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Production of Ethanol from Agricultural and Wood Wastes Degraded by Cellulases of *Trichoderma harzianum*

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ABSTRACT: The potentials of *Trichoderma harzianum* in the hydrolysis of agricultural and wood residues as natural lignocellulosic materials; for the production of ethanol were studied. Corn cob, groundnut shell and sawdust were used as cellulosic waste materials to induce the production of cellulases by *T. harzianum*. The enzyme produced was precipitated using Ammonium sulphate, characterized and used to hydrolyse alkali pre-treated cellulosic wastes. The product of hydrolysis was fermented to ethanol using the yeast *Saccharomyces cerevisae*. Among the three cellulosic waste materials tested, the endoglucanase and exoglucanase activity of 0.1494±0.0056 and 0.0820±0.0013 respectively, obtained in culture medium containing corn cob were significantly high ($p < 0.05$), when compared to those of groundnut shell (0.0532±0.0024 and 0.0229±0.0006) and sawdust (0.0235±0.0038 and 0.0117±0.0002). The optimum condition for the activity of the enzyme was 50^{o}C and pH 5.0. Hydrolysis of corn cob with the cellulase yielded 2.83 mg ml^{-1} of sugar which produced 1.04% v/v ethanol on fermentation. Comparatively, the sugar yield from hydrolysis of groundnut shell and sawdust was too low for use in fermentation to ethanol. Although cellulases of *T. harzianum* degraded the three lignocellulosic waste materials to sugar, highest yield was obtained with corn cob. Consequently, corn cob was established as the most viable feedstock in the production of ethanol using the yeast *S. cerevisae*.

KEYWORDS: Cellulose, endoglucanase, exoglucanase, sugar, ethanol.

1.0 Introduction

Ethanol is a product of fermentation of sugar with the enzyme zymase, from microorganisms such as yeast and bacteria. Most agricultural biomass containing starch can also be used as substrate for ethanol fermentation by microbial processes. These include corn (maize), wheat, oats, rice, potato and cassava which must first be converted to sugar by hydrolysis. These sugar and starch based feedstock are food crops and may not be sufficient to meet demands for ethanol production especially in biofuel industry (Greene, 2004). Wastes generated from wood processing such as sawdust and wood pieces; and agricultural residues including shafts, peels, shells, cobs, pulp, chaff and pods are great sources of lignocellulosic biomass which is renewable, inexpensive, and yet unexploited. These wastes have great potentials as feedstock in the production of bio-ethanol.

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Lignocelluloses are composed of a mixture of carbohydrate polymers i.e. cellulose, hemicelluloses and lignin. Lignin is a complex aromatic polymer which tightly binds cellulose and hemicelluloses together to give wood and other lignocelluloses a rigid structure. Lignin also interferes with the hydrolysis of the other polymers by acting as physical barrier that prevents the contact of relevant enzymes to the substrates (Umamaheswari *et al.*, 2010). The breakdown of lignocellulosic biomass to fermentable sugars therefore involves delignification to liberate cellulose and hemicelluloses from their complex with lignin and depolymerisation of the carbohydrate polymers (Mossier *et al.*, 2005). Delignification using physical, physico-chemical, chemical, and biological pre-treatment methods have been shown to achieve efficient enzymatic hydrolysis; avoid the degradation or loss of carbohydrate; avoid the formation of by-products that are inhibitory to the subsequent hydrolysis and fermentation processes; and be cost-effective (Sun and Cheng, 2002).

Thousands of fungal species are able to

utilize cellulose as sole carbon source. Among them, members of the genus *Trichoderma* elaborate high cellulase activity (Herr, 1979). *Trichoderma reesei* is very prominent in industrial production (Miettinen-Oinonen and Suominen, 2002; Liming and Xueliang, 2004; Singhania *et al*., 2007). Other species of the genus *Trichoderma* that produce cellulase include *T. longibrachiatum* (Omojasola *et al*., 2008), *T. harzianum* (Alam *et al.,* 2009), *T. koningii* and *T. viride* (Benkun *et al*., 2007). Several industrially useful strains have been developed and characterised, e.g. Rut-C30, RL-P37 and MCG-80 (Seidl *et al.,* 2008).

