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Isolation, Screening and Optimization of Xylanase Producing Fungi from Rhizosphere Soil of Cassava Tuber

Ayodeji Adedapo Oyinlola, Felix Akinsola Akinyosoye
Department of Microbiology, School of Life Sciences, Federal University of Technology Akure, PMB 704, Akure, Nigeria

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Abstract
Microbial xylanases have attracted a great deal of attention, due to their biotechnological potential in various industrial processes. In this study, the isolation, screening and optimization of xlanase-producing fungi from rhizosphere soil of cassava tuber under submerged fermentation were carried out. Altogether, eight fungal strains were isolated from the rhizosphere soil of cassava. All the fungal isolates were screened positive for xylanase activity on mineral salt medium supplemented with araboxylan as sole carbon source. The process parameters were optimized using one factor at a time technique. The identities of the isolates authenticated as Debaryomyces nepalensis and Penicillium polonicum by molecular techniques were regarded as good xylanase producers and they were selected for optimization studies. In order to maximize enzyme synthesis from fungi, the effect of nutritional and environmental conditions on xylanase production was investigated. The optimal incubation periods for maximal xylanase production by Penicillium polonicum and Debaryomyces nepalensis were 120 and 144 hours respectively while the optimal pH and temperature for xylanase production were 5.0 and 50^{o}C respectively by Penicillium polonicum and Debaryomyces nepalensis. The best carbon sources for xylanase production from both fungi were found to be xylan. As a result of this, both fungal species have significant potential as sources of xylanases for industrial and biotechnological applications.

1. Introduction

Plant rhizosphere is the soil under the powerful influence of the plant root system where roots release large quantity of metabolites from living root hairs or fibrous root systems [1]. Plant roots release water soluble chemicals including amino acids, sugars, and organic acids as they develop through the soil, providing food for microorganisms.. High levels of exudates in the rhizosphere attract a plethora of microorganisms to a larger extend than elsewhere in the soil [2]. Overall enzyme activity of the rhizophere can depend on enzymes localized in root cells, root remains, microbial cells and microbial cell debris. Plant roots of rhizophere have been considered a source of extracellular enzymes in soil. Rhizosphere microorganisms release extracellular enzymes for the initial degradation of higher molecular polymers such as cellulose, hemicelluloses and chitin [3-4].

Xylanase is the main enzyme that catalyzes the hydrolysis or breakdown of xylan, the major constituent of hemicelluloses. Xylans are the second most abundant natural polysaccharide after celluloses, with which they form major components of plant cell wall [5]. Xylans are the major hemicelluloses in hardwood and also predominate in annual plants and cereals. There are four main categories of xylans: arabinoxylans, glucuronoxylans, glucuronoarabinoxylans and galactoglucuronoarabinoxylans. Each xylan molecule's side chains are responsible for its solubility, physical conformation, and reactivity with other hemicellulose components, influencing the mode and amount of enzymatic cleavage [6].

Plant xylan must be broken down by a combination of hydrolytic enzymes with different specificities and modes of action because of its heterogeneity and complex chemical structure. The xylanolytic enzyme system that carries out the xylan hydrolysis include; endoxylanase (endo-1, 4-β-xylanase, E.C 3.2.1.8), β- Xylosidases **(**xylan-1,4-β-xylosidase; EC 3.2.1.37), α–glucuronidases (α-glucosiduronase, (EC 3.2.1.139), α–arabinofuranosidases (α-larabinofuranosidases, E.C.3.2.1.55**)** and acetylxylan esterase (EC 3.1.1.72) [7-9]. All of these enzymes act cooperatively to convert xylan into its constituent sugars or simple sugars such as xylose, xylobiose, xylotriose and arabinose, which actually play key role in the regulation of xylanase biosynthesis

According to Markets and Markets Watch (2015), [10] the global industrial enzyme market was valued at $ 4.2 billion in 2014 and is projected to grow at the rate of 7.0% to reach $6.2 billion worldwide by 2020. Increased investments, industrial enzyme research and development, increased demand for consumer goods and biofuel, and the need for cost reduction and resource optimization are the driving forces for the industrial enzyme market. Because of its numerous biotechnological and industrial applications, xylanases have recently received a lot of attention.

Xylanase from fungi is receiving more attention owing to their excellent catalytic ability, specificity stability, non toxicity, eco-friendly nature, cost effectiveness, and ease of production [11-12]. Due to the demand of xylanase, high premium is placed on the search for novel xylanase producing fungi which have natural adaptability towards industrial process conditions. As a result, the goal of this work was to isolate, screen, and select filamentous fungi with high xylanolytic activity. Optimization of culture conditions such as fermentation period, carbon and nitrogen sources, pH and temperature for higher production of xylanases, under submerged fermentation, were then carried.

