International Journal of Innovative Research and Review ISSN: 2347 – 4424 (Online)
An Online International Journal Available at http://www.cibtech.org/jirr.htm
2016 Vol. 4 (3) July-September, pp.69-80/Odelade et al.
Research Article

BIOETHANOL PRODUCTION BY SIMULTANEOUS PROCESSES OF SACCHARIFICATION AND FERMENTATION USING WILD AND MUTANT STRAINS OF *ASPERGILLUS NIGER* AND *SACHROMYCES CEREVISIAE*

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ABSTRACT
Cellulase is the key enzyme of potential use for industrial saccharification of cellulosic materials into simple sugars and also can be bioconverted to bioethanol which is presently the alternative to the depleting resources. The production of cellulase and glucose from sawdust via saccharification process using intact spores of a local strain of wild *A. niger* (isolated from soil samples) is inevitable. Cellulase and glucose released by each of the wild A. niger and its mutant strains per day were analyzed. Fermentation of the hydrolyzed samples to ethanol was done using the locally isolated *S. cerevisiae*. The purified fermented products were analyzed by primary distillation process at 80 °C – 92 °C. All the mutant strains secreted significantly more enzymes and reducing sugars than the parent strain. All mutant strains (i.e An 20, An 40 and An 60) showed maximum CMCase activities of (4.52, 4.90 and 4.54 U/ml) respectively for cellulase activities and the parent strain (An wild) gave activities of 2.0 U/ml. The intact spores of wild A. niger and its mutant strains were able to produce glucose where all the mutant strains (i.e An 20, An 40 and An 60) gave maximum glucose productions of (20.5, 22.3 and 20.7 µmoles/L) respectively and parent strain gave 9.15 µmoles/L. These levels of cellulase activities and glucose productions of the mutant strains was about 2.0 fold higher than those in parent strain when analysed statistically at $p < 0.05$. It show that mutagenesis is significant because it improved the cellulolytic activities of the local strain of *A. niger* and also enhanced better yield of the metabolites.

Keywords: *Saccharification, Fermentation, Cellulosic Materials, Mutant Strains, Bioethanol, Mutagenesis*

INTRODUCTION
The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component (Salem *et al.*, 2008). Plant biomass comprises an average of 23% lignin, 40% cellulose and 33% hemicelluloses by dry weight (Ahmed *et al.*, 2009). Cellulose is the most abundant renewable natural resource in the world and a potential source for the production of industrial useful materials such as fuels and chemical (Facchini *et al.*, 2011). Its annual biosynthesis by both land plants and marine algae occurs at a rate of 0.85×10^{11} tonnes per annum (Niranjane *et al.*, 2007). Also, Lignocellulosic wastes are the largest group of wastes present on this plant causing environmental pollution (Rani and Nand, 2000). It is estimated that the photosynthetic process produced 1.5 x 10 ton (150 billion tons) of dry material annually with respect to carbon of which about 50% is cellulose (Persson *et al.*, 1991). Cellulose, which is the most abundant renewable resource, is a polysaccharide composed of β-D-glucopyranosyl units joined by 1,4-glycosidic bonds because cellulose can be utilized to produce ethanol, it is a promising alternative energy source for the production of fossil fuels. Cellulose is degraded by cellulases to reducing sugars and fermented by yeast or bacteria to ethanol (Duff and Murray, 1996).
Many microorganisms have been evaluated for the production of cellulase including bacteria such as *Pseudomonas fluorescens, Bacillus subtilis, Escherichia coli, Serratia marcescens*. Filamentous fungi

have been used for more than 50 years in the production of industrial enzymes (Salem *et al.,* 2008). Many fungal strains secrete higher amounts of cellulases than bacterial ones. Cellulases from *Trichoderma* and *Aspergillus* species have been investigated in detail over the past few decades (Tao *et al.,* 2010). *Aspergillus* sp is an important commercial source of cellulases for food textile and pharmaceuticals industries (Naikia and Tiku, 2010). Production of cellulases by fungi in SSF using agricultural wastes has been reported. Therefore, investigation on the ability of fungal strains to utilize inexpensive substrates and improvement of enzyme productivity are important. The lignocellulosic biomass, especially agricultural wastes, is known to be an excellent carbon sources for microbial enzyme production (Gao *et al.,* 2007). The utilization of cheaper and indigenous substrate for cellulase production has contributed somewhat to economical recovery (Pandey *et al.,* 2010), Various agricultural substrates, by products and microbial cultures have been used successfully in solid state fermentation for cellulase production (Deunas *et al.,* 1995). In recent years, the interest in cellulases has increased due to many potential applications, for example, in the production of bio-energy and bio-fuel, in the textile industry and pulp and paper industry (Zhou *et al.,* 2008).

Cellulosic waste may be converted to products of commercial interest such as glucose, soluble sugars, enzymes, alcohol, and single cell proteins and the key element in the saccharification process of lignocellulosics to these industrially useful products relies on participation of cellulolytic organisms and their cellulase enzymes. The production of cellulase enzyme is a major factor in the hydrolysis of cellulosic materials but it is usually produced in small quantities by the parent organisms. The spectacular successful examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection (Vu *et al.,* 2011). Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics (Karanam and Medicherla, 2008). Hence, there is need to enhance the wild *A. niger* to obtain strains capable of producing high amount of fermentable sugar which can thereby lead to increase in bioethanol production. Therefore, the production of mutant strains of *A. niger* which could produce high amount of glucose for the bioethanol production is inevitable.