Several species and strains of bacteria, yeast and moulds have been reported as microorganisms involved in the fermentation of sugar to ethanol (Lin and Tanaka, 2006). Yeast, under anaerobic conditions, metabolizes glucose to ethanol primarily by way of the Embden-Meyerhof pathway (Lin and Tanaka, 2006).
Baker's yeast (*Saccharomyces cerevisiae*), has long been used to produce ethanol from hexoses. It can yield up to 18% ethanol; it utilizes both monosaccharide such as glucose and disaccharides including sucrose and it is generally recognized as safe (GRAS) for consumption (Kiran *et al.,* 2003*;* Lin and Tanaka, 2006).

This study compared the yield of ethanol from three waste biomass; corn cob, groundnut shell and sawdust; that were hydrolysed using *Trichoderma harzianum*; and fermented by *Saccharomyces cerevisae*.

2.0 Materials and Methods

2.1 Culturing of the Organism

Trichoderma harzianum was cultured on Potato Dextrose Agar plate. Culture was maintained on agar slant and refreshed by sub-culturing every 2 weeks. The yeast was cultured on yeast peptone dextrose agar (YPDA) plates. Dried yeast sample (1 g) was measured into 10ml sterile distilled water in MacCartney bottle. Bottle was shaken rigorously for even distribution of the cells. An aliquot (0.1 ml) of the cell suspension was then used to inoculate yeast peptone dextrose agar medium. Plates were incubated at 28±2ºC for 48 hours. Colonies were purified by sub culturing on YPDA and pure cultures maintained on agar slants at 4ºC. (Zakpaa *et al*., 2009)

2.2. Screening for Cellulolytic Activity

The agar diffusion method of Hankin and Anagnostakis, (1977) was employed to screen the mould for cellulolytic activities. The medium comprised of 1% carboxyl methyl cellulose (CMC) incorporated into Mandels Mineral Salt (Mandels *et al.,* 1974) and solidified with agar (15 g/l). Medium was autoclaved and distributed into Petri dishes. Each plate was inoculated by streaking once across the middle of the plate and incubated at 28±2ºC. Cellulolytic activity was detected after growth, by flooding the plates with 1% congo red solution for 15 minutes. The dye was drained and plates were flooded with 1N sodium chloride solution for another 15 minutes. Clearance around growth of isolate represented cellulase production. The diameter of zone of clearance was measured at five different locations and the mean was used to represent cellulase activity of the organism.

2.3 Pre-treatment of Waste Biomass

The waste materials used in this study were groundnut shell, corn cob and sawdust. Fresh groundnut was purchased from a local retailer at Ipata market in Ilorin. Groundnut was treated by separating the nut from the shell. The shell was thoroughly washed in water to remove dust and sun dried for two days. Shell samples were further dried in electric oven at 65ºC until constant weight was attained. Corn cobs were obtained from retailers of grains also at Ipata market, Ilorin. The cobs were rinsed in water, drained and sundried for two days. Cobs were further treated by breaking to small pieces with the aid of wooden mortar and pestle. The broken cobs were dried in electric oven at 65ºC to constant weight. Saw dust was collected from a wood processing factory at Tanke area, Ilorin and dried in electric oven at 65ºC to constant weight. The dried samples were grinded into powder using electric grinder Phillips HR2810/A, and sieving through a mesh sieve

(pore size, 100µm). The powdered samples were separately treated with 0.25N sodium hydroxide solution to swell the cellulose and make it available for enzyme hydrolysis following a method described by Vyas *et al* (2005).

2.4 Production of Enzyme

The organism was grown in Mandels Mineral Salt medium (Mandels *et al*., 1974), containing 0.2% (w/v) CMC in Erlenmeyer flasks (250 ml) for 48 hrs in order to develop the inoculums. The fermentation medium, 100 ml in separate 250 ml Erlenmeyer flasks, comprised of Mandels Mineral Salt (Mandels *et al*., 1974), into which 10 % (w/v) each of carboxyl methyl cellulose, alkali pre-treated and untreated groundnut shell, corn cob and sawdust, had been incorporated. Each flask was inoculated with 5 ml of spores' suspension from inoculums culture (4.2×10^6 spores ml^{-1}). Flasks were incubated at 28±2°C on a MKV Orbital/gyratory shaker by Lh Fermentation, England at 150 rpm for 10 days (Omojasola and Filani, 2008). Enzyme filtrates were obtained after cultures were centrifuged at 13,000 x g for 20 minutes in a table top high speed refrigerated centrifuge H1850R, China at 4°C. The enzyme filtrates was stored at -10°C and used within two weeks.