2. Materials and Methods

2.1 Collection of Soil Sample

Soil samples were collected from the school farm, Federal University of Technology, Akure, Nigeria (FUTA). The soil samples were collected by carefully uprooting the cassava plant and collecting adhering soil into sterile polythene bags. It is then transported to the Microbiology laboratory for microbial analysis.

2.2 Isolation and enumeration of fungi from the soil samples

Serial dilution technique was used for isolation of fungal isolates from soil samples in which 1g of soil was suspended in 10 ml of sterile distilled water and considered as a stock. Using sterile distilled water, various dilutions from 10^{-1} to 10^{-7} were prepared from this stock. One milliliter of the diluted sample was pour plated into Petriplates containing Potato Dextrose agar medium containing 0.7% (w/v) araboxylan. The plates were incubated at 27°C for 72hours [13].

2.3 Purification of fungal isolates

Corresponding Author,
E-mail address: oyinlolaayodeji31@gmail.com; felixkinsola@yahoo.com**;** Phone No--+234- 7064284120

The isolated fungi obtained were sub-cultured using inoculating needle on solidified sterile set plate of PDA supplemented with 0.7% (w/v) araboxylan and incubated at 27℃ for 72hours. The process of sub-culturing was repeated until pure fungal culture was obtained and was maintained on PDA slants and stored in the refrigerator at 4℃ [13].

2.4 Identification of fungal isolates

Fungal isolates were identified up to species level by macroscopic characterization and microscopic examination using standard mycological text**.**

2.5 Screening of Fungal Isolates for Xylanase Production

The identified fungi were screened for xylanolytic activity using both plate culture (qualitative) and submerged fermentation (quantitative) methods and the selection was based on the producing organisms with the highest xylanolytic activity index.

2.6 Qualitative screening of isolates for Xylanase Production

All fungal isolates were subjected to screening for their xylanase activities using the plate assay method on minimal agar medium containing 0.5% araboxylan as the only carbon source and this was done by boring a hole of 1cm at the middle of the agar or medium inside the plate with a cork borer, then the fungal isolate was placed inside the hole [14]. The following components (g/L) were present in the xylan-agar medium: 0.05g $MgSO_4 \cdot 7H_2O$, 0.005g $CaCl_2$, 0.005g $NaNO_3$, 0.009g $FeSO_4 \cdot 7H_2O$, 0.002g $ZnSO_4$, 0.012g $MnSO_4$, 0.23g KCl, 0.23g KH_2PO_4, 2g peptone, 19g agar. The inoculated plates were incubated for 5 days at 30ºC. Plates were flooded with 0.4% (w/v) congo red and after 10 minute, washed with 1 M NaCl and were observed for zone of clearance around the fungal growth. Fungal strains, which produced distinct cleared zones around their mycelia, were selected [15-17].

2.7 Secondary (quantitative) screening of isolates for xylanase production

The xylanolytic fungal isolates from primary screening were further screened in xylanase production medium or mineral salt medium containing 1.0% (w/v) araboxylan as the sole carbon source. The pure fungal isolates were individually inoculated into production medium (100ml) contained in Erlenmeyer flasks (250 ml) after sterilizing it in the autoclave at 121°C for 15 min and were incubated at 30°C in rotary incubator shaker at 120 rpm for 6 days. Samples were taken after every 24 hours. The culture medium was filtered using Whatmann no.1 filter paper, the filtrate was cold centrifuged at 10000 rpm for 10 min to remove the fungal cells and unwanted particles. The clear supernatant was used as the crude extracellular xylanase [18].

2.8 Enzyme Assay

Xylanse activity in the supernatant was assayed by determining the concentration of reducing sugar liberated by the activity of the crude enzyme on its substrate xylan using DNS reagent [19]. Reaction mixture containing 0.5 ml of culture enzyme with 0.5ml of 1% araboxylan solution prepared in 0.05 M citrate phosphate buffer (pH 5.0) incubated at 30°C for 15 min [20]. After incubation, the reaction was terminated by the addition of 1.0 ml of 3, 5-dinitrosalicylic acid (DNS) and heated in the boiling water bath for 10minutes for brick red color development. After cooling, the reducing sugars liberated were measured with spectrophotometer (Gulfex Medical and Scientific England, Spectrumlab 23A, model number 23A08215) at 540 nm and expressed as xylose equivalent. Under conventional assay conditions, one unit of endo-1, 4-xylanase was defined as the quantity of enzyme required to release 1 mol of xylose per minute [21].