MATERIALS AND METHODS

Collection of Sample

Saw dust obtained from locust bean tree was collected from Adeleke sawmill, in Ogbomoso, Oyo state, Nigeria. The sample was preserved at 20 ^{0}C for 72 hours. This was sieved through a mesh size of 20 mm and the filtrate was used as microbial substrates and rinsed in distilled water.

Pre-treatment of Substrate

The substrate was treated chemically with 1 % NaOH for 120 minutes to enhance its hydrolysis by the microorganism according to the method of Solomon *et al.,* (1999). It was rinsed with distilled water until a neutral pH was reached and was air dried. The dried powder obtained was stored in amber bottles at 25 ^{o}C till required.

Culturing of Microorganisms

Isolation of Aspergillus Niger

The fungal culture *A. niger* was screened from decomposed sawdust soil sample collected from saw mill Adeleke area, Ogbomoso. Serial dilution was carried out on the soil sample collected; 1 ml was taken from 10^{-2} and 10^{-4} serial dilution test tubes and inoculated into the sterile petri dishes and the PDA media as prepared. The solidified plates were incubated at 30 ^{o}C for a period of 4 days. The grown organisms were identified according to the method of Nagamani *et al.,* (2006).

Generation of Mutant Strains of Aspergillus Niger

Mutant strains of the wild *A. niger* was generated by inoculating test tube containing 3 ml of sterile distilled water each with a loopful of *A. niger*.

These test tubes were exposed to ultraviolet light 234 nm wavelength at different time intervals (20, 40 and 60 minutes). The exposed test tubes containing the organism was poured on solidified PDA plates and incubated at 30 ^{o}C for the period of 4 days.

Screening for Cellulolytic Abilities of Aspergillus Niger and its Mutant Strains

The agar diffusion method of Hankin and Anagnostakis, (1977) was employed to screen for cellulolytic activities of the fungi.

Preparation of Inoculum

The PDA plates containing *A. niger* were incubated at 30 ^{o}C for 72 hours until the mycelium sporulates black conidia. Inoculum was produced in 250 ml Erlenmeyer flasks containing 0.2 g of yeast extract and 1% sucrose in 100 ml of distilled water, was autoclaved at 121 ^{o}C for 15 minutes. The flasks containing the medium (discs) were incubated for another 72 hours at 25 ^{o}C on a shaker.

Isolation of Saccharomyces Cerevisiae

The isolation was carried out according to the method of Kregervan (1984).

Preparation of Yeast Inoculum

A loopful of the yeast colony was transferred from the agar plate into 100 ml sterilized medium containing 1 % sucrose and 0.2 g of yeast extract broth was incubated at 25 ^{o}C on a mechanical shaker coupled with heater for 48 hours.

Fermentative Production of Bioethanol

The fermentative production of bioethanol was carried out in two steps namely by saccharification and fermentation. The chemically pre-treated substrate was used for all the experiments.

Saccharification of Substrates by Aspergillus Niger and its Mutant Strains

For saccharification of substrates *A. niger* spores suspension was employed. *A. niger* was selected for saccharification. In this study, an attempt was made to design an economical process by the use of intact fungal organism as a source of cellulase enzyme instead of commercially available enzyme. As A. niger grows on the cellulosic substrates, it hydrolyzes cellulose of the substrate and release simple sugars which can be fermented to produce bioethanol by *S. cerevisiae*. The organisms grow on synthetic media containing (in 1 L of distilled water; 3 g of $NaNO_3$; 0.5 g of KCI; 0.5 g of $MgS0_4.7H_2O$; 1.0 g of KH_2PO_4; 0.01 g of $FeSO_4.7H_2O$; with 1.0 % (w/v) of sawdust as sole carbon source. Each liter of the respective media contained 1.0 ml of a supplement of trace metal containing 0.1 % (w/v) $ZnSO_4$ and 0.05 % (w/v) $CuSO_4$. The pH was determined and was adjusted. 180 ml of the respective media were autoclaved at 121 ^{o}C for 15 minutes, cooled and inoculated with 20 v/v of the inoculum. The cultures were incubated at 25 ^{o}C with shaking intermittently. Cells were harvested at every 24-hour intervals for 6 days for cellulase activities.

Cellulase Assay of Aspergillus Niger and its Mutant Strains

Cellulase activities were determined using carboxymethyl cellulose as a substrate.

Fermentative Production of Bioethanol by Saccharomyces Cerevisiae

For fermentative production of bioethanol under stationary condition, cells of *S. cerevisiae* was employed. At the six days of saccharification 10 % (v/v) of *S. cerevisiae* inoculum suspension was added to all the flasks. The process was carried out for a period of six days at 25 ^{o}C. During the fermentation process all the bottles were shaken intermittently and samples were taken at every 24 hinterval for the estimation of bioethanol.

Distillation and Determination of Quantity of Ethanol

The Distillation and determination of quantity of ethanol was carried out according to the method of Oyeleke and Jibrin (2009). The fermented broth was dispensed into a round-bottom flask fixed to a distillation column enclosed in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. A heating mantle with the temperature adjusted to 80 °C was used to heat the round-bottomed flask containing the fermented broth. The distillate collected was measured using a measuring cylinder, and expressed as the quantity of ethanol produced in g/L by multiplying the volume of distillate collected at 80 °C by the density of ethanol (0.8033 g/ml). g/L is equivalent to the yield of 100 g of dried substrate.

Tests for Bioethanol

Lucas test for primary, secondary and tertiary alcohols was followed to further confirm the distillate as bioethanol. An equimolar mixture of $ZnCl_2$ and HCl was added to a known volume of the distillate to

further confirm if it's a primary bioethanol. Biological tests such as morphological, biochemical and physiological characteristics were carried out.