2.5 Assay for Cellulase Activity

Endoglucanase activity was determined following the method of the International Union of Pure and Applied Chemistry (Ghose 1987). The reaction mixture comprised of 0.5 ml carboxyl methyl cellulose (0.2% w/v) in 0.05 M citrate buffer, pH 4.8 and 0.5 ml crude enzyme in test tubes. Enzyme blank was prepared by boiling the crude enzyme. The mixture was incubated at 50°C for 1 hour. After incubation 3ml of dinitro-salicylic acid (DNSA) reagent was added to stop the reaction. The reactants in test tubes were boiled for 5 minutes in a boiling water bath and transferred to cold water bath. Absorbance was read at 540 nm in a Genesys-20 Thermo Scientific Spectrophotometer, Japan. The amount of reducing sugar produced by the action of crude enzyme was read off from glucose standard curve. Exoglucanase activity was determined according to the method of Mandels *et al.,* (1976). An aliquot (1.0 ml) of crude enzyme was added to 1.0 ml of citrate buffer, pH 4.8 in a test tube. Filter paper strip (1 cm x 6 cm) served as the substrate and was added to the tube. The tube was incubated at 50°C for 1 hour. DNSA reagent (3 ml) was added to the reactant to stop the reaction. The mixture was boiled for 15 minutes in rigorously boiling water bath and cooled immediately by transferring to cold water bath. Absorbance was read at 540 nm and concentration of reducing sugar obtained from glucose calibration curve. In each case, one unit of enzyme activity was defined as the amount of enzyme that released 1µm of reducing sugar per millilitre of substrate suspension per minute.

2.6 Optimization of Conditions for the Activity of Crude Enzyme Filtrates

The optimum temperature for the activity of the cellulases was determined. Culture filtrates (0.5 ml) was added to 0.5ml carboxyl methyl cellulose (2% w/v) in 0.05M citrate buffer, pH 4.8 in a test tube. Mixture was incubated in water bath at temperatures ranging between 20 to 80°C for 1 hour. Endoglucanase activity was determined as described earlier. Endoglucanase activity was also determined at varying pH values ranging from pH 3.0 to 7.0. The enzymes were assayed in 0.05M citrate buffer in the pH range of 3.0 to 6.0 and in 0.05 M phosphate buffer in the pH range 6.5 to 7.0 (Macris 1984). All the tubes were incubated at 50°C.

2.7 Partial Purification of Enzyme Filtrates with Ammonium Sulphate

Solid $(NH4)_2SO_4$ was added to clarified culture fluid to obtain 20% saturation. After centrifugation (10,000 x g, 4°C, 10 min) the sediment was discarded, and ammonium sulphate was added to the supernatant to obtain 80% saturation. The mixture was again centrifuged, and the precipitate was dissolved in 0.05 M phosphate buffer at pH 7.0 (Macris, 1984). Endoglucanase and exoglucanase activity

was determined following the methods described earlier.

2.8 Hydrolysis of waste biomass

The method of Mandels *et al*., (1974) was employed. Citrate buffer (100ml) containing alkaline treated waste biomass, (10%w/v) in 250ml Erlenmeyer flask was inoculated with 10ml of the partially purified enzyme filtrate and incubated at 50^{o}C for 7hrs. Samples (1 ml) was withdrawn aseptically from each flask at 1 hour interval and analysed for reducing sugar by the DNSA method to determine the optimum time in hours for cellulase digestion of the wastes. The hydrolysate was then used for fermentation to produce ethanol.

2.9 Fermentation of the products of waste biomass digestion to alcohol

2.9.1 Inoculum Preparation

Yeast peptone dextrose broth (100 ml) in 250 ml Erlenmeyer flask was inoculated with pure colonies of yeast from agar slant with the aid of an inoculating loop. This was incubated at 28±2^{o}C on a gyratory shaker at 150 rpm for 48 hours. Cell population was enumerated with the aid of a haemocytometer (Zakpaa *et al*., 2009).

