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STUDIES ON XYLANASE PRODUCTION BY *Aspergillus niger* ON TOMATO POMACE MEDIUM

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ABSTRACT

There is a need for locally produced xylanase because of its vast importance and high cost of importation. Xylanase is used for many industrial processes such as for baking, bleaching paper pulp, bioethanol production and juice clarification. This study was therefore carried out to examine the potentials of tomato pomace as part of the growth medium for xylanase production. The objectives are to identify the specific activities of xylanase from the basal salt medium and the tomato pomace medium and to determine the Partial Purification of xylanase obtained from tomato pomace medium inoculated with A.niger This study isolated xylanase from *A. niger* on tomato pomace medium. The xylanase was partially purified and characterized. *A. niger* was obtained from deteriorated banana (*Musa acuminata*) fruit. A 72-h-old culture of *A. niger* was employed as the inoculum. It was inoculated onto Tomato pomace medium and a basal salt. Xylanase production was carried out after four days at room temperature (27 ^{o}C). Xylanase activity was determined by measuring the released reducing sugar (xylose). The specific activities of xylanase from the basal salt medium and the tomato pomace medium were 3.6 U/mg and 2.0 U/mg respectively. Partial purification of xylanase was by Ammonium sulphate precipitation. Optimum substrate concentration of 0.5mg/ml and a purification fold of 4.3 were obtained. The Michael is Menten constant (Km) from the Line-weaver burk plot was approximately 0.50mg/ml. This study established appreciable activity of xylanase from the *A. niger* used. It is therefore a potential organism for the utilization of tomato waste for xylanase production.

Keywords: *Aspergillus niger*, Xylanase, Tomato, pomace, Banana (Musa acuminate)

INTRODUCTION

Xylanase is one of the microbial enzymes that has aroused great interest due to its biotechnological potential in many industrial processes, such as in xylitol and ethanol production (Sharma and Kumar, 2013), in the cellulose and paper industry (Harris and Ramalingam, 2010) and in the production of oligosaccharides (Aragon *et al*., 2013). Other uses of xylanases include in the food industry (Harris and Ramalingam, 2010, poultry, pork, and caprine feeding (Bhatt *et al*., 2012), coffee extraction, preparation of soluble coffee, protoplastation of plant, production of alkyl glycosides for use as surfactants and washing of precision devices and semiconductors

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(Tallapragada and Venkatesh, 2011). Enzymatic treatment provides the same level of output as conventional methods that utilize harsh chemicals (Adrio and Demain, 2014). Despite the vast importance of xylanase, a lot is being spent on its importation. There is therefore a need for the use of local materials that are readily available for the production of xylanase. Large percentage of the tomato (*Lycopersicon esculentum* Mill) fruits produced annually in Nigeria is been lost to postharvest deterioration caused by microorganisms (Ajayi and Olasehinde, 2009). This tomato fruits can be used for the production of tomato pomace .Tomato pomace is a mixture of tomato skin, pulp, cores, culls, liquor and crushed seeds. It contains crude and soluble protein, lipid, amino acid, carotenoids, minerals, high concentration of fibre and crude fat (Maheri-Sis *et al*., 2012). This can be incorporated into a growth medium for the production of tomato pomace medium (Bhatt *et al*., 2012).

Xylanases are extracellular enzymes produced by microorganisms such as (saprophytic and phytopathogenous) bacteria (Ellis and Magnuson, 2012), mycorrhizic fungi (Sridevi and Charya, 2011) and some yeasts. *A. niger* has been identified as one of the fungi responsible for the production of xylanase. The presence of microorganism that degrades hemicelluloses, particularly the xylan constituent which is a major constituent of hemicelluloses had been reported (Harris and Ramalingam, 2010). `

Aim

This study therefore investigated the production of xylanase obtained from culture filtrates of *A. niger* using tomato pomace medium. The enzyme was partially purified and characterized.