Statistical Analysis

Results of cellulase, glucose and bioethanol obtained were analysed using analysis of variance (ANOVA) and means were compared for significance at $P < 0.05$ using Duncans multiple range analysis.

RESULTS AND DISCUSSION

Results

Fungal and Yeast Isolates

Aspergillus niger was isolated from decomposed sawdust soil sample while *S. cerevisiae* from palm wine using PDA. The wild strain of *A. niger* is a slow growing organism showing profused growth with heavy sporulation on the third day of incubation with a well defined colony. The white coloured hypae and the black coloured conidiophores were observed from the isolate after 4 days of culturing and it was confirmed as *A. niger*. The grown yeast isolate was identified as *S. cerevisiae* by studying some of the morphological, biochemical and physiological characteristics. The results of the biochemical tests did on the yeast isolate shows that it was glucose, fructose, sucrose, raffinose and starch fermenter because it shows positive result. It shows negative result on lactose which means it is not lactose fermenter while it shows either positive or negative result for galactose. It also produces acid and ester but did not hydrolyse urea. Also, the yeast isolate gave positive result for peptone, aspargine and ammonium sulphate and negative result for nitrate.

Pre-treatment of Substrate

The abundantly and cheaply available renewable cellulosic substrate- sawdust was utilized. Sawdust appears to be a suitable cellulosic materials for cellulase production, for all the organisms. This may also be as a result of adaptation; since the organisms were isolated from soil sample sawdust. In the present study, Alkali treated sawdust was used for the production of cellulase by *A. niger* and its mutant strains, as shown in figure 1. The cellulase produced broke down the cellulose in the sawdust and produced glucose and *S. cerevisiae* in return converted the glucose to bioethanol in the fermentation process.

Figure 1: Pretreated Sawdust Used as the Substrate

Cellulolytic Activities of A. Niger and its Mutant Strains when Screened on Carboxyl Methyl Cellulose (CMC) Agar Medium

The cellulolytic activities of the fungi were demonstrated on agar plates. Clearance zones measuring 4.6, 8.3, 8.0 and 7.6 mm were obtained after staining the cultures of *A. niger* wild, *A. niger* exposed to ultra violet radiation for 20 mins (An 20), it was exposed to ultra violet radiation for 40 mins (An 40) and Aspergillus niger exposed to ultra violet radiation for 60 mins (An 60) on agar medium containing carboxyl methyl cellulose with congo red solution respectively (figure 2 and 3). Ability to reduce

viscosity when grown in medium containing carboxyl methyl cellulose was taken as a measure of cellulase activity of the fungi. Activities were determined for the wild organism and all the mutant strains. The results show that all the mutant strains gave better cellulolytic activities than the wild parent organism.

Results obtained from the experiment were analyzed using Analysis of Variance (ANOVA), while comparisons were made using the Dunnet's test. A P-value <0.05 was adopted as the level of significance. The cellulase activities of wild and mutant strains of *A. niger* are in the range from 1.0 -2.37 U/ml and 0.9 – 4.90 U/ml respectively after the 4th days of cultivation using sawdust as substrate (figure 4). Statistically, the result obtained from the cellulase activities shows that after 1st, 2nd and 3rd of cultivation all the mutant strains were significantly not different from the wild at $p< 0.05$. After 4th day, the cellulase activities of all the mutant strains i.e An 20, An 40 and An 60 have increased significantly ($p< 0.05$) which gave 4.52, 4.9 and 4.54 U/ml respectively when compared with the wild type that gave 2.0 U/ml. Furthermore, An 20 after the day 5 of cultivation shows a significantly higher cellulase activities at $p< 0.05$ of 4.9 U/ml when compared with the wild that gave 2.37 U/ml while all other mutant strains i.e. An 40 and An 60 that gave 3.78 and 3.10 U/ml respectively were not significant different ($p< 0.05$) to the wild of 2.37 U/ml.

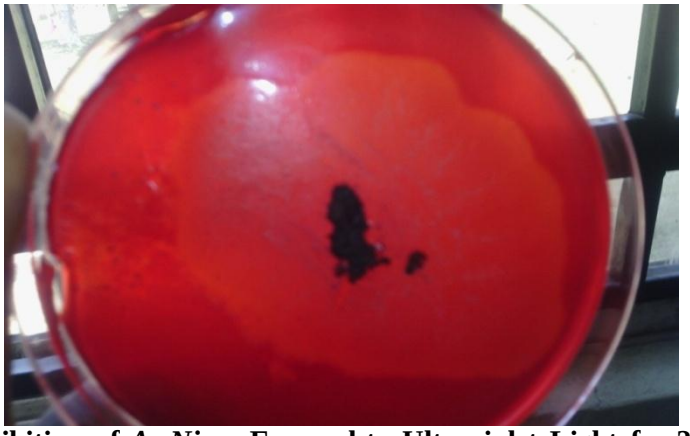

Figure 2: Zone of Inhibition of *A. Niger* Exposed to Ultraviolet Light for 20 min on CMC Agar Plate

