Optimization of Amylase Production in Three Fungal Species

ABSTRACT

Aim: Amylase is an important enzyme that is employed in starch processing industries, used in the hydrolysis of polysaccharides such as starch into simple sugar constituents. In this study, we investigated the abilities of several isolated amylolytic soil fungi to produce amylase.

Materials and Methods: Soil samples collected from the botanical garden, Department of Plant Science and Biotechnology, University of Jos was serially diluted and screened for the presence of amylase producing fungi. Optimization studies was performed across different parameters; Incubation period (7 days), different temperatures (25-60°C), different pH (5-9), different starch concentration (0.2-2%), carbon source (sucrose, maltose, lactose).

Results: A total of 15 isolates belonging to 7 genera were isolated. Soil samples were analyzed for their ecological parameters. The plate assay showed that three species *T. viride*(62mm), *P. citrinum*(50.25mm), and *A. niger* (67mm) had the largest zones of clearance and highest amylolytic activity thus were selected for further studies. For submerged fermentation, optimum amylolytic activity was observed at 24 hours of incubation for all three species *T. viride*(7.92 IU/ml), *P. citrinum*(5.04 IU/ml), and *A. niger* (7.00 IU/ml). Maximum enzyme activity was observed at incubation temperature of 45°C (17.10 IU/ml) for *T. viride,* 50°C (33.60 IU/ml) for *P. citrinum,* and 50°C (14.30 IU/ml) for *A. niger.* The maximum enzyme activity was at pH 9 (20.40 IU/ml) for *T. viride,* pH 11 (18.50 IU/ml) for *P. citrinum,* and pH 7 (25.80 IU/ml) for *A. niger. T. viride*and *P. citrinum*recorded an optimum enzyme activity of 15.40 IU/ml and 13.20 IU/ml respectively when sucrose was used as a carbon source while *A. niger* recorded an optimum activity of

7.28 IU/ml when maltose was used. Starch concentration of 2% showed the highest enzyme activity of 16.52 IU/ml, 15.4 IU/ml and 14.00 IU/ml, for *T. viride, A. niger* and *P. citrinum,* respectively.

Conclusion: *Trichodermaviride*, *Penicilliumcitrinum*, and *Aspergillus niger* showed potential of producing amylase which is useful in the biodegradation of biological wastes.

Keywords: Amylase, *Trichodermaviride*, *Penicilliumcitrinum*, *Aspergillus niger,* Amylolytic activity, Soil fungi.

1. INTRODUCTION

Enzymes are biological catalysts which bring about chemical reactions in living cells. They are produced by the living organisms and are usually present in only very small amounts in the various cells (about 0.1%) [1]. Amylases are enzyme that breaks down starch or glycogen. Amylase can be obtained from numerous sources such as plant, animal and microbes [2].

The new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in exploration of extra cellular enzymatic activities in several microorganisms [3, 4]. In recent times, amylases (α- amylase, β- amylase and γ- amylase) received a great deal of attention due to its perceived technological significance and economic benefits [4]. Amylolytic enzymes constitute one of the most significant groups of commercial enzymes. These enzymes have widespread utilizations in industrial processes, such as food industries; major consumers of amylases, detergent, leather, and pharmaceutical industries [5]. Amylase of fungal origin was found to be more stable than the bacterial enzymes on a commercial scale, and attempts has been made to improve culture conditions and suitable strains of fungi. Major benefit of using fungi for amylase production is the economical bulk production capability [2]. Potentially pathogenic amylolytic fungi are widely spread worldwide and includes diversity of filamentous fungi [6]. Submerged fermentation (SmF) is defined as fermentation in the presence of excess water, it is beneficial because process control and sterilization is easier to obtain depending on the fungi strain and the culture conditions the enzyme can be constitutive or inducible, showing diverse production patterns [2].

Due to the growing demand for amylase in various industries, there is huge interest in developing enzymes with better properties such as, raw starch degrading amylases suitable for industrial applications and their cost effective production techniques [7]. This study was therefore carried out to screen for amylolytic soil fungi strains capable of producing high amylase for industrial use.