Objectives

1. To identify the specific activities of xylanase from the basal salt medium and the tomato pomace medium
2. To determine the Partial Purification of xylanase obtained from tomato pomace medium inoculated with A.niger

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RESEARCH METHODOLOGY

Collection of Samples

The tomato fruits employed for this research work were the Roma VF variety of tomato fruits. The fruits have smooth and unridged surface. It is a determinate cultivar and is high yielding. The fruits are oval in shape. It is a common type of cultivar in the Northern region of Nigeria where large acreages of tomato are grown under irrigation. It is not known to be susceptible to cracking (Jaiyeoba and Raji, 2012). They were obtained from the Sango Ota main market in Ogun State Nigeria. They were brought to the microbiology laboratory of the Department of Biological Sciences for further processing.

Preparation of Tomato Pomace Medium

Slightly deteriorated tomatoes were obtained from the Sango Ota main market .They were washed with tap water, dried in an oven at 60^{o}C for 24 h and stored at room temperature until needed. Prior to use, the tomato pomace was milled, sieved and particles smaller than 1 mm and greater than 3 mm were discarded**.** Dried tomato pomace was prepared into a basal salt medium for cultivation of *A. niger* since its constituents are sources of carbon and nitrogen. Ten grammes of the dried tomato pomace was weighed and mixed with the basal salt medium in a 1:10 (w/v) ratio according to the method described by Bhatt *et al*. (2012). Experimental and Control flasks were incubated without shaking at 25^{o}C.

Composition of Basal Salt Medium

KH_2PO_4 (2.0g/L) , $(NH_4)_2SO_4$ (1.4g /L), $MgSO_4.7H_2O$ (0.3g/L), $CaCl_2$ (0.3g/L), Urea (0.3g/L), Tween 80 (1ml/L), $FeSO_4.7H_2O$ (5mg/L), MnSO4 (1.6mg/L), $ZnSO_4$ (1.4mg/L), $CoCl_2$ (2.0mg/L)

Sources and Identification of Isolates

The isolate of *Aspergillus niger* used for this research was obtained from deteriorated banana (*Musa acuminata*) fruits. It was identified using the techniques contained in the illustrated Handbook of Fungi (Hanlin, 1990). The identification of fungal isolates was carried out by observation of the cultural and morphological characteristics. Each fungal isolate was cultivated on malt yeast extract agar and characteristics such as nature of growth, colour of colony and sporulation pattern were carefully observed. Mature cultures of each fungus which have obviously sporulated were employed for microscopic examination. The fungus was taken from

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centre as well as from the advancing margin of the growth region with a sterile inoculating needle and stained on a glass slide with lactophenol in cotton blue. Morphological characteristics including types and arrangement of spores produced were carefully examined under light microscope.

Culture Conditions and Preparation of Inocula

The isolate was subcultured and maintained on potato dextrose agar plates. Further sub-culturing was carried out on the same medium. A 96-h-old culture of *A. niger* was employed for this investigation. The culture was grown in a basal salt medium according to a modified method of Adejuwon *et al*. (2013) whereby the basal salt medium without the tomato pomace served as the control

Inoculation of Media

One milliliter of the aqueous spore suspension containing approximately $5x10^6$ spores per ml of the isolate was inoculated into conical flasks containing 100ml growth medium. The spores were counted using the Neubauer counting chamber.

Extraction of Enzyme from Culture Media

Enzyme solution was obtained from both media by sieving the culture content with muslin.

Enzyme Assay

The xylanase activity was determined determined according to the methods of Ghanem *et al* (2000) whereby Oat spelt xylan (Sigma Co., USA) was used as substrate. The reducing sugars produced were determined by the dinitrosalicylic acid method described by Ajayi *et al*. (2013) using the D-xylose as standard. The control reactions containing 1.0 ml of the enzyme solution and 0.5 ml of 0.5% (w/v) oat spelt xylan (pH 5.0 Citrate phosphate buffer) were incubated in a water bath at 40 °C for 15 min, the reaction was terminated by adding 3.0 ml of dinitrosalicylic acid reagent. After incubation in a boiling bath for 5 min, the liberated reducing sugars were measured at a wavelength of 540 nm.