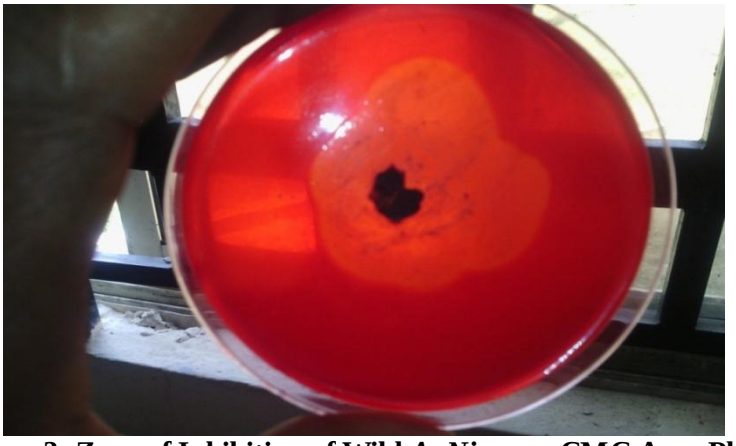

Figure 3: Zone of Inhibition of Wild *A. Niger* on CMC Agar Plate

Statistical Analysis of Cellulase Activities of A. Niger and its Mutant Strains

A. niger mutant strain exposed to ultraviolet radiation for 40 minutes produced highest enzyme of 4.90 U/ml followed by *A. niger* mutant strain exposed to ultraviolet radiation for 60 minutes which gave 4.54

U/ml and *A. niger* mutant strain exposed to ultraviolet radiation for 20 minutes which gave 4.52 U/ml after 4th day of cultivation. After 5th day of cultivation An 20 shows higher significant different of enzyme activities by producing the highest amount of cellulase 4.90 U/ml (in figure 4). The result shows that there was progressive increase in enzyme activities from the day 1 to the day 4 of incubation for An 40 and An 60 while it extended to day 5 for An 20 and the wild type. The yield of enzymes produced decreased for all the organisms after the day 6 of incubation (as shown in figure 4).

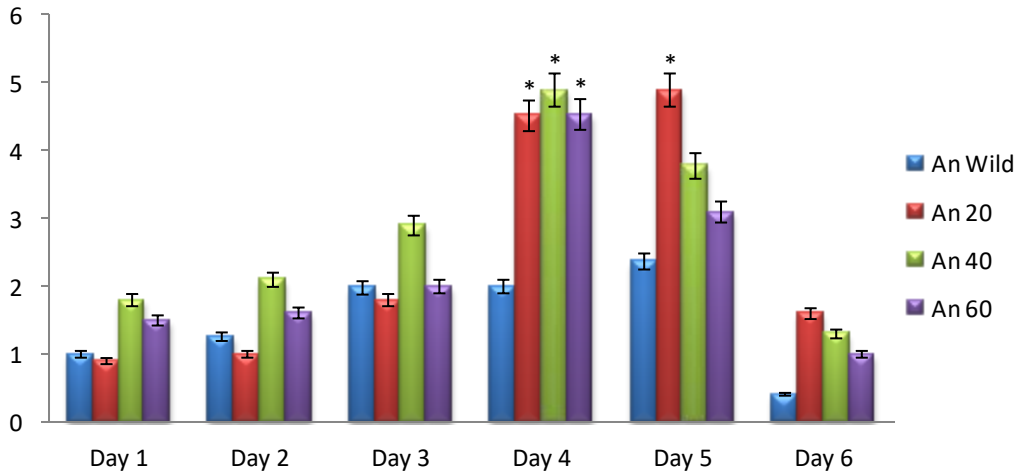

Figure 4: Cellulase Activities (U/ml) for Wild A. Niger and its Mutant Strains

* p< 0.05 significantly different from the wild type. Values are expressed as mean ± SD

Statistical Analysis of Glucose Produced by Aspergillus Niger and its Mutant Strains

Results obtained from the experiment were analyzed using Analysis of Variance (ANOVA), while comparisons were made using the Dunnet's test. A P-value <0.05 was adopted as the level of significance. The glucose produced by the intact spore of wild and mutant strains of A. niger are in the range from 4.40 – 19.0 µmoles/L and 4.34 – 26.61 µmoles/L respectively after the 4 th days of cultivation using sawdust as substrate (figure 5). Statistically, the result obtained from the glucose produced shows that after day 1, 2 and 3 of cultivation all the mutant strains were significantly not different from the wild at p< 0.05. After day 4, glucose produced by all the mutant strains i.e An 20, An 40 and An 60 have increased significantly (p< 0.05) which gave 20.5, 22.3 and 20.7 µmoles/L respectively when compared with the wild type that gave 9.15 µmoles/L. An 20 after day 5 of cultivation shows a significantly higher glucose production at p< 0.05 of 26.61 µmoles/L when compared with the wild that gave 19.06 µmoles/L while all other mutant strains i.e An 40 and An 60 that gave 20.65 and 20.1 µmoles/L respectively were not significantly different (p< 0.05) from the wild the gave 19.06 µmoles/L (figure 5).

Aspergillus niger mutant strain exposed to ultraviolet radiation for 40 minutes produced highest glucose 22.3 µmoles/L, followed by A. niger mutant strain exposed to ultraviolet radiation for 60 minutes 20.7 µmoles/L and followed by A. niger mutant strain exposed to ultraviolet radiation for 20 minutes 20.5 µmoles/L after day 4 of cultivation. After the day 5 of cultivation An 20 shows higher significant difference in glucose production by producing the highest amount of glucose of 26.61 µmoles/L (figure 5). The result shows that there was progressive increase in the glucose produced from day 1 to day 4 of incubation for An 40 and An 60 while it extended to day 5 for An 20 and the wild type (figure 5). The yield of glucose produced decreased for all the organisms after day 6 of incubation (as shown in figure 5).

