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ISOLATION AND SCREENING OF α-AMYLASE AND GLUCOAMYLASE PRODUCING FUNGI AND THEIR APPLICATION IN BIOETHANOL PRODUCTION

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ABSTRACT
Isolation of α-amylase and glucoamylase producing fungal strains was investigated. Samples (120 in number) were collected aseptically from rice mill industrial areas, cassava processing grounds, potato farms, corn processing industries and refuse dumping sites within Abakaliki metropolis in Ebonyi State of Nigeria. The samples were first grown on solid agar (PDA) and sub-cultured to get their pure cultures. The pure cultures were then grown for 3 days on PDA and screened for starch hydrolysis using Iodine-potassium iodide method. Out of the 120 cultures, 26 showed some degrees of starch hydrolysis. The 26 positive fungal isolates were further screened for enzymatic activities which were measured quantitatively with spectrophotometric methods. However, only 3 isolates (2a, 3 and 6b) were finally selected based on their high α-amylase and glucoamylase activities. Alpha-amylase production by co-cultivation of the selected isolates showed higher activities than single cultures. For instance, the co-culture of isolate 2a and 3 was higher (40.32±0.489 U/ml) than individual activities of isolate 2a (30.55±0.710 U/ml) or isolate 3 (32.44±0.442 U/ml). Bioethanol production was achieved by Simultaneous Saccharification and Fermentation (SSF) process using the selected fungal isolates and *Saccharomyces cerevisiae.*The bioethanol concentrations were measured quantitatively with boiling/iodometric method. Isolate 3 with the yeast gave the highest concentration of the ethanol (10.913±0.874 g/L) after 96hours followed by isolate 2a and yeast (9.817±0.400 g/L) and then isolate 6b and yeast (8.540±0.308 g/L). Finally, the selected fungal isolates were identified as *Aspergillus species* (isolate 3), while isolates 2a and 6b were *Mucor* and *Rhizopus species* respectively.

KEYWORDS: Alpha-amylase, Glucoamylase, Bioethanol, Fungi, Isolation, Activities.

INTRODUCTION

Amylases are of great importance in fermentation and food industries for hydrolysis of starch and other related oligosaccharides (Akpan *et al*, 1999; Pederson and Nielson, 2000). They are enzymes produced by variety of organisms, ranging from bacteria to plants and animals. Bacteria and fungi secrete amylases to the outside of their cells to carry out extracellular digestion. Amylases degrade starch and other related polymers to yield products characteristic of individual amylolytic enzymes. Starch is a glucose polymer linked together by α-1,4 and α-1,6 glycosidic bonds. Amylases act by hydrolyzing the glycosidic bonds between adjacent glucose units of the polymer (Bernfeld, 1951).

Based on the points of attack on the glucose polymer chain, alpha-amylases can be classified into two categories, liquefying and saccharifying (Nigam and Singh, 1995). The fungal α-amylase belongs to the saccharifying category and attacks the second linkage of the straight chain of starch molecule from the non-reducing terminal (i.e.C4 end), resulting in the splitting off of two glucose units at a time (Fadel, 2000). The resulting product is a disaccharide called maltose. The starch chains are thus broken down into smaller units. Glucoamylase (amyloglucosidase) consequently hydrolyses α - 1, 4 - glycosidic bonds from non- reducing ends of starch molecules, resulting in the production of glucose. To a lesser extent, glucoamylase also has the ability to hydrolyze α- 1, 6 linkages, also resulting in glucose as the end product (Mertens and Skory, 2006).

Fungal α-amylase and glucoamylase may be used together to convert starch to simple sugars, which in turn serve as a feedstock for production of bioethanol or in the production of high fructose syrups (Seviek *et al*, 2006). While fructose syrups are important sweetener in food processing industries like beverages, bakeries and confectionaries, bioethanol has great potential as a renewable, non-toxic and clean alternative fuel that reduces dependency on fossil energy. Fermentation of sugars derived from energy crops and grains is an economical and efficient method for bioethanol production. The use of these sugars for producing bioethanol leads to opportunities for farmers by increasing demand for their products, resulting in a boost in rural economies (Olfert *et al*, 2007). Bioethanol as an alternative source of energy has received special attention over the world due to depletion of fossil fuels. According to United States Department of Energy, for every unit of energy put towards ethanol production, 1.3 units are returned (Hill *et al*, 2006). Ethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases (Altıntas *et al*, 2002).