3. Optimization of Nutritional and Environmental Conditions for Xylanase Production

The optimization of different parameters was carried out using one factor at a time technique to know the best condition for production of xylanase.

3.1 Effect of carbon sources on xylanase production

The effect of different carbon sources such as glucose, soluble starch, sucrose, lactose, fructose and xylan, at 1.0% (w/v) on xylanase production from the best xylanase fungi were determined. After inoculation with 1.0 ml standardized spore suspensions of the isolates, the flasks were incubated at 30°C for 5-6days. At the end of incubation, the culture supernatants were assayed for xylanase activities using the method earlier described [22-24].

3.2 Effect of nitrogen sources supplementation on xylanase production

The effect of various nitrogen sources, at 0.5% (w/v**)** on xylanase production was determined by supplementing the mineral salt medium with different nitrogen sources (ammonium nitrate, ammonium sulphate, sodium nitrate, peptone, potassium nitrate and yeast extract) and incubated at 30°C for 5-6days. The cell-free supernatant obtained was assayed for xylanase activity as previously described [22-24].

3.3 Effect of different incubation temperature on xylanase production

The influence of temperature on xylanase production was also determined by incubating the basal medium inoculated with best xylanase producing organisms at 30°C to 80°C for 120- 144 hours. The cell-free supernatant obtained was assayed for xylanase activity as previously described [22];[23];[24].

3.4 Effect of initial pH on xylanase production

The effect of initial pH on xylanase production was determined by varying the pH values of the basal medium from pH 3.0 to 8.0. Fungal inoculated fermentation medium was incubated appropriately at 30°C for 5-6days and the cell-free supernatant obtained was assayed previously described [22].

3.5 Effect of fermentation period on xylanase production

To ascertain the optimum incubation time for maximum production, the best two xylanolytic isolates were grown on the basal medium and incubated at 30∘C for 10 days at an interval of 24 hours, samples were withdrawn, followed by xylanase assay as earlier described [22].

4. Result and Discussion

In this present study, a total of eight filamentous fungal isolates were associated with the rhizosphere soil of cassava tuber. Based on cultural, morphological and microscopic characteristics, isolates were presumptively identified as Debaryomyces nepalensis, Fusarium oxysporium, Microsporium persicolor, Aspergillus terreus, Aspergillus fumigatus, Geotrichum candida, Alternaria alternata and Penicillium polonicum (Table 1). This is in agreement with the findings of [25-30]. [31]isolated arrays of filamentous fungi from the rhizosphere soil of Cassava. The fungi associated with rhizosphere soil of cassava as reported in this study could be as a result of nutrients secreted by plant roots in form of exudates contributing to their growth and enzyme activity.

Table 1. Morphological and microscopic characteristics of fungal isolates

Isolate	Morphological and microscopic characteristics	Probable Identities
1.	Produces hyaline (clear), septate hyphae which show dichotomous branching, cells are cylindrical in shape, off white to cream coloured colonies with a butyrous texture with a variety, suede-like or ground glass appearance. Ascopores are spherical and have a warty wall.	Debaryomyces nepalensis
2.	Produces white cottony colonies with the aerial mycelia becoming tingle in purple. The reverse was non descript pale to yellow. Hyphae are hyaline (clear/non pigmented);are septate (show divisions or walls within	Fusarium oxysporium

	hyphae).	
3	Colonies are glabrous, downy, wooly or powdery, yellow-cinnamou colour, the reverse is red-brown, produces septate hyphae, hyaline, oval to clavate in shape, smooth and thick rough cell wall	Microsporium persicolor
4	Colonies are beige to buff to cinnamon. Reverse is yellow and yellow soluble pigments are frequently present. Colonies are finely granular with conidia production, hyphae are septate and hyaline	Aspergillus terreus
5	Colonies are rapid growing, flat, filamentous and velvety, woolly or cottony in texture. The colonies are initially white and become pinkish. The reverse plates are usually pale to yellowish. Chains of single-celled conidia are produced in basipetal succession from a specialised condiogenous cell called a phialide.	Penicillium polonicum
6	Colonies are smoky gray-green with a slight yellow reverse. Rapid growth, texture is woolly to cottony. Hyphae are septate and hyaline.	Aspergillus fumigatus
7	Produces woolly to cottony, flat, spreading colonies. White colonies and reverse is brown. Hyphae are hyaline, septate and branched	Geotrichum candida
8	Grow rapidly, flat colony, downy to woolly and covered by grayish, short, aerial hyphae. The reverse side typically brown to black due to pigment production, has septate, brown hyphae, condiphores are also septate and brown in colour	Alternaria alternate