2. Materials and Methods

2.1 Soil Sample Collection

Soil samples were collected from the botanical garden of the Department of Plant Science and Biotechnology, University of Jos. The pH of soil samples was determination using the method of Okonkwoeje [8] by suspending 30g of the soil sample in 100ml of distilled water and pH calculated using a pH meter. 30g of soil from each soil sample collected was dried to a constant weight in hot air oven set at 110°C and percentage of moisture content assessed using the method Ogbonna and Pugh [9].

2.2 Isolation of Fungi from the Soil Samples

Fungal colonies were isolated from test soil samples using serial dilution method; An aliquot of 10^{-4} was taken from each test tube and spread on Petri dishes containing solidified Potato Dextrose Agar (PDA) supplemented with 5ml penicillin and streptomycin sulphate and incubated at 25°C for 5 days. Plates were observed daily for fungal growth which were later sub-cultured to obtain pure cultures. Identification of the isolates was done using

schemes of Domsch*et al.* [10] by examining the nature of colonies, microscopic inspection of mycelia, arrangement and the nature of the fruiting bodies.

2.3 Screening of Fungi for Amylolytic activity

All isolated fungal species were screened for amylase production based on modified method of Ogbonna *et al*. [11] using selective medium containing (g/l): KH_2PO_4; 0.7, NH_4NO_3; 5, KCl; 0.2, $MgSO_4.H_2O$; 0.2, $FeSO_4.H_2O$; 0.5, soluble starch; 10, distilled H_2O; 1000ml, agar; 15, at pH of 5.0. Isolates were inoculated at three points on Petri plates and incubated at 25 ± 2°C for 5 days. The plates were removed after 5 days. At the end of the incubation, all plates were flooded with iodine solution and kept for 15minutes to observe for clear zones of hydrolysis around the inoculated culture. Mean diameters of three replicates were measured (in mm) for each fungal species. *Aspergillus niger, Trichodermaviride*and *Penicilliumcitrinum*were selected for further studies due to their maximum relative diameter of clear zones as compared to other species.

2.4 Effects of incubation period on amylase production and enzyme assay

The isolated fungi species were grown on submerged fermentation medium using a modified method of Ogbonna et al. [11] consisting of (g/l): KH_2PO_4; 0.7, NH_4NO_3; 5, KCl; 0.2, $MgSO_4.H_2O$; 0.2, $FeSO_4.H_2O$; 0.5, distilled H_2O; 1000ml, 1% (w/v) soluble starch at a pH of 5.0. The medium was incubated at 25°C for 7 days. 1ml of crude enzyme were harvested at 24 hours' interval and centrifuged at 8000rpm for 15 minutes at 40°C. Supernatant was filtered using Whatman filter paper and filtrate used as enzyme source for assay. The amylase activity in the culture filtrate was assessed by the analysis of reduced sugars liberated during the hydrolysis of 1ml of 1% (w/v) starch in citrate phosphate buffer (pH 5.0) at 40°C for 20 minutes by a modified dinitro-salicyclic acid (DNS) method [12]. The amylase enzyme activity was defined as the quantity of glucose liberated per ml per unit time in the reaction mixture [12].

2.5 Optimization studies on amylase production

Effects of temperature, pH, carbon sources and substrate concentration on amylase activity were studied. The present study investigated different temperature (25, 37, 45, 50 and 60°C) different pH (5, 7, 9, 11), different carbon sources (1% of maltose, sucrose and lactose), different concentration of substrate (0.2, 0.5, 1.0, 1.5, and 2.0%). Standard amylase assay by Toye [12] was performed to determine optimum condition for amylase activity.

3. RESULTS

The results for ecological parameters (pH and moisture content) of the soil samples are presented on Table 1. Sample A, B and C recorded a pH of 5.78, 6.11 and 6.20 respectively with sample A being more acidic than the others and result for moisture content of the experimental soil samples A, B and C were 19.30%, 17.93%, and 16.35% respectively. The result showed that the soil samples were within the pH ranges that support fungal growth.