Yeast cells (*Saccharomyces cerevisiae*) are facultative anaerobes and under anaerobic conditions can ferment glucose to ethanol. *Saccharomyces cerevisiae* is ideal for ethanol production due to several properties including fast

growth rates, efficient glucose repression, effective ethanol production and tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels. Glucose is broken down to form pyruvate in most organisms via the glycolytic pathway and this pyruvate can result in the production of ethanol under anaerobic conditions. The energy for growth of cells during ethanol production is provided by the glycolytic and fermentation pathways (Piskur *et al.*, 2006).

The efficiency of bioethanol production largely depends on the availability of suitable substrate, yeast strain and method employed. Starchy substrates are promising due to their economic viability, availability and renewability (Coughlan, 1985). Starch, a macromolecular polymer of glucose units, is a significant component of domestic and commercial waste and a useful resource that can be converted into ethanol. Yeast is unable to consume raw starch and hence, the starch must first be broken down into simple sugars (Birol *et al*., 1998). This task is achieved by two enzymes: α-amylase, which hydrolyzes α-1, 6 linkages and glucoamylase, which hydrolyze α-1,4 linkages in starch molecules. These enzymes are expensive and contribute significantly to bioethanol production costs. Amylase enzymes are not yet produced in Nigeria, making them too expensive for our industries. More so, in Ebonyi State, Abakaliki precisely, much research work are yet to be done in the area of amylase production. Meanwhile, fungal organisms are known for their ability to produce extracellular enzymes, α-amylase and glucoamylase capable of hydrolyzing starch substrates (Syu and Chen, 1997). These challenges were actually the driving forces that necessitated this research, to isolate and screen for effective amylase producing fungal strains from our environment. And so doing, we could therefore explore the possibility to ameliorate the cost of amylase enzymes for our industries and thus, develop a cheaper process for conversion of starch materials to bioethanol.

MATERIALS AND METHODS

Materials used include: Agar media {Potato Dextrose Agar (PDA)}, distilled water, petridishes, autoclave, thermometer, centrifuge, refrigerator, water bath, incubator, pH-meter, weighing balance, Bunsen burner, wire loops, test tubes and racks, bijou bottles, universal containers, Erlenmeyer flask, spectrophotometer and other laboratory apparatus.

Sample collection

Samples were aseptically collected from cassava processing sites, rice milling industries, corn processing areas, potato farms and refuse dumping grounds within Abakaliki metropolis in Ebonyi State, South-East of Nigeria. The upper layers of the sites were scraped off with a sterilized spatula and the samples beneath were collected using aseptic bags. These were immediately transported to the laboratory and stored at 4^{o}C.

Preparation of Culture Media

Potato Dextrose Agar (PDA) was prepared according to the manufacturer's specification. That is, 39g of agar media powder was dissolved in Erlenmeyer flask containing 1L (1000ml) distilled water. The flask was covered with cotton-plug and foil and sterilized by autoclaving at 121^{o}C for 15 minutes. After cooling to about 45^{o}C, about 12 ml of the media was poured into different sterile petridishes and then left undisturbed until the agar solidified. The plates were maintained at aseptic condition.

Isolation of Fungal Organisms

The samples from different sources were suspended in 10 ml of sterilized distilled water, followed by serial dilution of each sample into four different flasks. A loopful of each sample from the diluents was streaked on the solidified PDA media plates and incubated at 37^{o}C for 72 hours for the fungal growth. The pure cultures were identified by their morphology and colony characteristics and sub-cultured. The organisms were maintained on PDA slants and stored at 4^{o}C.

Screening of Isolates for Starch Hydrolysis

The isolates were screened for starch hydrolyzing ability. The fungal isolates were inoculated on 1 % starch PDA plate. After 3-4 days of fungal growth, the plates were flooded with iodine solution. Starch reacted with iodine to form a dark blue starch-iodine complex that covered the entire agar. When starch was broken down into sugars, there were clear zones surrounding streaked lines which indicate starch hydrolysis (Alfred, 2007).