The 8 fungal isolates were screened using the formation of clear zones surrounding the microbial colonies as screening criteria. All isolates showed positive reaction at varying levels to xylanase production as indicated by clear zones in xylan agar media plates as shown in Table 2. The presence of membrane bound hydrolyses, which cause the formation of a hydrolysis zone on the substrates being hydrolyzed, could justify the clear zones around the isolates on xylan agar media [32].

Table 2. Qualitative xylanase activities of the Isolates

Isolate	Organism	Clear zone (cm)
1	Debaryomyces nepalensis	1.5 ± 0.03
2	Fusarium oxysporium	1.1 ± 0.03
3	Penicillium polonicum	1.5 ± 0.07
4	Aspergillus terreus	0.8 ± 0.03
5	Microsporium persicolor	0.8 ± 0.07
6	Aspergillus fumigatus	1.2 ± 0.07
7	Geotrichum candida	1.0 ± 0.03
8	Alternaria alternata	0.7 ± 0.07

The xylanolytic fungal isolates from primary screening were further screened in xylanase production medium under submerged fermentation condition using araboxylan as the sole source of carbon as shown in Figure 1. All the isolated exhibited varied xylanase activities with the highest activity (2.396 ± 0.169 U/mL) recorded for Debaryomyces nepalensis and lowest activity (1.338 ± 0.060 U/mL) for Alternaria alternata. Thus, Screening of microorganisms is very important to find the appropriate starting material for enzyme production [22]. Debaryomyces nepalensis and Penicillium polonicum were identified as good producers of enzyme and further studies were carried out with these two isolates for optimization of fermentation conditions.

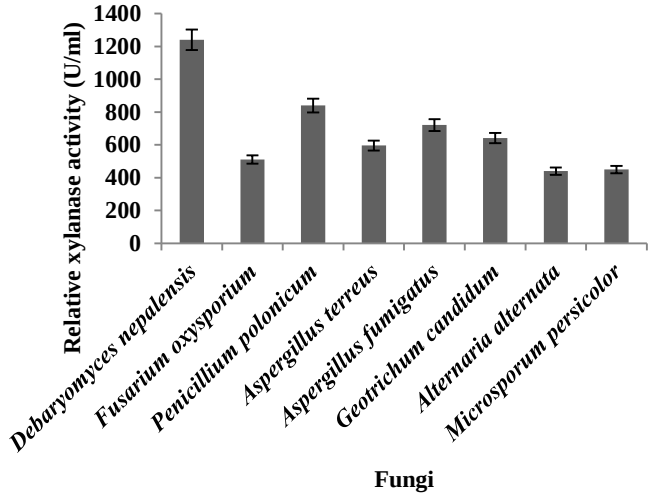

Fig.1: Quantitative xylanase activities of fungal isolates

Production of microbial enzymes is dependent upon various nutritional and cultural factors such as initial pH, temperature, carbon and nitrogen sources and hence these were studied in order to optimize xylanase production from the selected fungi [33].

The effect on different carbon source on xylanase production by the two fungal isolates is presented in Figure 2. Debaryomyces nepalensis and Penicillium polonicum utilized different carbon sources in the fermentation medium for xylanase production with the maximum enzyme yield (1.913 ± 0.038 U/mL and 1.737 ± 0.129 U/mL) being observed with the use of xylan as carbon source respectively, followed by other sugars. The findings are in agreement with [22] and [34]who stated that xylanase synthesis is favored by xylan from A. flavus and A. tubingensis respectively, linking to the fact that growth and enzyme productions by organism are greatly influenced by nutrients present in the growth medium but contradicts reports that pure sugars only gave good growth but poor xylanase production [35].