The number of fungi species isolated from the soil samples are recorded in Table 2. A total of 25 fungal isolates were obtained from analysis of the soil samples consisting 7 genera and 15 species. 5 species identified belonged to the genus *Aspergillus,* 3 to the genus *Penicillium*, 2 belonging to *Trichoderma*and *Fusarium*genera,

and 1 species each to *Cladosporium, Mucor, Rhizopus*genera. Samples A & C recorded 9 species each while sample B recorded 7 species.

The results from plate assay (Table 3) indicated that all the fungal isolates were amylolytic and the diameters of the clearance zones indicated the starch degrading ability of the fungi species. *A. niger*, *T. viride*and *P. citrinum*recorded the largest zones of clearance of 67, 62 and 50.25mm respectively. *R. stolonifer, Mucor*sp and *A. oryzae*had diameter zones of clearance of 46.25, 44.75 and 40.75mm respectively and the least zones of clearance were observed for *Cladosporium*sp and *Penicilliumchrysogenum*with clearance zones of 21 and 13.25mm respectively. Plate 1-4 shows the zones of hydrolysis of some of the fungi isolates. Having produced the highest diameter zones of clearance, *T. viride, A. niger*, and *P. citrinum*were selected for further studies.

The effects of incubation period are presented on Table 4. The test fungi *T. viride, A. niger*, and *P. citrinum*showed a maximum amylase activity of 7.92 IU/ml, 7.00 IU/ml and 5.04 IU/ml respectively on day one (24 hours) of incubation.

Figure 1 shows the result of the effects of incubation temperature on the growth and enzyme activity indicated that the organisms had enzyme activity at all the incubation temperatures. The optimum amylolytic activity for *P. citrinum*(33.60 IU/ml) and *A. niger* (14.30 IU/ml) was observed at 50°C while that for *T. viride*(17.10 IU/ml) was observed at 45°C. The results for optimum pH range for amylase activity is reported in Figure 2. The enzyme activity showed a gradual in with an increase in pH value. The maximum amylase activity of *T. viride*(20.40 IU/ml) was at pH 9*, A. niger* (25.80 IU/ml) was at pH 7, and *P. citrinum*(18.50 IU/ml) was at pH 11. Figure 3 shows the result of amylolytic activities of test fungi using different carbon sources. Optimum enzyme activity of 15.40 IU/ml and 13.20 IU/ml was observed on sucrose by *T. viride*and *P. citrinum*respectively. *A. niger* recorded an optimum amylase activity of 7.28 IU/ml on maltose source.

The results of different concentration of soluble starch assayed for enzyme activity is reported in Figure 4. The optimum enzyme production was recorded at 2% of substrate concentration for *T. viride*(16.52 IU/ml)*, P. citrinum*(15.40 IU/ml), *and A. niger* (14.00 IU/ml).