2.9.2 Fermentation

The fermentation broth comprised of the product of hydrolysis of the waste biomass as the fermenting sugar; supplemented with 2% (w/v) each of peptone and yeast extract. The broth (80 ml) was filled into a 100 ml sealable bottle, sterilized in an autoclave and inoculated with 10 ml of the inoculums culture of yeast at 3.8 x 10^{6} cells ml^{-1}. The bottles were sealed with the aid of an adhesive tape and incubated at 28±2^{o}C for a period of 8 to 48 hours. Bottles were removed at 8 hour intervals to determine the amount of ethanol produced and the residual sugar in the medium.

2.10 Determination of ethanol concentration by the acidified dichromate/thiosulfate titration method

The fermented broth was assayed for ethanol using the acidified dichromate/thiosulfate titration method described by College of Science, University of Canterbury with some modifications. A 250 ml Erlenmeyer flask containing 10 ml of the fermented broth was covered with rubber stopper. Ten millilitre acid dichromate solution (0.01 M in 5.0 M sulphuric acid) was placed in 250 ml Erlenmeyer flask. This was connected to 10 ml of fermented broth in another 250 ml Erlenmeyer flask which was placed in water bath set at 80^{o}C. The set up was allowed to stand for 3 hours during which ethanol produced by fermentation of the broth would have evaporated into the acid dichromate solution. All the flasks were fixed with rubber stopper and sealed with wax to avoid leakages After the incubation period, the setup was dismantled, 100 ml distilled water and 1.0ml potassium iodide (1.2 M) was added to the dichromate solution. This was titrated with sodium thiosulphate (0.03 M) until the brown colour turned to yellow, at which point 1% starch solution (1 ml) was added as indicator of iodine, and further titrated until the blue colour fades. Three flasks consisting of 10 ml acid dichromate were setup as blanks and titrated first so as to monitor the volume of thiosulphate required. The difference between the volume of thiosulphate used in the titration of the blank and that of the sample was used in calculating the amount of ethanol produced.

2.11 Determination of Residual Sugar in the Fermentation Medium

The residual sugar after each period of fermentation was determined following the DNSA method of Miller *et al.,* (1959). Dinitro-salicylic acid (DNSA) reagent (1 ml) was added to an aliquot (1 ml) of the fermentation medium in a test tube and properly mixed. The mixture was boiled for 5 minutes and cooled under running tap water. Five millilitres of 40% Rochelle salt solution was added to the mixture and absorbance was read in a Genesys-20

Thermo Scientific Spectrophotometer, Optimal, Japan at 540 nm. Amount of reducing sugar was read off a curve of glucose standard and expressed as mg ml^{-1}

2.12 Statistical Analysis

All data were subjected to analysis of variance and the sample means tested for significant differences using the multiple intervals test (Duncan). This was carried out using the statistical package SPSS 15.0.

3.0 Results and Discussion

In this study, *Trichoderma harzianum* produced a diameter of zone of clearance measuring 16.0±1.2 mm on potato dextrose agar plates containing 1% CMC. This is an indication that the fungus produces cellulolytic enzymes. The cellulolytic activities of *T. harzianum* has been reported (Saddler *et al.*, 1985; Kolar *et al.*, 1989; Kocher *et al*., 2008; Ahmed *et al.*, 2009; Alam *et al.*, 2009). In general, fungi of the genus *Trichoderma* are reputed as prominent cellulase producers (Mandels *et al.*, 1974; Sternberg, 1976; Macris, 1984).