Screening of Isolates for Amylase Enzymes Production

The isolates were screened quantitatively for the production of α-amylase and glucoamylase in a liquid culture medium constituted of (in gram per litre) soluble starch, 20; KH_2PO_4, 14; NH_4NO_3, 10; KCl, 0.5; $MgSO_4.7H_2O$, 0.1; $FeSO_4.7H_2O$, 0.01. This was adjusted to pH6.9. About 100ml of the medium was distributed to each 250ml Erlenmeyer flasks and sterilized by autoclaving at 121^{o}C for 15 minutes. They were allowed to cool down to room temperature (25^{o}C). The isolates were inoculated into the prepared liquid medium and incubated with shaking at 37^{o}C for 3-5 days. The entire content of the culture were centrifuged and filtered aseptically to get the crude enzymes as culture filtrates. These were used to hydrolyze fresh starch solution and assayed for α-amylase and glucoamylase activities.

Measurement of Alpha-amylase activity

The α-amylase activities of the isolates were assayed using the Caraway-Somogyi, Iodine- Potassium Iodide (IKI) method according to Cheesbrough (2005).

Measurement of Glucoamylase activity

The glucoamylase activity of each of the isolates was determined by the method of Trinder, (1959) known as the glucose oxidase/peroxidase (GOD/POD) method.

Effects of Mixed-Culture of the Fungal Isolates on Alpha-amylase and Glucoamylase Production

The three selected fungal isolates were co-cultivated (two isolates in one culture) in a liquid medium to compare the efficacies of mixed and single cultures of the isolates on the production of α-amylase and glucoamylase.

Identification of Selected Fungal Isolates

The selected fungal isolates were identified by morphological characteristics according to the taxonomic key of Alexopoulos *et al.* (1990). Both the macroscopic and microscopic characteristics were observed on the fungal isolates. In microscopic identification, the spores were dispersed in lactose phenol blue solution on a slide and the spore arrangement was examined microscopically,

while in macroscopic examination, the colour, texture and shape were observed.

Bioethanol production from potato flour by the selected fungal isolates and *Saccharomyces cerevisiae*

Bioethanol was produced from 80 g/L potato flour at pH 4.0 and 37^{o}C. The potato tubers were peeled, sliced, dried, ground and sieved to produce fine flour and then gelatinized. The isolates were innoculated together with *Saccharomyces cerevisiae* and cultivated for 5 days, while the concentrations of the ethanol produced and glucose liberated were measured every 24 hours. The ethanol concentration was determined with the boiling/iodometric method of Gwarr (1987).

RESULTS

Screening of fungal isolates for amylase production

A total of 120 microorganisms were isolated. Among the isolates, 26 of them produced α-amylase and glucoamylase. Table 1 presented the results of the 26 positive isolates when tested based on their α-amylase and glucoamylase activities. All the experiments were run parallel in triplicates; each value represents the mean and standard deviation of three experiments. The results of the screening showed that among the 26 producing isolates, 10 of them showed higher enzyme activities than others and were taken for further screening. They include, isolates 2a, 3, 6b, 12, 13, 25, 31, 44, 50 and 118.

TABLE 1: Alpha-amylase and glucoamylase activities of 30 fungal isolates after 72 hrs cultivation at 25 ^{o}C, pH 6.9 and 10 g/L starch.

Isolate	Alpha-amylase activity(u/ml)	Glucoamylase activity(u/ml)
3	28.81±0.305	1.327±0.103
6	08.70±0.363	0.110±0.019
7	18.73±0.946	0.068±0.014
8	07.85±0.316	0.148±0.011
11	23.45±0.668	0.085±0.011
12	21.40±0.159	0.385±0.042
13	22.85±0.165	0.087±0.005
20	07.45±0.551	0.149±0.011
25	26.70±0.474	0.996±0.099
31	23.30±0.140	0.084±0.004
40	21.32±0.507	0.249±0.036
41	08.20±0.511	0.125±0.008
44	23.10±0.072	0.284±0.007
47	11.70±0.505	0.174±0.008
50	25.90±0.245	0.299±0.025
67	16,25±0.313	0.193±0.009
71	14.22±0.440	0.107±0.010
75	14.60±0.537	0.155±0.022
78	13.95±0.221	0.027±0.008
79	07.82±0.236	0.081±0.005
85	08.19±0.137	0.094±005
97	12.80±0.369	0.073±0.007
107	11.70±0.195	0.066±0.008
118	23.30±0.435	0.257±0.016
2a	29.80±0.269	1.006±0.010
6b	27.65±0.161	0.984±0.097