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Partial Purification of Enzyme (Ammonium Sulphate Precipitation)

Ammonium Sulphate (Analytical grade) was added to the crude enzyme preparation to 90% saturation according to the method described in Encor biotechnology Inc. (2012). The solution was kept at 4^{o}C for 24hr and the resulting precipitate was removed by centrifugation at 4000 rpm using a centrifuge, Model (800D) for 15 min. The supernatant was discarded. The precipitate was re-dissolved in 0.05M Citrate phosphate buffer. The enzyme was dialysed overnight against four changes of the buffer. Dialysis was performed in acetylated cellophane tubing prepared using dialysis tubing (Gallenkamp) as described by Ajayi *et al*. (2013).

Characterization of the Enzyme

The effect of a number of factors on the activity of the partially purified enzyme was examined.

Effect of temperature

The reaction mixtures containing 0.5ml of the substrate and 1ml of the enzyme were incubated at different temperatures, 20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C and 40^{o}C. Incubation was for 15 min at each temperature. Xylanase activity was determined.

Effect of heat

The effect of heat on the stability of the enzymes was examined. Samples of the crude enzymes was heated at 70 ^{o}C for different periods of time (0, 2, 5, 10, 15, 20, 25, 30 min) respectively. The reaction mixture consisted of 0.5ml of the substrate and 1ml of the enzyme. Xylanase activity was determined.

Effect of pH

The substrate, soluble oat spelt xylan (Sigma) was dissolved in acetate phosphate buffer of different pH values ranging from pH 3.0 to pH 7.0. The reaction mixture consisted of 0.5 ml of the substrate and 1ml of the enzyme. Incubation was at 40 ^{o}C for 15 min. Xylanase activity was determined.

Effect of substrate concentration

Different concentrations - 0.1%, 0.3%, 0.5%, 0.7%, and 0.9% of soluble oat spelt xylan in citrate phosphate buffer (pH 5) was prepared and employed as substrate. The reaction mixture was 1ml enzyme and 0.5 ml substrate incubated at 40^{o}C for 15mins and analysed for xylanase activity.

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RESULTS

Table 1: Xylanase activity in Tomato Pomace Medium and Basal Salt Medium

S/N	MEDIUM	ISOLATE	XYLANASE ACTIVITY (Specific activity) (Units/mg)
1	Basal Salt Medium	*Aspergillus niger*	3.6
2	Tomato Pomace Medium	*Aspergillus niger*	2.0

Table 2: Partial Purification of xylanase obtained from tomato pomace medium inoculated with A.niger

S/N	Fraction	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg)	Yield (%)	Purification fold
1	**CRUDE EXTRACT**	3621	1810.5	2.0	100	1
2	**AMMONIUM SULPHATE PRECIPITATION**	3571	225	8.6	97.6	4.3

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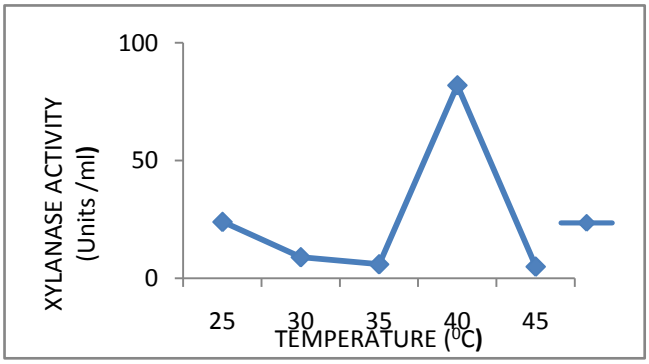

Figure 1: Effect of Temperature on partially purified Xylanase obtained from tomato pomace medium inoculated with Aspergillus niger .

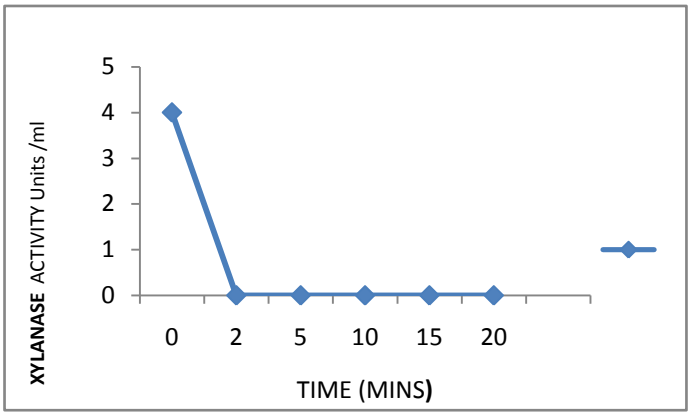

Figure 2: Effect of time of heating on partially purified Xylanase obtained from tomato pomace medium inoculated with Aspergillus

