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*Full Length Research Paper*

# Production of bioethanol from agro-waste hydrolyzed with cashew nut shell extract

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**This study examines the lignocellulytic activities of the ethanol extract of *Anacardium occidentale* empty nut shell on some agricultural waste. The enzyme activity assay was carried out on the extract obtained. This was measured as micromole sugar released per min. Pretreatment and natural acid saccharification was done using the extract. The result obtained showed that the enzyme activity (µ/ml) for β-1,4-exoglucanase, β -1,4-endoglucanase and xylanase was maximum 3.70 ±0.43, 0.95 ±0.03 and 2.32 ±0.10, respectively. While maximum reducing sugar yield for the waste was from sugarcane chaff (491mg/g) and rice husk gave the lowest amount of 46mg/g. Bioethanol produced was highest in sugarcane chaff (20.70±1.40g/L) at 72 hr of incubation using the yeast *Pichia caribbica* (IMI 398400) and lowest in rice husk (3.22±3.22g/L) with the yeast *Kluyveromyces marxianus* (IMI 398399). This study showed that the ethanol extract of cashew nut shell is capable of producing cellulases and xylanase enzyme. The fermentation of hydrolysates obtained from the pretreatment and natural acid saccharification can give considerable amount of bioethanol thus assisting in effective waste management.**

**Keywords:** *Anacardium occidentale*, Agro-waste, Cellulose, Bioethanol, Yeast, Pichia caribbica, *Kluyveromyces marxianus.*

## INTRODUCTION

Cashew (*Anacardium occidentale* Linn), which is cultivated extensively as a cash crop in Africa, is the world's number one tree nut (Alexander, 2008). The plant grows well in waste sandy places and has been satisfactorily used for reclamation of sand-dune near the sea (Hutchinson and Dalziel, 1958). The three main cashew products traded in the international market are: raw cashew nuts, cashew kernels and cashew nut shell liquid (Azam-Alli and Judge, 2001). The cashew nutshell liquid (CNSL), a byproduct of processing cashew, is mostly composed of anacardic acids (Alexander, 2008). The shell oil has local uses in tattooing, to remove warts, to put in a carious tooth; it is used for refractive leprosy and ulcer. The oil also has industrial applications in manufacture of brake lining, industrial belting, clutching, for reinforcing synthetic rubber (Ogunsina and Bamgboye, 2007). The kernel extracted from the husk is edible and contains 40-57% oil, 20% protein. Recently, Trox *et al.* (2010) found appreciable levels of certain bioactive compounds such as beta-carotene, lutein, zeaxanthin, alpha-tocopherol, gamma-tocopherol, thiamin, stearic acid, oleic acid and linoleic acid in raw cashew nut kernels. Several compounds including esters, terpenes, hydrocarbons, carboxylic acids, aldehydes, alcohols, ketones, lactones and norisoprenoids have been isolated from *A. occidentale,* characterized and quantified by gas chromatography-mass spectrometry analyses (Bicalho and Rezende, 2001).

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Hugh amount of agricultural waste such as crop residue, herbaceous plants, forest residue and animal waste are produced annually around the world. The need to process these waste into beneficial use cannot be over-emphasized. Producing biofuel from these agro-waste have variously been reported (Patle and Lal, 2007; Taherzadeh and Karimi, 2007; Akin-Osaniaye *et al.,* 2008; Oyeleke and Jibrin, 2009; Mathiyazhagan *et al.,* 2011; Mohd *et al.,* 2011). However, the lignocellulose waste must be pretreated chemically or enzymatically for the release of fermentable sugars. Much work have been carried out regarding the hydrolysis of waste with chemicals (Adesanya *et al.,* 2008; Akin-Osaniaye *et al.,* 2008; Ahmed *et al.,* 2009) and with enzymes from microorganisms (De Vasconcelos *et al.,* 2004; Chaudhary and Qazi, 2008; Oyeleke and Jibrin, 2009 among others). However, the possibility of using plant based extract for the hydrolysis of agro-waste has not been given much attention. This study was therefore initiated to explore the possibility of using cashew nut shell extract as a substrate for hydrolyzing agro-waste. It was also geared to isolate new fermenting yeast from locally available sources.

## MATERIALS AND METHODS

### Collection and Processing of Substrates Used

The agro-waste (sugarcane chaff, sweet potato peel) were collected from waste dumping sites in Lagos metropolis while rice husk was collected from rice mill in Ifo Local Government Area of Ogun state, Nigeria. The samples were transported to the laboratory, washed, dried and ground to powder form using a Warring blender (Binatone).These particles were then sieved to obtain average particle sizes of 300 µm in diameter.