Further Screening of Ten Isolates

The 10 out of the 26 isolates with the higher amylase activities were further screened. Figure 1 shows the result for α-amylase activities while figure 2 shows the results of the screening for glucoamylase activities. Among the ten isolates, three were observed to exhibit higher enzyme productivity in the following order (isolate 3 > 2a > 6b), and they were used for further studies.

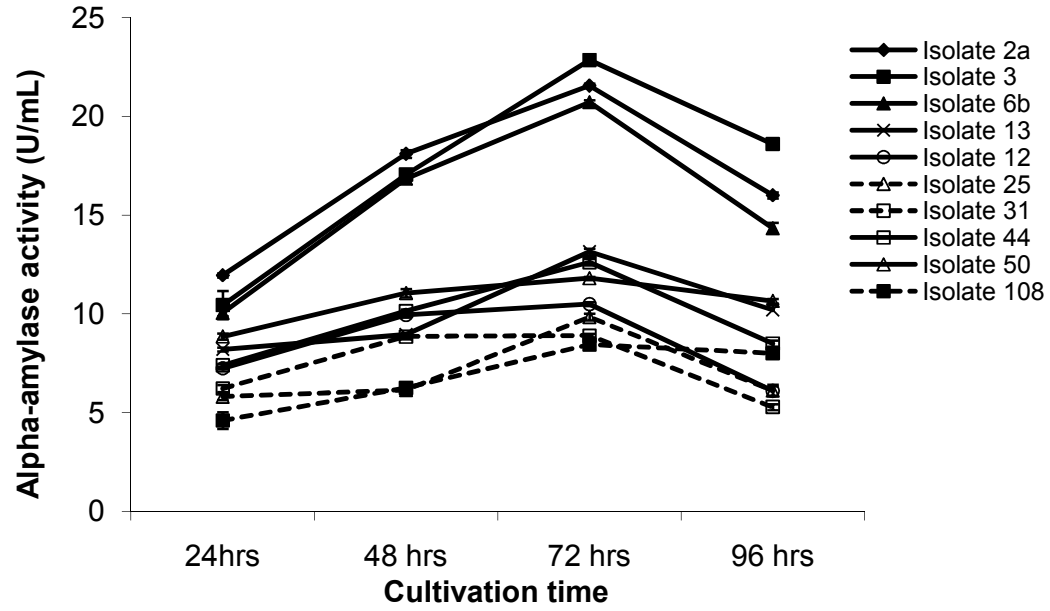

FIGURE 1. Alpha-amylase activities of 10 selected fungal Isolates cultivated at 25^{o}C, pH6.9 and 10g/L starch concentration