Fermentative Production of Bioethanol by Saccharomyces Cerevisiae and Distillation Method for the Extraction of Bioethanol

Locally isolated *S. cerevisiae* from palm wine was used to bioconvert the produced glucose released from respective wild A. niger and its mutant strains to bioethanol. The locally isolated *S. cerevisiae* was found to be able to ferment the released glucose to bioethanol and Figure 7 shows the data obtained from the

conversion of fermentable sugar released by the wild type and its mutant strains in glucose fermentation to bioethanol by *S. cerevisiae* (figure 6). It shows that the highest bioethanol production of 17.55 g/L was obtained when S. cerevisiae converted the glucose released by the An 40, followed by An 60 and the least bioethanol production of 14.0 g/L was reached when S. cerevisiae converted the glucose released by the An 20 after day 4 of fermentation. The ethanol produced from the glucose released by all mutant strains were significantly different at $p < 0.05$ than the ethanol produced from the glucose released by the wild type. The bioethanol produced (20.55 g/L) when *S. cerevisiae* converted the glucose released by the mutant strain was shown in figure 7. An 20 is significantly higher than the bioethanol produced when *S. cerevisiae* converted the glucose released by the wild A. niger, An 40 and An 60 after day 5 of fermentation. The result shows that the amount of bioethanol produced is in accordance with the amount of glucose released by wild *A. niger* and its different mutant strains. As the concentration of glucose increases ethanol productions also increased. With increase in time of fermentation, ethanol production increased up to after day 5 of incubation. After day 6, the yield of ethanol decreased indicating that the maximum amount of sugar was consumed by day 6 of fermentation.

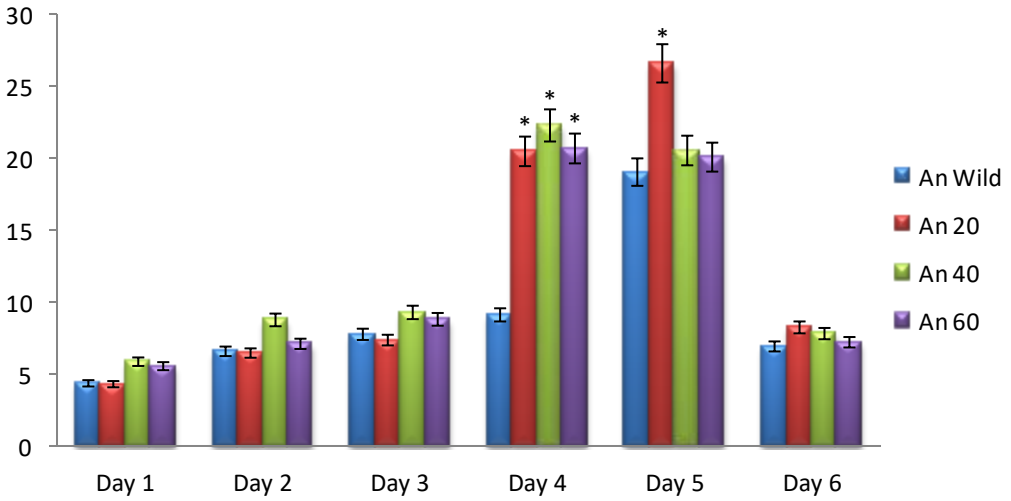

Figure 5: Reducing Sugar Released by Wild and Mutant Strains of A. Niger (µmoles/L)
* $p < 0.05$ significantly different from the wild type. Values are expressed as mean ± SD.

Figure 6: Production of Bioethanol by *Saccharomyces Cerevisiae* in the Fermentation Process

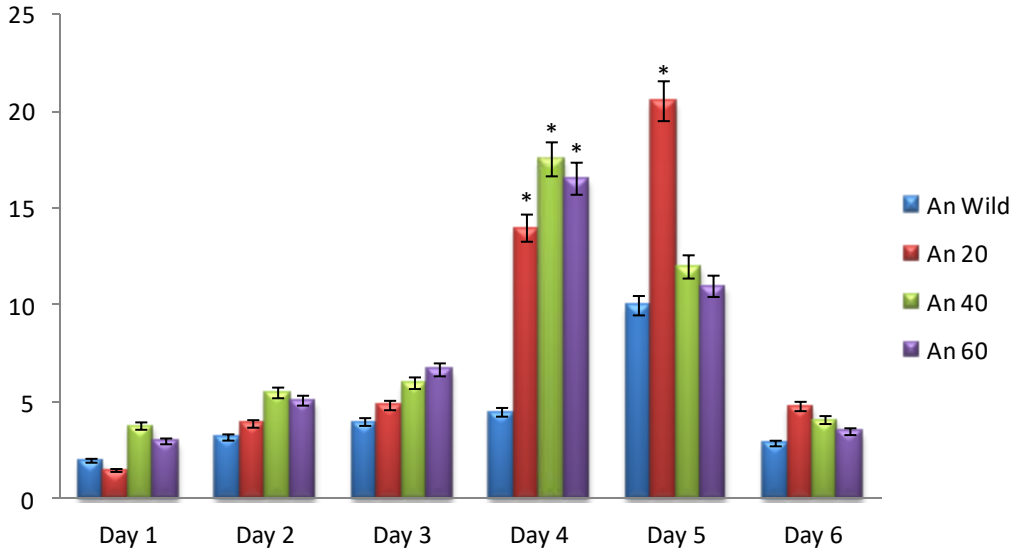

Figure 7: The Volume of Bioethanol Produced in the Fermentation of Sawdust (g/L) per Day
* $p < 0.05$ significantly different from the wild type. Values are expressed as mean ± SD.

pH Values of Both Saccharification and Fermentation

pH values of both enzyme activity and bioethanol production were monitored at different days of incubations as shown in Table 1 and 2. Table 1 illustrates the effect of pH on release of sugars from the substrate. Saccharification increased from pH 6.4 to a maximum pH of 7.5 after which it decreased up to 6.1.