The endoglucanase and exoglucanase activity profile of the crude enzymes produced in Mandels' Mineral Salt medium supplemented with different cellulosic materials is presented on Table 1. Corn cob significantly induced production ($p < 0.05$) of cellulases with higher activity compared to the other substrates tested. This may be due to the relatively high cellulose and low lignin component of corn cob (Sun and Cheng, 2002; Zych, 2008). Production of cellulases with the use of various lignocellulosic materials as inducers has been reported (Liming and Xueliang 2004; Benkun *et al.*, 2007). Busto *et al.* (1996) reported the synthesis of higher endoglucanase in *Trichoderma reesei* induced by amorphous cellulose compared to cellobiose, lactose, sucrose or other commercial celluloses. Bagasse, corncob and sawdust were used as lignocellulosic substrates for the production of cellulase enzyme using *Aspergillus flavus* after ballmilling and pretreatment with caustic soda (Ojumu *et al*., 2003). Jaafaru and Fagade (2007) reported production of high cellulase activity in *Aspergillus niger* with corn cob and groundnut shell, compared to other substrates including saw dust, cotton wool, and newspaper. Production of cellulases with high activity by *A. niger* and *T. longibrachiatum* was also achieved with the use of wastes of pineapple and oranges as substrate (Omojasola *et al.*, 2008; Omojasola and Jilani, 2008).

Cellulases produced by the fungus used in this study had optimum activity at 50^{o}C and a pH between 4.5 and 5.5 (Figures 1 and 2). This indicates that the enzyme is thermophilic and may not be affected by a slight increase in temperature during hydrolysis. The enzyme is also active in acidic condition and of low spectrum. Exposure to pH in the extreme of 4.5-5.5 may denature the enzyme thereby making it inactive. Varying optimum temperature and pH for the activity of cellulases has been reported (Singhania *et al.*, 2007; Acharya *et al.*, 2008; Omojasola, *et al*., 2008; Liu *et al.*, 2010; Saliu and Sani, 2012).

Partial purification of the crude enzyme with ammonium sulphate significantly enhanced the activity of cellulases produced by *T. harzianum*. Activity was enhanced by 171% in the cellulases produced in alkaline treated substrates indicating that the process may have not only successfully removed impurities, but may have also concentrated the enzyme. However, enhancement of activity was not as significant for the enzyme produced in the untreated substrates ($p < 0.05$) (Table 2). This may be due to the relatively low activity of the crude cellulases from the substrates. Chen *et al*. (2004) obtained a high CMCase with cellulases purified with 40 – 60% ammonium sulphate. The partial purification process may not be sufficient for the enzyme filtrates of the fungi isolates; it is however a step that is necessary for the full purification process (Macris, 1984; Chen et al., 2004). The level of improvement achieved in the activity of the enzyme is encouraging and an indication of the potentials of the cellulases of the fungi in the hydrolysis of waste biomass.

Hydrolysis of corncob by the partially purified cellulases of *T. harzianum* yielded

highest concentration of sugar compared to groundnut shell and sawdust (Figure 3). This may be due to the relative high cellulose content of corn cob which translates to higher substrate availability and consequent higher enzyme activity. Among the three substrates, only the hydrolysates of corn yielded enough sugar for fermentation to ethanol. The time course for ethanol production and fermentation of sugar

Table 1: Cellulase activity profile of the crude enzymes produced by *Trichoderma harzianum*

Substrate	CMCase (IU ml^{-1})	FPase (FPU ml^{-1})
Treated Corn Cob	0.1494 ± 0.0056^{a}	0.0820 ± 0.0013^{a}
Untreated Corn Cob	0.0326 ± 0.0018^{c}	0.0278 ± 0.0010^{f}
Treated Groundnut Shell	0.0532 ± 0.0024^{d}	0.0229 ± 0.0008^{e}
Untreated Groundnut Shell	0.0234 ± 0.0033^{b}	0.0172 ± 0.0006^{d}
Treated Saw Dust	0.0235 ± 0.0038^{b}	0.0117 ± 0.0002^{c}
Untreated Saw Dust	0.0218 ± 0.0021^{b}	0.0061 ± 0.0007^{b}
Carboxyl methyl cellulose	0.0745 ± 0.0010^{e}	0.0289 ± 0.0009^{e}

Each value represents mean of three independent tests. Means displayed with homogenous superscript within the same column are insignificantly different $P > 0.05$