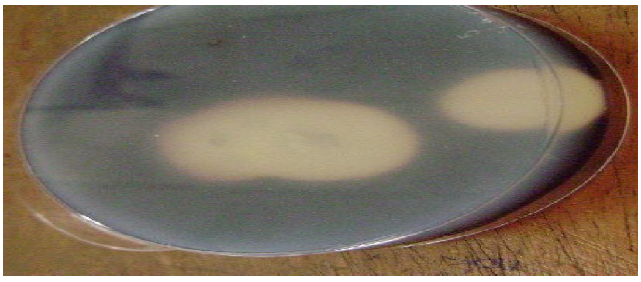

Plate 1: ***Penicilliumcitrinum***

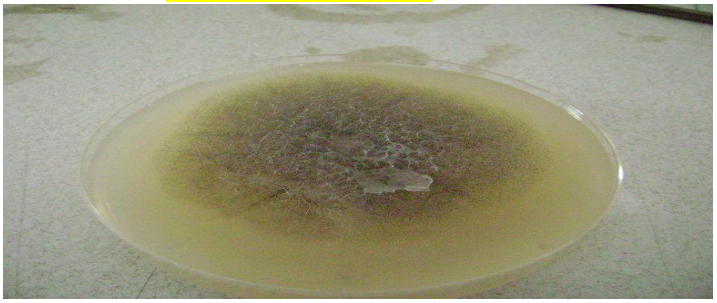

Plate 2: ***Aspergillus niger***

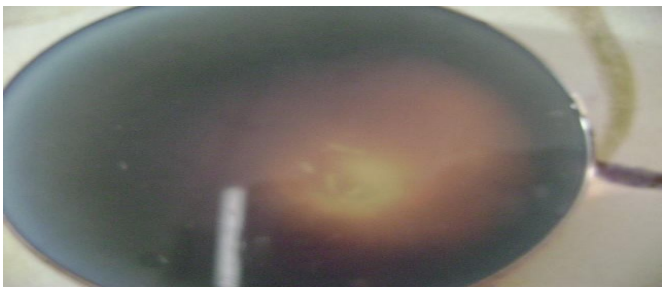

Plate 3: ***Trichodermaviride***

Plate 4: ***Mucorsp***

Table 1: Ecological Parameter of the Experimental Soil Samples

Parameter	Soil Sample A	Soil Sample B	Soil Sample C
Moisture %	19.30 ± 1.60	17.93 ± 1.82	16.35 ± 1.26
pH	5.78 ± 0.06	6.20 ± 0.03	6.11 ± 0.04

Table 2: Fungal Species Isolated from Experim Samples

S/NO	Fungal isolates	Locations			Total
		A	B	C	
1.	*Aspergillus flavus*Link	-	+	-	1
2.	*A. niger Van Tieghem*	-	+	-	1
3.	*A. terreus* Thom	-	+	+	2
4.	*A. parasiticus*	+	+	-	2
5.	*A. oryzae*	+	+	-	2
6.	*Cladosporium*sp	+	-	+	2
7.	*Mucor*sp	-	-	+	1
8.	*Penicilliumchrysogenum*Thom	+	-	+	2
9.	*Penicilliumcitrinum*	+	-	+	2

10.	*Penicilliumsp*	+	-	-	1
11.	*Rhizopussp*	-	+	-	1
12.	*Trichodermaviride*pers. *Ex Gray*	+	+	+	3
13.	*Trichodermasp*	+	-	+	2
14.	*Fusariumsp*	+	-	+	2
15.	*Fusariumsp*	-	-	+	1
	Total	9	7	9	25
	% frequency of occurrence	3.33,1.75,3.33.			

+ (Present), - (Absent).Sp (Species).

Table 3: Determination of Amylolytic Activity of the Fungal Isolates Using Starch as sole source of Carbon

Fungal isolate	Growth on starch Agar	Clear zone	Diameter (mm)
Aspergillus niger	+ + +	+	67.00 ± 1.81
Trichodermaviride	+ +	+	62.00 ± 1.46
Penicilliumcitrinum	+ + +	+	50.25 ± 1.30
Fusariumsp	+	+	31.50 ± 1.47
Rhizopussp	+	+	46.25 ± 1.08
A. terreus	+	+	30.25 ± 0.18
A. oryzae	++	+	40.75 ± 0.88
Mucorsp	+	+	44.75± 0.58
P. chrysogenum	+	+	13.25 ± 1.33
Cladosporiumsp	+	+	21.00 ± 0.96

The symbol (+ + +), (+ +), and (+); indicates high, moderate and low activity respectively, Sp (Species)