Table 2 illustrates the effect of pH on bioethanol production from the substrate. During the fermentation process the pH increased from 5.6 to a maximum pH of 9.9 after which it decreased up to 5.2. Since the cellulase enzyme activities of all the mutant strains were significantly different from the wild or parent organism after day 4 of incubation statistically, therefore, all the mutant strains (i.e An 20, An 40 and An 60) after day 4 of incubation have optimum pH values of 8.0, 7.5 and 6.8 respectively. After the day 5 of saccharification, the optimum pH value for An 20 was 6.1. Also, for bioethanol production the optimum pH values for the ethanol produced when S. cerevisiae converted the glucose released from each of the mutant strains (i.e An 20, An 40 and An 60) to bioethanol were 9.9, 9.3 and 5.3 respectively after day 4 of fermentation. However, for the ethanol produced by S. cerevisiae from the glucose released by An 20 after day5 of incubation which was significantly at $p < 0.05$ different from wild type and other mutant strains the optimum pH value was 9.9.

Table 1: pH Values of Enzyme Activities in Saccharification at Different Days

Organism	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
An wild	7.2	7.3	7.5	7.4	6.5	6.2
An 20	7.2	7.4	7.6	7.7	8.0	6.1
An 40	6.4	7.2	7.3	7.3	7.5	6.5
An 60	7.1	6.5	6.6	6.6	6.8	6.3

Table 2: pH Values of Ethanol Released in Fermentation at Different Days

Organism	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
An wild	8.8	9.0	9.4	9.6	9.7	9.7
An 20	9.2	9.4	9.8	9.8	9.9	9.9
An 40	8.7	8.8	9.0	9.2	9.3	9.7
An 60	5.6	5.4	5.0	6.0	5.8	5.2

Table 3 shows the amount of fermented sawdust left after the fermentation. The biomass reduced from 1.7 g at day 1 of fermentation to 0.4 g at day 5 for *A. niger* mutant strain at 20 min which gave the highest yield of cellulase, glucose and bioethanol. At the 6th day of fermentation the biomass remained 0.4 g because the biomass did not ferment again.

Table 3: Amount of Biomass Remained after Fermentation of Sawdust in Gram (g)

Organism	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
An wild	1.60	1.40	1.10	0.80	0.60	0.60
An 20	1.70	1.60	1.52	1.50	0.40	0.39
An 40	1.51	1.40	1.20	1.04	0.51	0.50
An 60	1.80	1.41	1.15	1.00	0.57	0.56

An Wild = *Aspergillus niger* wild, An 20 = *Aspergillus niger* mutant strain exposed to ultraviolet light for 20 min, An 40 = *Aspergillus niger* mutant strain exposed to ultraviolet light for 40 min and An 60 = *Aspergillus niger* mutant strain exposed to ultraviolet light for 60 min.

Discussion

Aspergillus niger was used in this research work to produce cellulase when it saccharified the agrowaste i.e sawdust which in turns degrade the cellulose in the sawdust to glucose. Although a large number of microorganisms (fungi, bacteria and actinomycetes) are capable of degrading cellulose, only a few of them produce significant quantities of cell-free enzyme fractions capable of complete hydrolysis of cellulose in vitro. Among the cellulolytic mircofungi, the genera *Trichoderma* and *Aspergillus* are notable cellulase producers (Chu *et al.,* 2002).

Mutagenesis is an easy tool to achieve genetic modification of an organism. In the present study, mutagenesis was carried out by exposing the fungal culture *A. niger* to ultraviolet radiation of wavelength 234 nm at different time interval to generate mutant strains of A. niger. A total of 3 strains (designated as An 20, An 40 and An 60) that showed survival rate of 8.3 mm, 8.0 mm and 7.6 mm and *A. niger* wild which gave 4.6 mm clearance zones when cultured on CMC agar were selected from the figures 2 and 3. This method has been used to measure the initial hydrolysis rate for endoglucanases using soluble cellulose derivatives (Zhang *et al.,* 2006) and is strongly recommended along with determination of reducing sugar (Ghose, 1987).

The results presented in Figure 4 indicated the production of Cellulase enzyme by the mutant strains generated via ultraviolet radiation and the wild *A. niger*. All the three mutant strains (An 20, An 40 and An 60) secreted significantly more enzymes than the parent strain when analyzed statistically by ANOVA at $p< 0.05$. All the mutant strains (An 20, An 40 and An 60) proved to be the most promising extracellular cellulase as they showed maximum CMCase activities (4.52, 4.90 and 4.54 U/ml respectively) which were significantly at $p< 0.05$ higher than the parent strain activities that gave CMCase activity of 2.0 U/ml after day 4 of incubation. These levels (i.e the CMCase activities) of the all the mutant strains were of about 2.0 fold higher than those in parent strain statistically. These findings indicated that the treatment of the parent fungal organism with ultraviolet radiations at different time intervals was suitable for improving cellulase production by the fungal strain. That is, it was appropriate for the fungi that showed improved cellulase production.