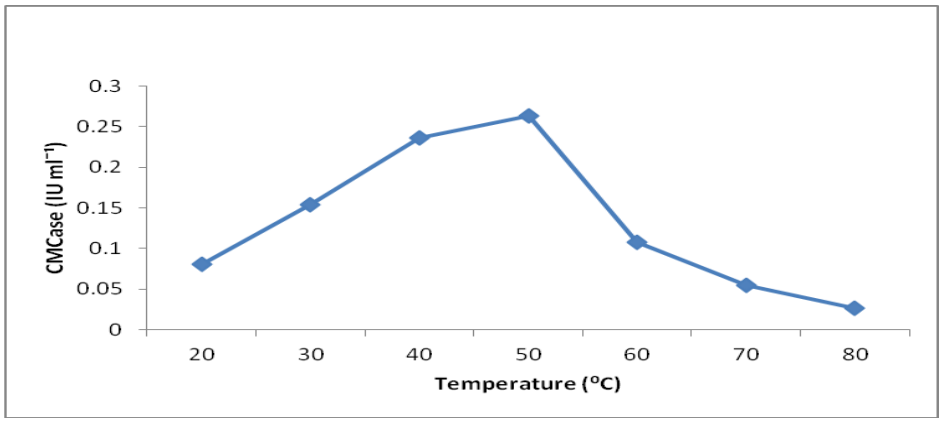

Figure 1: Effect of temperature on the endoglucanase activity of crude enzyme of *T. harzianum*

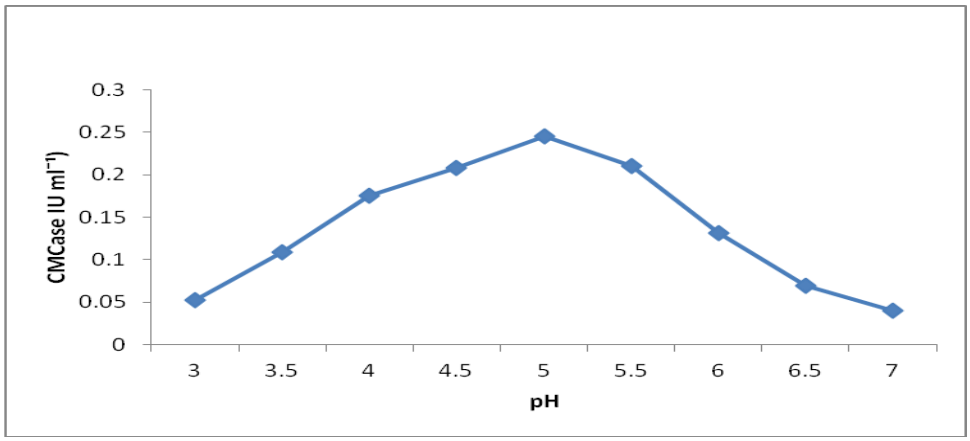

Figure 2: Effect of pH on the endoglucanase activity of crude enzyme of *T. harzianum*

Table 2: Cellulase activity profile of the partially purified enzymes of *Trichoderma harzianum*

Substrate	CMCase (IU ml^{-1})	FPase (FPU ml^{-1})
TCC	0.1556 ± 0.0196^{d}	0.0913 ± 0.0005^{a}
UCC	0.0339 ± 0.0039^{a}	0.0289 ± 0.0013^{f}
TGS	0.0574 ± 0.0019^{b}	0.0240 ± 0.0013^{e}
UGS	0.0278 ± 0.0049^{a}	0.0186 ± 0.0003^{d}
TSD	0.0308 ± 0.0021^{a}	0.0123 ± 0.0004^{c}
USD	0.0253 ± 0.0010^{a}	0.0064 ± 0.0003^{b}
CMC	0.1130 ± 0.0093^{c}	0.0361 ± 0.0013^{g}

Each value represents mean of three independent tests. Means displayed with homogenous superscript within the same column are insignificantly different $P > 0.05$