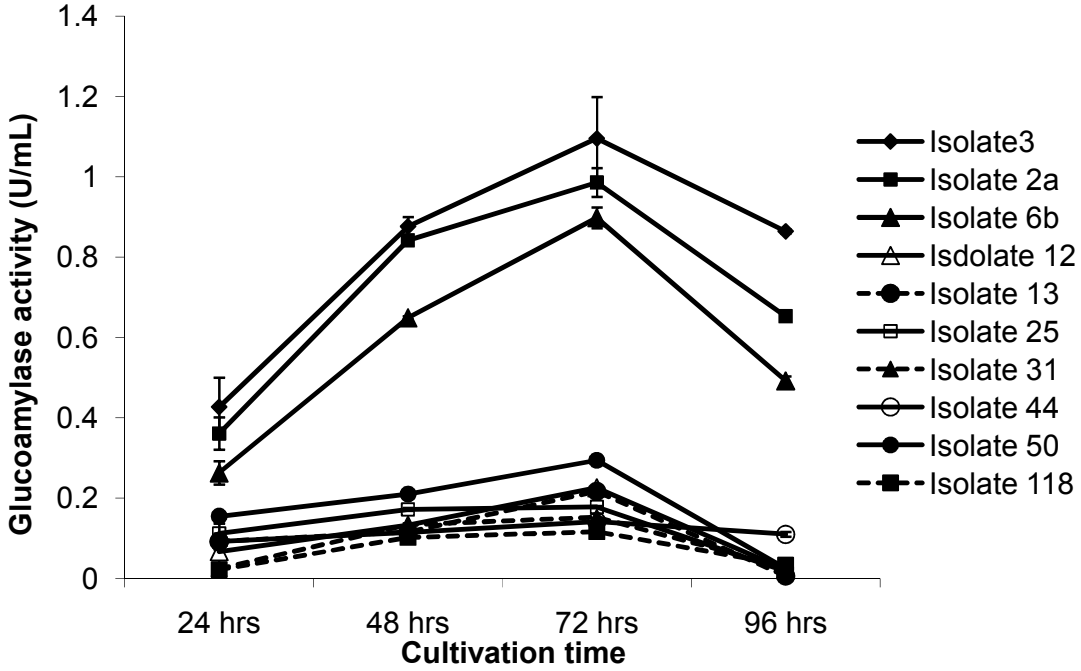

FIGURE 2. Glucoamylase activities of 10 selected fungal isolates cultivated at 25^{o}C, pH6.9 and 10g/L starch concentration

Effects of mixed cultures of the 3 selected isolates on amylase production

Figures 3 and 4 showed the influence of mixed cultures on the amylase enzymes production by the selected fungal isolates. The mixed cultures were more effective in production of α-amylase and glucoamylase than their component single cultures (2a+3 > 2a or 3). The mixed cultures of isolates 2a+3 were the highest producers while the single culture of isolate 6b was the least producer for both α-amylase and glucoamylase.

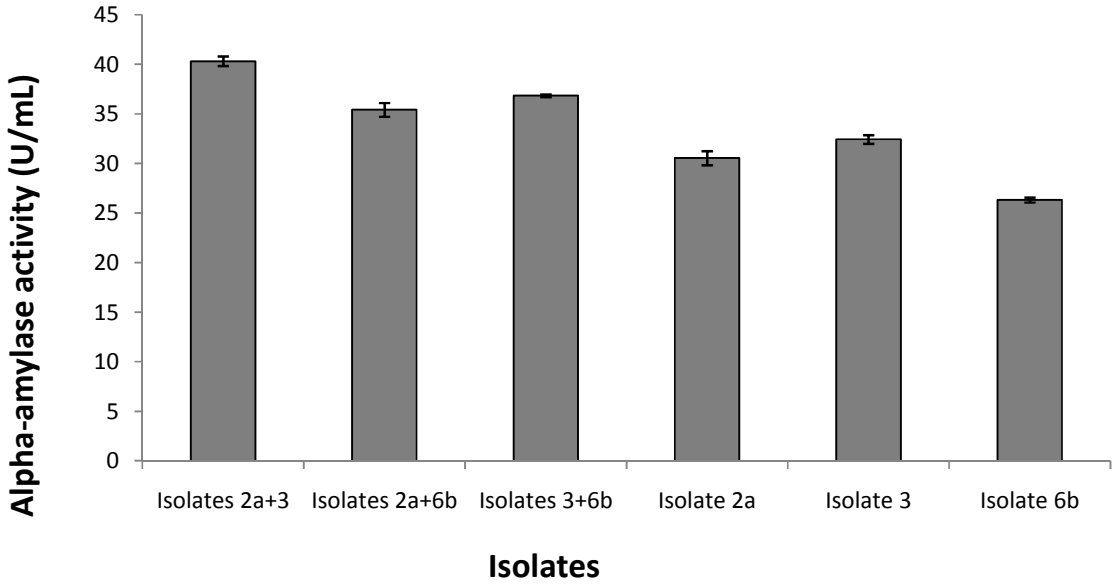

FIGURE 3: Effect of Mixed Culture of selected Fungal Isolates on the production of Alpha-Amylase at 25 ^{o}C, pH 4 and 80 g/L Starch concentration