Ability to degrade cellulose when grown in medium containing carboxyl methyl cellulose was taken as a measure of cellulase activity of the isolate. This method has been used to measure the initial hydrolysis rate for endoglucanases using soluble cellulose derivation (Zhang *et al.,* 2006) and is strongly recommended along with determination of reducing sugar (Ghose, 1987). Cellulase is an induced enzyme and its production increased with increase in fungal biomass over incubation period and as simple sugar in the substrate is released (Lynd *et al.,* 2002). Enzyme and glucose productions were affected by incubation time, at the short time and longer time of cultivation period, the enzyme production was reduced. According to Melo *et al.,* (2007), report shows that the enzyme level declined with prolonged incubation, this could be due to loss of moisture or denaturation of the enzyme resulting from variation in

pH during fermentation and also reported that the decrease of enzyme activities may be due to the accumulative effect of cellulobiose. The time of the highest cellulase activity depends upon the substrate and fungus (Ojumu *et al.*, 2003).

In this present study, intact spores of *A. niger* was used to produce glucose. The results presented in Figure 5 indicated the production of glucose by the mutant strains generated via ultraviolet radiation and wild *A. niger*. All the three mutant strains (An 20, An 40 and An 60) secreted significantly more reducing sugars than the parent strain when analysed statistically by ANOVA at $p< 0.05$. All the mutant strains (An 20, An 40 and An 60) proved to be the most promising glucose producer as they showed maximum glucose productions (20.5, 22.3 and 20.7 µmoles/L respectively) which were significantly at $p< 0.05$ higher than the parent strain activities that gave glucose production of 9.15 µmoles/L after day 4 of incubation. These levels (i.e glucose productions) of the all the mutant strains were of about 2.0 fold higher than those in parent strain statistically. These findings indicated that the intact spores of A. niger was able to produce glucose and the treatment of the parent fungal organism with ultraviolet radiations at different time intervals was suitable for improving glucose production by the fungal strain. That is it was appropriate for the fungi that showed improved glucose production. Generally, during enzymatic hydrolysis, cellulose is degraded by cellulases to reducing sugars that can be fermented by yeast to ethanol (Sun and Cheng, 2002).

Bioethanol production is a widely studied process. Different workers have studied various raw materials and different methods for bioethanol production but, recently it has been observed that lignocellulosic materials are focused for bioethanol production. Hence, cheap and abundant available agrobased waste i.e sawdust was selected for bioethanol production. In this present study, locally isolated *S. cerevisiae* from palm wine was used to ferment the produced glucose to ethanol. The amount of bioethanol produced (i.e 14. 0, 17.55 and 16 55 g/L) when *S. cerevisiae* converted the glucose released by each of the mutants (i.e An 20, An 40 and An 60) respectively and the parent organism (4.5 g/L) to ethanol after day 4 of fermentation was shown in figure 6. The result show that each bioethanol product produced when *S. cerevisiae* converted the glucose released by each of the mutant strain (i.e An 20 An 40 and An 60) were significantly at $p< 0.05$ higher than the bioethanol product produced when *S. cerevisiae* converted the glucose released by the wild organism statistically. Then, with elongation of cultivation time the yield of bioethanol decreased. Highest amount of ethanol was produced after day 5 of fermentation (20.55 g/L) which is in accordance with the release of total sugars.

The pH of the medium revealed that the optimum values for enzyme and bioethanol productions were obtained at pH 8.0 and 9.9. This is in conjunction with another study which had maximum enzyme activity pH at 7.5 using strain of *A. niger* (Coral *et al.*, 2002). Optimal pH is very important for growth of the microorganism and its metabolic activities. As the metabolic activities of the microorganisms are very sensitive to changes in pH. One of the most important factors for any fermentation process is pH and it depended on microorganisms because each microorganism possesses a pH range for its growth and activity (Lonsane *et al.*, 1985). Increasing and decreasing in pH on either side of the optimum value resulted in decrease in growth product fermentation (Kokab *et al.*, 2015).

This research work has led to the isolation of *A. niger* with cellulolytic ability and a good strain of *S. cerevisiae* that could convert reducing sugars to bioethanol using sawdust as a substrate. Mutant strains of wild *A. niger* were created through the exposure to ultraviolet radiation, with a very good improved activities especially *A. niger* exposed to uv radiation for 20 minutes (i.e An 20). All the mutant strains An 20 An 40 and An 60 produced significantly higher cellulase and glucose at $p< 0.05$ than the wild *A. niger* which was after day 4 of cultivation which consequently led to an increase in the productions of bioethanol when all the data were analysed statistically. After day 5 of cultivation, An 20 showed remarkable result as it gave cellulase activity and glucose production that were significantly higher at $p< 0.05$ than other mutant strains and the wild type which led to increase in the bioethanol produced when S. cerevisiae converted its glucose to ethanol.

Comparing the results obtained with some literatures, when crude cellulosic wastes like sawdust are used as raw material, intact microorganism such as *A. niger* can be enhanced for its use for saccharification of

cellulose as a substitute to expensive pure cellulase enzyme. It can therefore be concluded that mutation of wild *A. niger* to mutant strains of An 20, An 40 and An 60 which enhanced the production of cellulase and glucose have biotechnological applications. Also, *S. cerevisiae* which converted their respective reducing sugars to bioethanol should be encouraged.

Conclusion

From this work it has been shown that: the spores of wild type of *A. niger* and its mutants can directly convert sawdust to fermentable sugar; the spores of all the mutant strains of *A. niger* produced more fermentable sugar from sawdust; leading to the production of more ethanol and the spores of A. niger exposed to uv for 20 min (i.e An 20) produced more cellulolytic enzymes and glucose than those of the wild type and other mutants even after the 5 th day of cultivation.

Compliance and Ethics

The authors declare that they have no conflict of interest.

REFERENCES

Ahmed S, Bashir A and Saleem H (2009). Production and purification of cellulose degrading enzymes from a filamentous fungus *Trichoderma harzianum*. *Pakistan Journal of Botany* **41** 1411- 1419.