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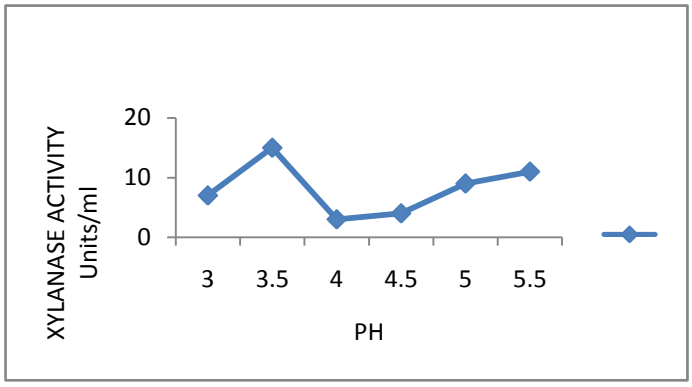

Figure 3: Effect of pH on partially purified Xylanase obtained from tomato pomace medium inoculated with Aspergillus niger **.**

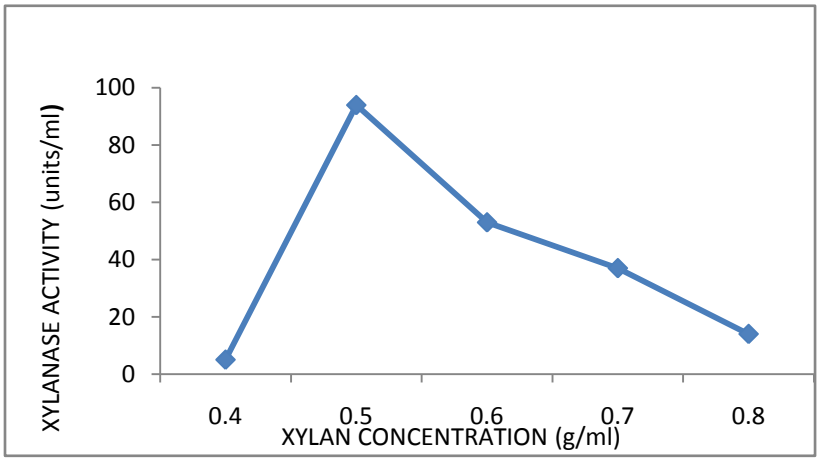

Figure 4 : Effect of Substrate Concentration on partially purified Xylanase obtained from tomato pomace medium inoculated with Aspergillus niger .

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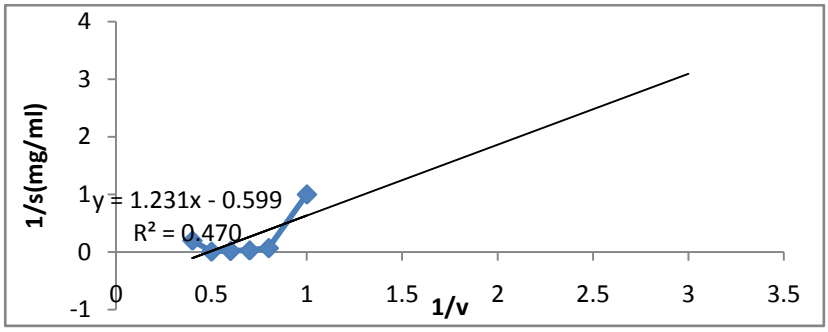

Figure 5: Line weaver- Burk plot for the hydrolysis of xylan by partially purified xylanase obtained from tomato pomace medium inoculated with Aspergillus niger