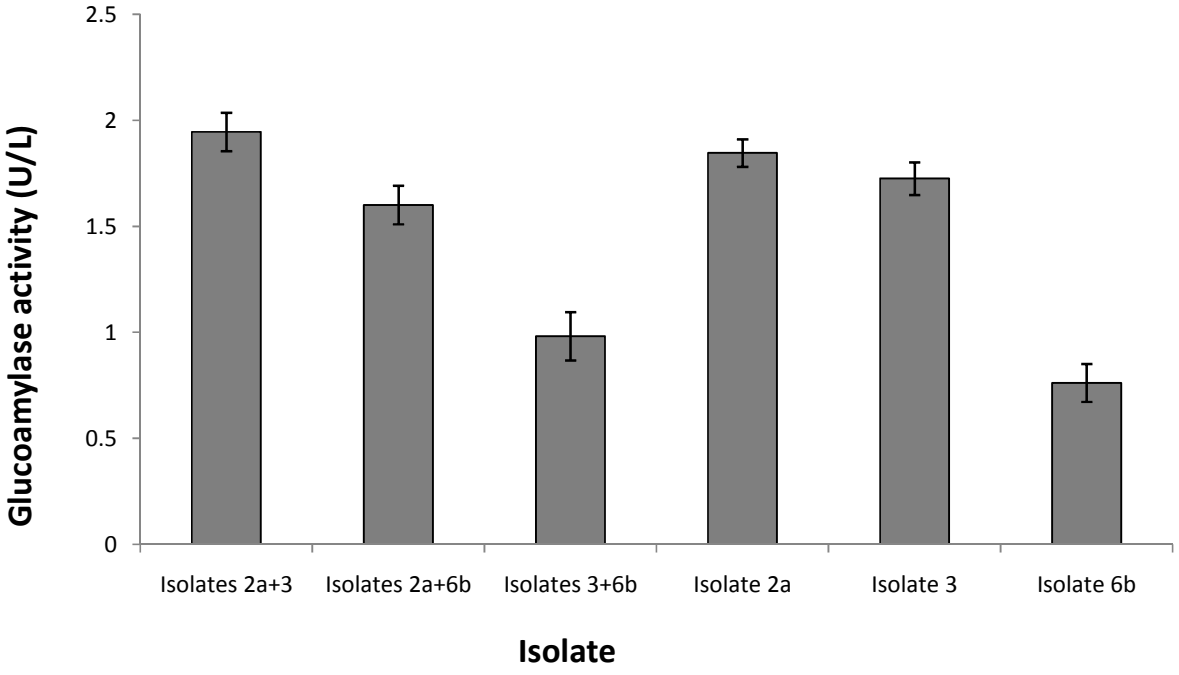

FIGURE 4: Effect of mixed culture of selected fungal isolates on the production of Glucoamylase activity at 25 ^{o}C, pH 4 and 80 g/L starch concentration

Bioethanol Production by Co-Cultivation of the Selected Fungal Isolates and Yeast Strain

The selected fungal isolates were co-cultivated with *Saccharomyces cerevisiae.* As the isolates hydrolyze the starch to sugars, the yeast cells convert the sugars to bioethanol. The maximum amounts of glucose liberated were observed after 72hrs while the highest quantities of alcohol were recorded after 96hrs. The results were shown in figure 5. The highest ethanol was produced by isolate 3. This was followed by isolate 2a while isolate 6b produced the least concentration of ethanol.