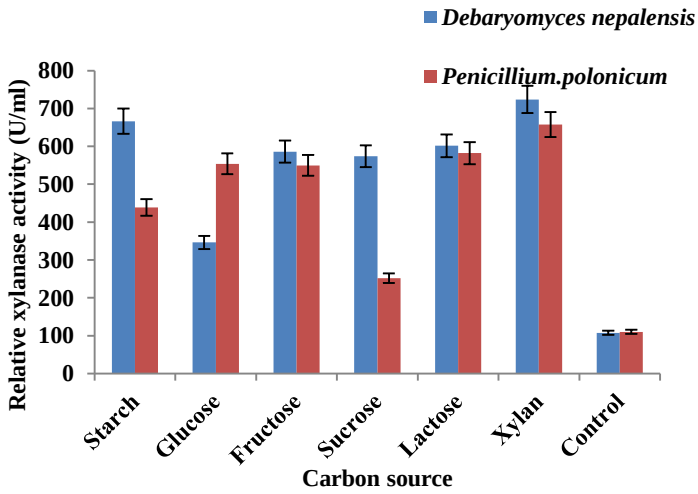

Fig.2: Effect of different carbon sources at 1% (w/v) on xylanase production from fungal isolates

Among the nitrogen sources evaluated for xylanase production from Debaryomyces nepalensis, peptone produced maximum xylanase yield (1.194 ± 0.271 U/mL), followed by ammonium nitrate (1.158 ± 0.156 U/mL), while ammonium sulphate (0.740 ±

0.103 U/mL) had the least xylanase yield Figure 3. Penicillium polonicum produced maximum xylanase activity (0.898 ± 0.050 U/mL) with the use of sodium nitrate as a nitrogen source, followed by the use of ammonium sulphate (0.683 ± 0.005 U/mL) while the yeast extract produced the least enzyme activity (0.554 ± 0.087 U/mL) as shown in Figure 3. This is in agreement to the findings of [36];[37] and [38] who reported peptone to be the best source of nitrogen source for the production of xylanase from A. niger and F. solani. and with also the findings of [39] and [23] who found sodium nitatre ($NaNO_3$) as the best nitrogen source for xylanase production by local A. *niger* and thermoalkalophilic Paenibacillus sp.

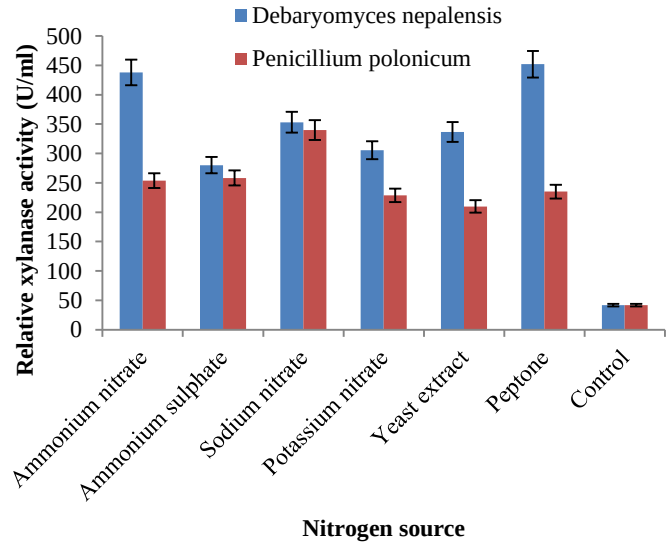

Fig.3: Effect of different nitrogen sources at 0.5% (w/v) on ylanase production from fungal isolates

Effect of different incubation temperature on xylanase activity from D. nepalensis, and P. polonicum is shown in Figure 4. The percentage relative activities of D.nepalensis, and P. polonicum increased at 30^{0}C and reached maximal at 50^{0}C (0.626 ± 0.027 U/mL and 0.610 ± 0.017 U/mL) respectively. Beyond 50^{0}C, there was decline in the xylanase activity. The fermentation temperature has marked effect on the level of xylanase production as it plays important role in the metabolic activities of microorganisms [40] and [41]. The same incubation temperature of 50^{o}C optimum for xylanase production by streptomyces sp., Bacillus velezensis AG20 K37 and Aspergillus oryzae were documented by [42] [43]; [44]. However, lower incubation temperature of 25°C and 30°C were observed to be the best for xylanase production by Trichoderma sp. [45] and A. niger [46] respectively.