Chu YF, Sun J, Wu X and Liu RH (2002). Antioxidant and anti-proliferative activities of common vegetables. *Journal Agricultural Food Chemistry* **50** 6910-6916.

Coral G, Arikan B, Unaldi MN and Guvenmes H (2002). Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type Strain. *Turkey Journal of Biology* **26** 209-213.

Deunas R, Tengerdy RP and Gutierrez M (1995). Cellulase production by mixed fungi in solid state fermentation of bagasse. *World Journal of Microbiology and Biotechnology* **11** 333-337.

Duff SJB and Murray WD (1996). Bioconversion of forest products industry waste cellulosics to fuel ethanol: A Review. *Bioresources and Technology* **55** 1-33.

Facchini FD, Vici AC, Reis VR, Jorge JA, Terenzi HF, Reis RA and Polizeli Mde L (2011). Production of fibrolytic enzymes by *Aspergillus japonicus* $C0_3$ using agro-industrial residues with potential application as additives in animal feed. *Bioprocess Bio-systematic Engineering* **34** 347-55.

Gao J, Weng H, Zhu D, Yuan M, Guan F and Xi Y (2007). Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid- state cultivation of corn stover. *Bioresource Technology* **99** 7623-7629.

Ghose TK (1987). Measurement of cellulase activities. *Pure and Applied Chemistry* **59**(2) 257-268.

Hankin L and Anagnostakis S (1977). Solid media containing carboxyl methyl cellulose to detect CM cellulase activity of microorganisms. *Journal of General Microbiology* **98** 109–15.

Karanam SK and Medicherla NR (2008). Enhanced lipase production by mutation induced *Aspergillus japonicas*. *African Journal of Biotechnology* **7** 2064-2067.

Kokab S, Asghar M, Rehman K, Asad MJ and Adedayo O (2015). Bio-processing of banana peel for á-amylase production by *Bacillus subtilis*. *International Journal of Agricultural Biology* **5** 36-39.

Kregervan R and Eds NJ (1984). *The Yeasts: A Taxonomic Study*, third revised and enlarged edition. (Elsevier, Amsterdam, Netherland) 004.

Lonsane BK, Ghildyal NP, Budiatman S and Ramakrishna SV (1985). Engineering aspects of solid state fermentation. *Enzymes and Microbiological Technology* **7** 258-265.

Lynd LR, Weimer PJ, van Zyl WH and Pretorius IS (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews* **66**(3) 506-577.

Melo IR, Pimentel MF, Lopes CE and Calazan GM (2007). Application of fractional factorial design to levan production by *Zymomonas mobilis*. *Brazil Journal of Microbiology* **38** 45-51.

Nagamani A, Kunwar IK and Manoharachary C (2006). *Handbook of Soil Fungi*, (IK International, New Delhi, India).

Naika GS and Tiku PK (2010). Characterization of functional intermediates of endoglucanase from *Aspergillus aculeatus* during urea and guanidine hydrochloride unfolding. *Carbohydrates Resources* **345** 1627-31.

Niranjane AP, Madhou P and Stevenson TW (2007). The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantean*. *Enzyme and Microbial Technology* **40** 1464-1468.
Ojumu TV, Solomon BO, Betiku E, Layokun SK and Amigun B (2003). Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in saw dust, bagasse and corn cob. *African Journal of Biotechnology* **2** 150-152.
Oyeleke SB and Jibrin NM (2009). Production of bioethanol from guinea corn husk and millet husk. *African Journal of Microbiological Research* **3** 147-152.
Pandey A, Soccol CR, Nigam P, Brand D, Mohan R and Roussoss S (2000). Biotechnology potential of agro-industrial residues, part II. Cassava bagasse. *Bioresources Technology* **78** 81-87.
Persson I, Tjerneld F and Hahn-Hägerdahl B (1991). Fungal cellulolytic enzyme production part of: Persson, I. Production and utilization of cellulolytic enzymes in aqueous two-phase systems. Thesis University of Lund, Sweden.
Rani D and Nand K (2000). Production of thermostable cellulase-free xylanase by *Clostridium absonum*. *Process Biochemistry* **36** 355- 362.
Saleem F, Ahmed S and Jamil A (2008). Isolation of a xylan degrading gene from genomic DNA library of a thermophilic fungus *Chaetomium thermophile* ATCC 28076. *Pakistan Journal of Botany* **40** 225-1230.
Solomon BO, Amigun B, Betikue TV, Ojumu T and Layokun SK (1999). Optimization of cellulose production by *Aspergillus flavus* Linn. Isolates NSPR 101 grown on bagasse. *JNSChE* **18** 61-68.
Sun Y and Cheng J (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresources and Technology* **83** 1-11.
Tao YM, Zhu XZ, Huang JZ, Ma SJ, Wu XB, Long MN and Chen QX (2010). Purification and properties of endoglucanase from a sugar cane bagasse hydrolyzing strain, *Aspergillus glaucus* XC9. *Journal of Agricultural Food Chemistry* **58** 6126-6130.
Vu VH, Pham TA and Kim K (2011). Improvement of Fungal Cellulase Production by Mutation and Optimization of Solid State Fermentation. *Microbiology* **39** 20-25.
Zhang YH, Himmel ME and Mielenz JR (2006). Outlook for cellulase improvement: Screening and selection strategies. *Biotechnological Advancement* **24** 452-481.
Zhou J, Wang YH, Chu J, Zhuang YP, Zhang SL and Yin P (2008). Identification and purification of the main components of cellulases from a mutant strain of *Trichoderma viride T* 100-14. *Bioresource and Technology* **99** 6826-6833.