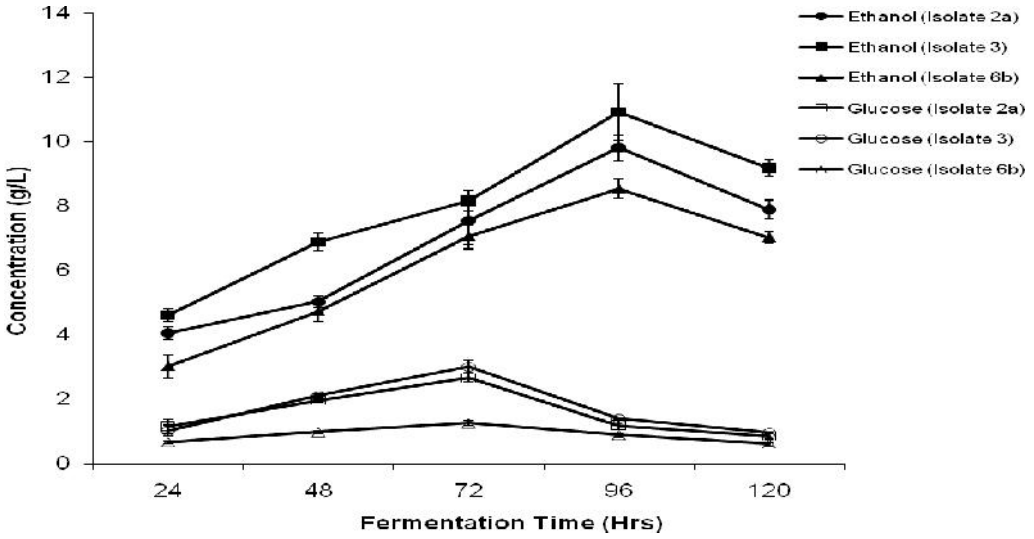

FIGURE 5: Ethanol production and Glucose Liberation from 80 g/L Starch by selected Fungal Isolates with *Saccharomyces cerevisiae* at pH4.0 and 37°C

Identification of the Selected Fungal Isolates

The summary of the results of both microscopic and macroscopic examinations for the identification of the selected fungal isolates were presented in table 2. The isolated fungal strains (isolates 3, 2a and 6b) were suspected to belong to *Aspergillus, Mucor and Rhizopus species* respectively.

TABLE 2: Identification of the selected fungal isolates

Isolates	Macroscopy	Microscopy	Fungus Suspected
3	White Filamentous growth that sporulate to black powdery growth	Long–septate hyphae with a conidiophore that bears brown-spores	*Aspergillus species*
2a	White and whorly aerial mycelial growth that later darken as it sporulated	Non-septate hyphae with straight sporangiophores with many spherical spores	*Mucor species*
6b	White long hyphae growth that darkens as it sporulates within 2 days.	Non-septate long-branched mycelium with terminal shaped sporangia	*Rhizopus species*.

DISCUSSION

In order to select the efficient starch hydrolyzing fungal strains, two processes were adopted. The first was to isolate 120 microorganisms from various sources of our environments and screened for fungal strains with ability to hydrolyze starch materials. The result of these yielded 26 amylase-producing isolates. Table 1 showed the results of amylase enzymes activities of the 30 isolates. The resulted fungal isolates were further screened for their α-amylase and glucoamylase activities. Among the isolates, 10 were selected for further examinations. Most productive three isolates emerged from this final screening stage. The ranking order of productivity of the three selected isolates, 3>2a>6b as shown in figures 1 and 2. These records have supported the earlier reports of Rehana, (1989) and Freer, (1993) that the soil is known to be a repository of amylase producers.

Higher amylase productivities were achieved mostly with mixed culture when compared with their component single cultures. For instance, isolate 2a was co-cultivated with isolate 3 and their mixed cultures proved more productive than their individual cultures of 2a or 3 both for α-amylase and glucoamylase (figures 3 and 4). This showed that some of these isolates might have acted synergistically in their enzyme activities (Ueda, 1981).

Simultaneous Saccharification and Fermentation (SSF) process was employed in the bioethanol production. Starch hydrolyzing fungal isolates and ethanol producing microbes (*Saccharomyces cerevisiae*) were co-cultivated for the production of bioethanol. Glucose liberated by the isolates during SSF process was measured maximum after 72hrs; however, the highest concentration of ethanol was achieved at 96hrs as shown in figure 5. These observations might be as a result of concurrent enzymatic activities of the existing microorganisms in the fermentation process. As the starch hydrolyzing fungi released the sugars, the ethanol producing microbes convert the sugars to ethanol. Table 2 showed the summary result of macroscopic and microscopic examinations for the identification of the selected fungal isolates. Isolate 3 was suspected to be *Aspergillus species* while isolates 2a and 6b were suspected to be *Mucor* and *Rhizopus species* respectively.

These observations supports the earlier reports of Toye, (2009) that some fungal strains including *Aspergillus, Mucor and Rhizopus species* and some bacteria were found to be among the amylase enzymes producers.

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