylanases, chitinases, and cellulases is generally activated by specific substrates and is repressed by the most energy-efficient carbon source, normally glucose [20]. During optimization of incubation time, the present study reports highly active xylanase acquired from *Streptomyces* after thirty two hours of incubation at 40°C (32hrs) with an absorbance of (0.512 umol), which can be attributed to the findings of Gautam *et al.*, (2011) who also observe maximum xylanase production (1424.69IU/gds) at 30°C which might be due to the stationary phase. Furthermore, the organism is also capable of growing at pH 7.0–7.5 and producing high titers of xylanases at pH 7.5. From this current finding it is evident that the growth medium significantly influences xylanase production.

CONCLUSION

It can be concluded that the isolated bacteria (*Streptomyces*) from Kalgo Fadama land soil is capable of producing microbial xylanase enzyme. And according to this research, the best carbon source and best incubation period of xylanase enzyme were glucose and 32hrs respectively

REFERENCES

Chadha BS, Kaur B, Basotra N et al (2019) Thermostable xylanases from thermophilic fungi and bacteria: current perspective. Bioresour Technol 277:195–20

Suzuki M, Yoshida K, Ashida K (1991) Purification and characterization of xylanase from the mid-gut gland of the apple snail (Pomacea canaliculata). Agric Biol Chem 55:693–700

Brennan Y, Callen WN, Christoffersen L et al (2004) Unusual microbial xylanases from insect guts. Appl Environ Microbiol 70:3609–3617

Brennan Y, Callen WN, Christoffersen L et al (2004) Unusual microbial xylanases from insect guts. Appl Environ Microbiol 70:3609–3617

Moon SH, Parulekar SJ. (1991). A parametric study of protein production in batch and fed-batch culture of B. firmus. *Biotechnol Bioeng* 37: 467–483.

Walia A, Guleria S, Mehta P et al (2017) Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. 3 Biotech 7:1–12. https://doi.org/10.1007/s13205-016-0584-6

Bajpai P (2014) Sources, production, and classification of xylanases. Xylanolytic Enzyme Academic Press (Imprint Elsevier), Tokyo, pp 43–5

Bhardwaj N, Chanda K, Kumar B et al (2017) Statistical optimization of nutritional and physical parameters for xylanase production from newly isolated Aspergillus oryzae LC1 and its application in the hydrolysis of lignocellulosic agro-residues. BioResources 12:8519–8538

Walia A, Guleria S, Mehta P et al (2017) Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. 3 Biotech 7:1–12. https://doi.org/10.1007/s13205-016-0584-6

Mienda BS, Idi A, Umar A (2011) Microbiological features of solid state fermentation and its applications—an overview. Res Biotechnol 2:21–2

Taibi Z, Saoudi B, Boudelaa M et al (2012) Purification and biochemical characterization of a highly thermostable xylanase from Actinomadura sp. strain Cpt20 isolated from poultry compost. Appl Biochem Biotechnol 166:663–679

Walia A, Guleria S, Mehta P et al (2017) Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. 3 Biotech 7:1–12. https://doi.org/10.1007/s13205-016-0584-6

Thomas L, Joseph A, Arumugam M, Pandey A (2013) Production, purification, characterization and over-expression of xylanases from actinomycetes. Indian J Exp Biol 51:875–884

Irfan M, Asghar U, Nadeem M et al (2016) Optimization of process parameters for xylanase production by Bacillus sp. in submerged fermentation. J Radiat Res Appl Sci 9:139–147. https://doi.org/10.1016/j.jrras.2015.10.008

Kumar L, Nagar S, Kumar D et al (2013) Production of an alkali tolerant extracellular xylanase from Bacillus pumilus VLK-1 in solid state fermentation and its use in tomato juice clarification. Int J Cell Sci Biotechnol 2:1–10

Fernande A, Dersch P (2018) Expression and export: recombinant protein production systems for Aspergillus. Appl Microbiol Biotechnol 87:1

Jensen JK, Busse-Wicher M, Poulsen CP et al (2018) Identification of an algal xylan synthase indicates that there is functional orthology between algal and plant cell wall biosynthesis. New Phytol 218:1049–1060

Kumar L, Nagar S, Kumar D et al (2013) Production of an alkali tolerant extracellular xylanase from Bacillus pumilus VLK-1 in solid state fermentation and its use in tomato juice clarification. Int J Cell Sci Biotechnol 2:1–10

Ninawe S, Lal R, Kuhad RC. (2006) Isolation of three xylanase-producing strains of actinomycetes and their identification using molecular methods. *Curr Microbiol 53: 178–182, 2006.*

Moon SH, Parulekar SJ. (1991). A parametric study of protein production in batch and fed-batch culture of B. firmus. *Biotechnol Bioeng* 37: 467–483.

Kumar L, Nagar S, Kumar D et al (2011) Production of an alkali tolerant extracellular xylanase from Bacillus pumilus VLK-1 in solid state fermentation and its use in tomato juice clarification. Int J Cell Sci Biotechnol 2:1–10

Rodríguez S, Santamaría IR, Fernández-Ábalos JM. (2005). *Identification of the sequences involved in the glucose-repressed transcription of the Streptomyces halstedii JM8 xys A promoter. Gene 351: 1–9.*