Fungal isolates			24	48	72	96	120	144	166
T. viride									
	Optical (600nM)	Density	0.28	0.26	0.13	0.01	0.07	0.07	0.02
	Enzyme IU/ml	Activity	7.92	7.28	3.64	0.28	1.96	1.96	0.56
P. citrinum									
	Optical (600nM)	Density	0.18	0.16	0.16	0.05	0.04	0.05	0.07
	Enzyme IU/ml	Activity	5.04	4.48	4.48	1.40	1.12	1.40	1.96
A. niger									
	Optical (600nM)	Density	0.25	0.18	0.15	0.01	0.04	0.01	0.01
	Enzyme IU/ml	Activity	7.00	5.08	4.20	0.28	1.12	0.28	0.28

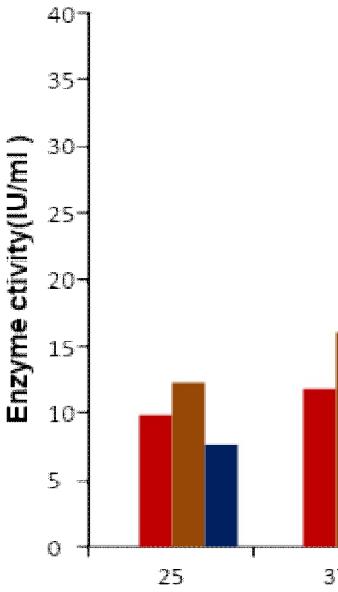

Figure 1: Effects of Different temperatures on Enzyme activity

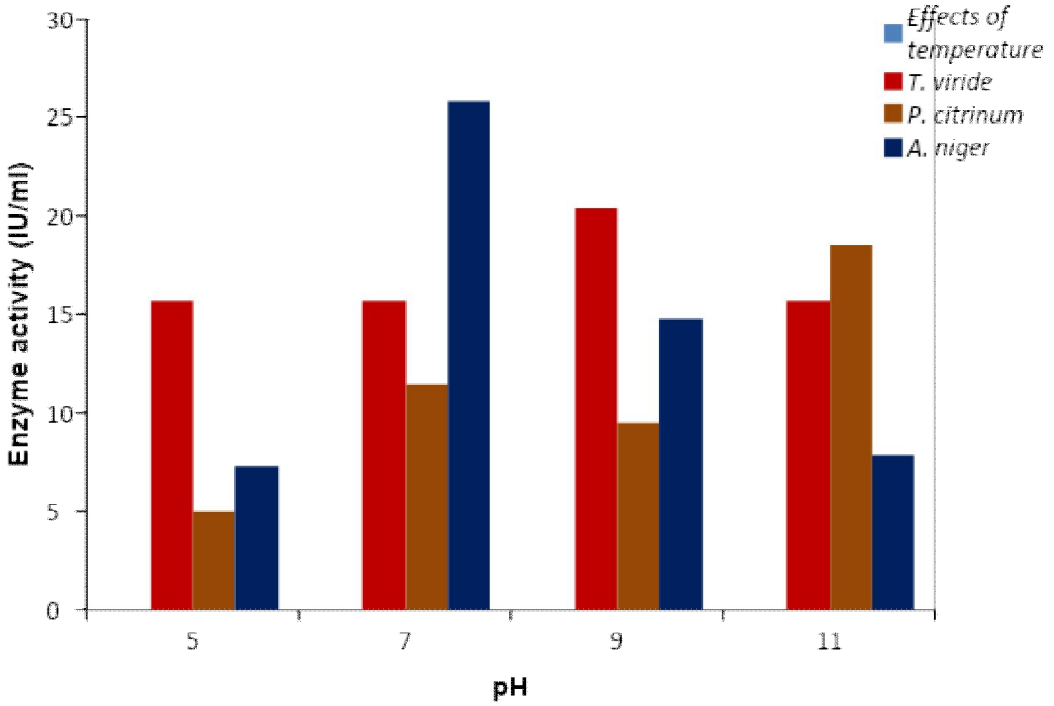

Figure 2: Effects of Different pH range on enzyme activity

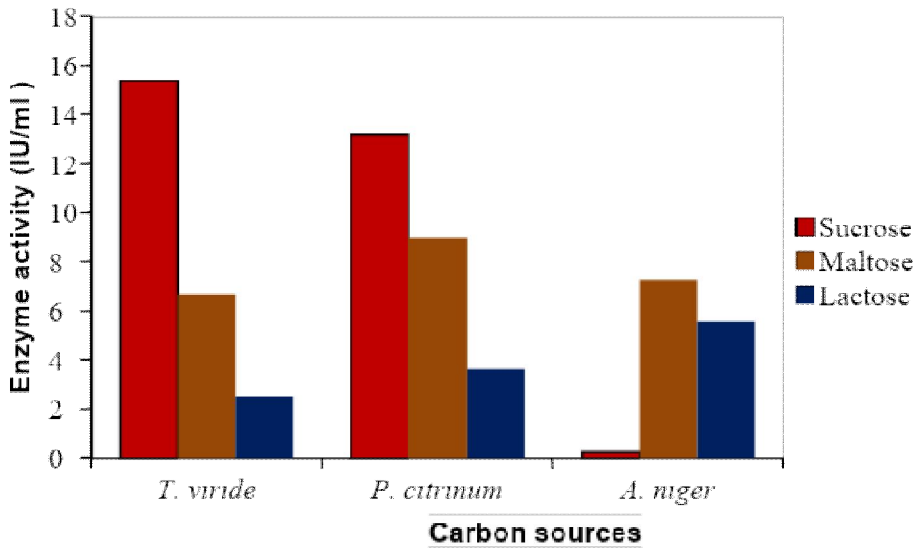