DISCUSSION

The result of this investigation shows that *A. niger* has the ability to degrade the xylan component of plant cell wall. Harris and Ramalingam (2010) reported the degradation of the xylan component of the plant cell wall and the presence of xylanase in the culture filtrate tested further confirms this degradation process by *A. niger* and also that xylanase is a pathogenic factor in the degradation of cell walls of plant. The basal salt medium showing less production of xylanase is an indication that the production of xylanase was likely induced by the presence of xylan in the cell wall of tomato fruits (Harris and Ramalingam, 2010). This diference was observed by the tomato pomace medium tested in comparison with the basal salt medium which therefore revealed the viability of tomato pomace in the production of xylanase. Umsza-Guez *et al*. (2011) reported the suitability of tomato pomace for xylanase production considering the important nutrients constituted in tomato fruits. The obtained data show that tomato pomace is a potentially promising substrate for the production of hydrolytic enzymes, particularly xylanase. This investigation also revealed that after the enzyme was partially purified the total protein content reduced to 225mg from 1810.5 mg. This indicates that the ammonium sulphate precipitation method was effective in isolating the enzyme or protein of interest. The result of this investigation also showed that temperatures at which the reaction mixture was incubated greatly affected the activity of the enzyme. The optimum temperature of 40^{o}C exhibited by xylanase from *A. niger* had earlier been reported by Sonia *et al*. (2005) whereby the optimum temperature range for xylanase produced by most fungi is in the range of 35 -40^{o}C . There was gradual increase in xylanase activity from 35 - 40^{o}C and considerable decrease below and above these temperatures. The result of this study further showed that substrate concentration had a

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marked effect on xylanase activity. This agrees with the findings of Sun *et al*. (2007). Increase in activity as a result of increase in substrate concentration may be attributed to the effective binding of the substrate to the active site. Voet *et al*. (2013) reported that further increase in substrate concentration above the optimum level will not produce any increase in the enzyme activity. Substrate concentration of 0.5mg/ml will be catalysed by xylanase with highest activity rate, but with decrease or increase in the substrate concentration the enzyme activity level drops. Worthington Biochemical Cooperation (2014) reported that enzyme activities are dependent on changes with the pH of the reaction mixture which not only influence the enzyme activity, but also affect the Michaelis Mentens constant (Km) and maximum velocity (Vmax). The optimum pH for the activity of xylanase was 3.5 and xylanolytic activity decreased remarkably after the optimum pH and later picked up at 5.0. (Worthington Biochemical Cooperation, 2014) reported the inhibition of enzyme activity due to changes in pH either to higher or lower values. This investigation showed that heating of the enzyme at 70^{o}C resulted in a total loss of activity within two minutes of heating. *Aspergillus* strains have been described to be susceptible to denaturation at heating temperatures above 50 ^{o}C (Umsza-Guez *et al*., 2011). The Km indicated the concentration of substrate to fill the half active sites of an enzyme. It is also a measure of strength of the enzyme-substrate (ES) complex. The double reciprocal plot revealed an approximate Km value of 0.5mg/ml. Lineweaver and Jansen (1951) reported that a high Km value indicates weak binding and vice versa. Previous researchers have implicated xylanases in fungal from other sources (Kulkarhi and Gupta, 2013; Umsza-Guez *et al*., 2011).

CONCLUSION

A conclusion has been established in the course of this research work that *Aspergillus niger* can be used to produce xylanase enzyme when it is grown on agricultural or industrial waste such as tomato pomace. The optimum condition for xylanase produced from tomato pomace medium have also been ascertained and they are 40^{o}C, 0.5mg/ml, 3.5 and 0.5mg/ml for temperature, substrate concentration, pH and Km (Michaelis Mentens constant). Tomato pomace (peel, seeds and pulp), a residue generated in large amounts by the agro-food industry, and found wasting in our markets is a good natural medium for fungal growth in submerged fermentation (SF). Its low cost makes it a potentially promising raw material for the production of high added value products, such as xylanase enzyme.

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RECOMMENDATION

Further studies on the application of these enzymes in the paper and pulp, food industry, in environmental science, such as, bio-fueling, effluent treatment, and agro-waste treatment will require a complete understanding of the functional and genetic significance of the xylanases and further purification of the xylanase. Hence the production of xylanase can be improved by finding more potent fungal or bacterial strains, or inducing mutant strains that can secrete greater amounts of the enzyme.

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