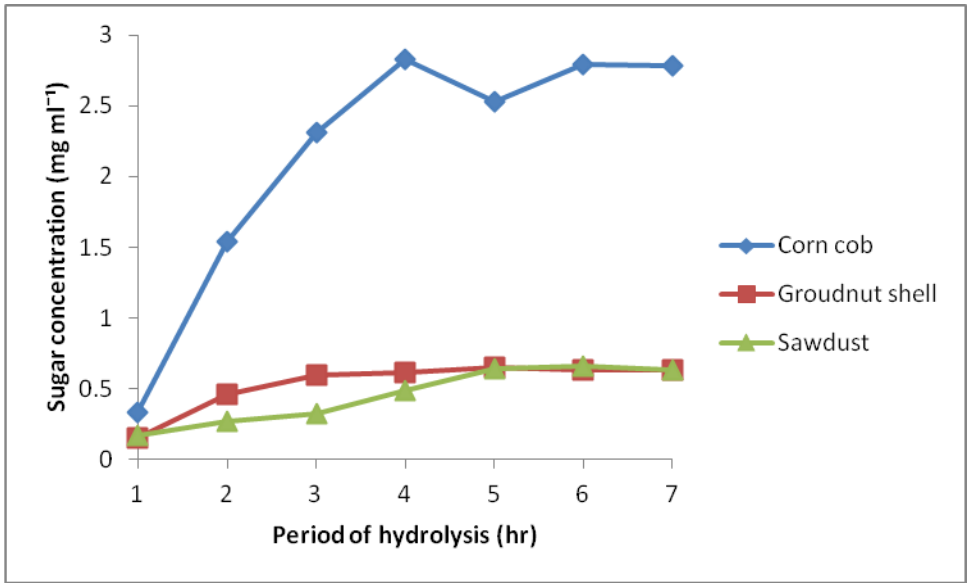

Figure 3: Time course for the hydrolysis of cellulosic waste materials with partially purified cellulases from fungal isolates

produced by hydrolysis of corncob with cellulases of *T. harzianum* is presented in Figure 4. The yeasts, *Saccharomyces cerevisae* used up the sugar in the fermenting fluid and produced ethanol. The concentration of sugar reduced as fermentation proceeded while ethanol concentration increased. Ethanol production was slow initially but increased exponentially after 32 hours of fermentation. Highest ethanol concentration was obtained at 48 hours. Though the 35% fermentation efficiency achieved falls short of the theoretical, it compares favourably with values reported by earlier researchers in cellulosic ethanol (Punnapayak *et al.*, 1999; Zakpaa, 2009). A higher yield has also been reported with the use of genetically engineered *S. cerevisae* (Cao *et al.*, 1996), other yeasts (Umamaheswari *et al.*, 2010) and the bacterium *Zymomonas mobilis* (Lawford and Rousseau, 2003).

In this study, the fungus *T. harzianum* was shown to possess the ability to efficiently degrade cellulosic wastes to produce fermentable sugar. Corn cob was the most viable of the three waste materials used and is therefore recommended for use as feedstock in bio-ethanol production with a combination of cellulase from *T. harzianum* and the fermenting organism *S. cerevisae.*

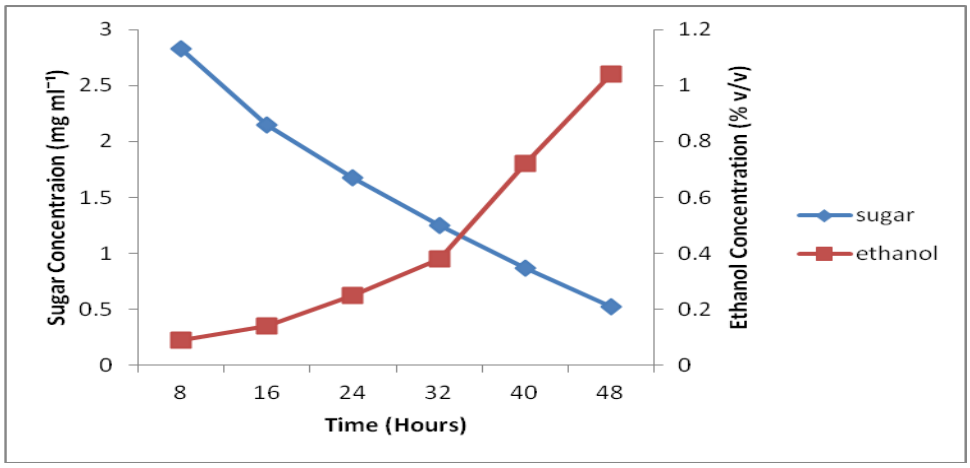

Figure 4: Time course for Ethanol Production and Fermentation of Sugar Produced by Hydrolysis of Corncob with Cellulases of *T. harzianum*

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