Figure 3: Effects of Different carbon sources on enzyme activity

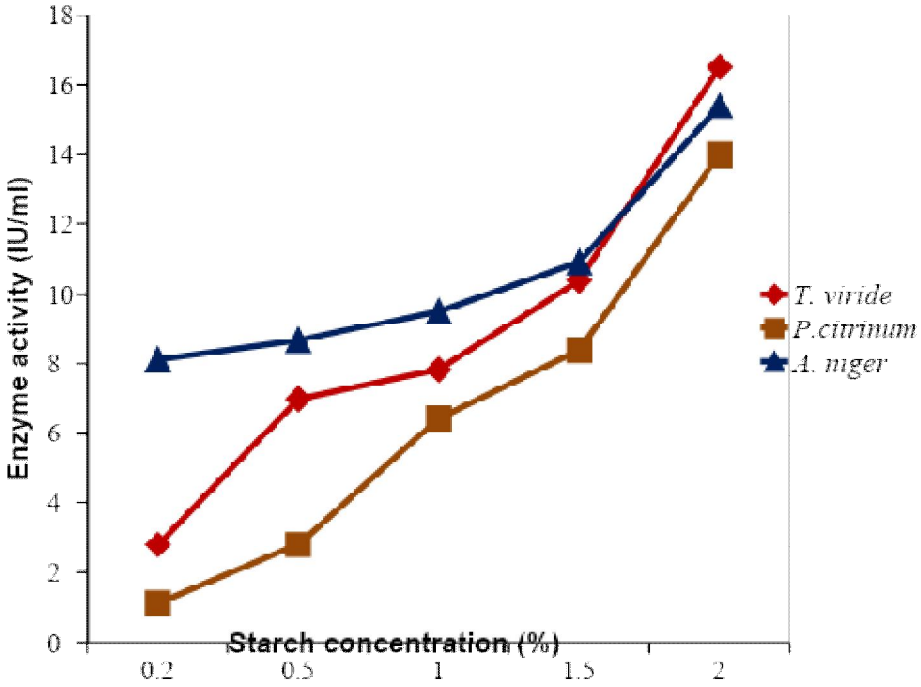

Figure 4: Effects of Different Concentrations of Soluble Starch on enzyme activity

4. DISCUSSION

Fungi are present in soils of different areas (Sarawis, 1990). A total of 25 isolates belonging to 7 genera, *Aspergillus, Trichoderma, Cladosporium, Fusarium, Mucor, Penicillium,* and Rhizopus were recorded and assessed for their amylolytic ability. *Aspergillus* was the most prevalent genera with *A. niger* as the dominant species. Studies by Soomro et al. [13] suggested that amylolytic soil fungi thrive better on nutrient rich (starch) substrate. *Aspergillus* species, *Penicillium*species and *Trichoderma*species are distributed worldwide [6].