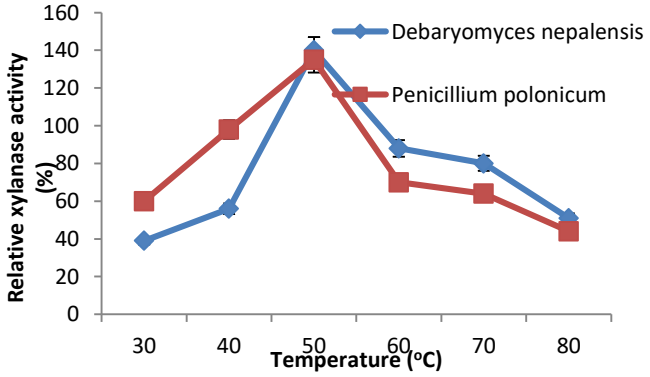

Fig 4: Effect of different temperature on xylanase production from fungal isolates

The effect of initial pH on xylanase production by D. nepalensis, and P. polonicum is shown in Figure 5. The xylanase activity increased with increased in initial at pH 3.0 and reached its maximum (0.624 ± 0.016 U/mL and 0.871 ± 0.062 U/mL) at pH 5.0 for D. nepalensis and P. polonicum respectively. The xylanase activity was observed to decline beyond pH 5.0 from both fungi. Xylanase production varied with the pH of the medium of enzyme production and it has a profound influence on the growth and metabolic activities of enzyme-producing microorganisms. This finding from this study corroborates with the findings of [47] and [48] who achieved best xylanase production by Aspergillus sp. ART500 at pH 5.0 and Aspergillus niger at pH 5.0. This report is in variance with the findings of [49]; [50]and [42] who separately reported highest xylanase production at pH ranged 7 – 8 for several fungi and Bacillus spp [51]. The reduction in xylanase production by the organisms under investigation in this study at alkaline region might be due to its inhibitory effect. Therefore, the nature of the substrates and sources of organisms might account for the differences in the pH regimes.

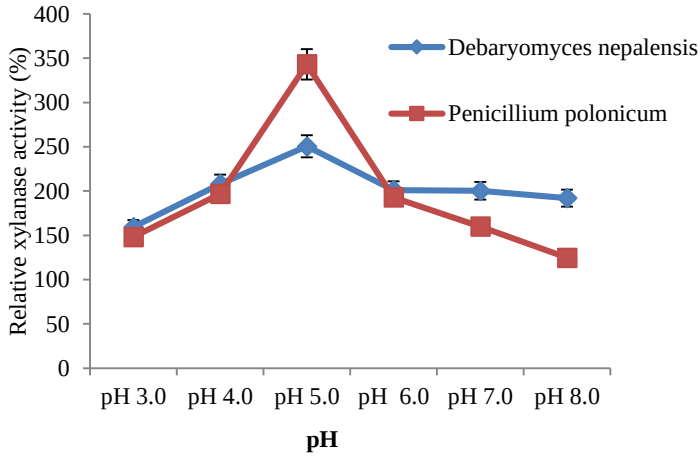

Fig. 5: Effect of different pH values on xylanase production from fungal isolates

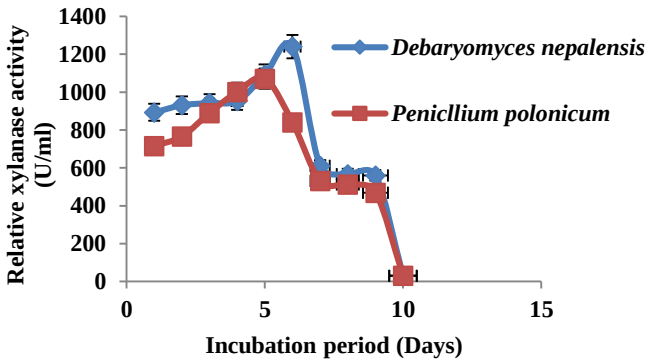

Fig. 6: Effect of incubation period on xylanase production from fungal isolates

Effect of incubation time on xylanase production by the best two xylanase producing fungi is presented in Figure 6. The xylanase activity increased with increase in incubation time until it reached the maximal (2.652 ± 0.033 U/mL) at 120 and (2.986 ± 0.083 U/mL) at 144 hours for Penicillium polonicum and Debaryomyces nepalensis respectively. Further incubation beyond optimal incubation time led to decreasing levels of enzyme production, probably due to decreasing levels of nutrients in the fermentation medium and accumulation of metabolic wastes leading to decrease growth and enzyme production [51]. This is in agreement to the findings of [46] and [52] who recorded maximal xylanase production from T. viride and A. niger at 144 hours and 120 hours incubation time, respectively. The insignificant xylanase

production by the organisms during the later stage of fermentation could be due to the scarcity of insoluble xylan particles in the medium [53].

Conclusions

Rhizosphere soil of cassava tuber can be used as a source of xylanolytic fungi . Also, *D*. nepalensis and P. polonicum can serve as an ideal source of xylanases due to their excellent characteristics for production under submerge fermentation

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