Soil sample A recorded a pH value of 5.78±0.06 and moisture content of 19.30±1.60, sample B recorded a pH value of 6.20±0.03 and moisture content of 17.93±1.82, and sample C recorded a pH value of 6.11±0.04 and moisture content of 16.35±1.26. The increase in the number of isolates in sample A could be attributed to the pH and water content of the soil which plays a significant role in microbial activities in natural environments such as soil. Fungi thrive best in acidic environment and dominate soil of low pH or slightly acidic pH where soils tend to be undisturbed [14]. It is reported that acidic and slightly alkaline soils promote biodegradation activity of microorganisms [15]. All the fungal isolates exhibited growth on the starch agar with clear hydrolytic zones when flooded with iodine solution and showed amylase activity. From Table 4, incubation time of 24 hours showed a maximum amylase activity for *T. viride*and *A. niger* and *P. citrinum.* The findings of this work are consistent to that ofNahas et al., [16] who reported a similar result of 1-2 days as optimum for amylase production. An increase in the incubation period resulted in a

decrease in amylase production by fungi isolates. This may be due to the fact that after the maximum time for maximum amylase production is reached, there result a production of other by product and depletion of the nutrients. These byproducts inhibit the growth of fungi and hence enzyme formation [17]. The effect of different incubation temperature on the activity of the amylases [Fig 1] indicated the best temperature for maximum amylase production for *P. citrinum*and *A. niger*is at 50°C while that of *T. viride*is at 45°C. Previous works by Sun *et el.,* [18] recorded an optimum temperature for α- amylase from fungi and yeast sources to be between 30 and 70°C. Penicillium species has recorded an optimum temperature between 30 and 60°C for amylase by earlier studies [19, 20]. Previous work by Sazzad et al [21] reported maximum temperature for *T. viride*at 50°C which is closely related to the findings of this research. This optimum activity temperature of the fungal amylase is similar to that of some known fungus α-amylases such as *Aspergillus niger, Aspergillus oryzae*and *Mucorpusillus.*

Different microbial organisms have recorded a vast range of pH for amylase production. The result (Fig 2) recorded an optimum amylase activity at pH 9 (20.4 IU/ml) for *T. viride*, pH 7 (25.8 IU/ml) for *A. niger* and pH 11 (18.5 IU/ml) for *P. citrinum*. The findings of this work is consistent with that of Gupta *et al.* [22] reported amylase production at pH 4.0- 11.0 although stability of amylases has been reported in narrow range. Amylase production increased with an increase in concentration of substrate. Substrate concentration of 2% starch yielded the highest amount of amylase in all three test fungi (Fig 3); *T. viride*, (16. 52 IU/ml), *A. niger* (14.0 IU/ml), and *P. citrinum*(15.4 IU/ml). Nguyen *et al*. [23] reported that starch and its hydrolytic products induce amylase production. From results of different carbon sources (Fig 4), *T. viride*and *P. citrinum*reported an optimum amylase activity of 15.4 IU/ml and 13.2 IU/ml respectively on sucrose while *A. niger* recorded an optimum amylase activity of 7.28 IU/ml on maltose. Maltose and sucrose have been reported as amylase inducers [24] (Moreira *et al*., 2001

5. Conclusion

Amylolytic fungi are very common and the most diverse amongst microorganism that degrade starch products. The test fungi (*P. citrinum, A. niger*, and *Trichodermaviride*) which are readily available, cheap and affordable from the environment showed the potential to produce amylase which has great importance in biotechnological applications in degradation processes.

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