### Isolation and characterization of microorganism

The yeast *Kluyveromyces marxianus* and *Pichia caribbica* were isolated from cassava tuber steep and maize steep respectively. They were identified in the Botany laboratory of the University of Lagos, Akoka, Lagos. The isolates were characterized based on standard procedures of colonial morphology, cultural characteristics and biochemical tests as described Seeliger (1956); El-Zaatari *et al.* (1990); Olutiola *et al.* (2000). The biochemical tests carried out include sugar fermentation test, germ-tube test, resistance to chloramphenicol and urea hydrolysis test. The identities were confirmed by comparing the characteristics with those of known taxa using the schemes of Rhode and Hartmann (1980) and Ellis *et al*. (2007). The pure isolates were also sent on malt extract agar (MEA) slants in 5.0 ml McCartney bottle to Centre for Agriculture and Bioscience International (CABI) Kew Garden, England for molecular identification, where the accession numbers were respectively given.

### Extraction of plant acid from *Anacardium occidentale*

This was done according to the method described by Sofowora (1982). Cashew nuts were bought at the fruit stall of Ketu market in Kosofe Local Government Area of Lagos State, Nigeria. The nuts were cracked using a nut cracker to separate the kernel from the shells. The solvent used was ethanol distilled from 360 h old palm wine (Nwachukwu *et al.,* 2006). The cashew shells were pounded into bits using a wooden pestle and mortal. The mashed cashew nut shell was soaked in ethanol at the ratio of 5:6 (w/v) for 72 h. The mixture was filtered through No. 1 Whatman filter paper. The filtrate was concentrated through the rotary evaporator under reduced pressure and controlled temperature and the pH was obtained as 3.2 using a pH meter.

### Study on enzyme production from plant extract

The study was done according to the modified method of Zaldivar *et al*. (2001). Cellulolytic enzymes production by the plant extract was determined using three carbon sources: carboxymethylcellulose (substitution degree 0.7, Sigma), microcrystalline cellulose and xylan.

### β 1, 4-endoglucanase activity

The β -1,4-endoglucanase activity was determined using carboxymethylcellulose as substrate and the formation of reducing sugars was measured by reaction with DNS. The reaction mixtures containing 10 mg CMC (carboxymethyl cellulose) in 1 ml of 0.05 M sodium acetate buffer (pH 5.0) and 1 ml cashew nut extract were incubated at 50°C for 30 min. The reducing sugar formed was measured with DNS. Three milliliter (3ml) of DNS reagent was added to 1ml of the test sample. The colour was developed by boiling the mixture in water bath for 5 min. Absorbance was read at 540 nm using spectrophotometer (UNICO 2100, Germany). Reducing sugar concentration was obtained from a standard glucose concentration curve using the DNS method of Miller (1959). This was done in triplicate.

### β -1,4-exoglucanase activity

The β-1,4-exoglucanase activity was assayed as above using microcrystalline cellulose (Avicel) as substrate and

the formation of reducing sugars was measured by reaction with DNS. The reaction mixtures containing 10 mg of microcrystalline cellulose in 1 ml of 0.05 M sodium acetate buffer (pH 5.0) and 1 ml cashew nut extract were incubated at 50°C for 30 min. The reducing sugar formed was measured with DNS as previously described above.

**Xylanase activity**

Xylanase activity was determined by measuring the release of reducing sugars from a solution of water soluble birch wood xylan (Fluka BioChemika, 95588) using the DNS method. The reaction mixtures containing 10 mg Xylan (Fluka BioChemika, 95588) in 1 ml of 0.05 M sodium acetate buffer (pH 5.0) and 1 ml cashew nut extract were incubated at 50°C for 30 min. The xylose formed was measured with dinitrosalicyclic acid (DNS) as described above.

**Pretreatment methods**

The modified method described by Ocloo and Ayernor (2010) was used. The ground cob and peels were slurried with distilled water using a solid to liquid ratio of 10% (w/v). The mixture was allowed to boil at 70°C until gelatinized.

**Natural acid saccharification (NAS)**

Twenty milliliter (20 ml) of the cashew extract was added to the gelatinized mash, stirred and the mixture allowed to cool gradually to 50°C for the conversion of the mash to sugars. The mixture was autoclaved after an hour at 121°C for 15 min to arrest enzyme action and immediately filtered using muslin cloth. The reducing sugar content in the hydrolysates were determined by DNS method of Miller (1959).

**Identification of specific simple sugars in the hydrolysates**

The hydrolysates were analyzed on an HP 6890 Series GC powered with an HP ChemStation Rev. A 09.01 (1206) and a flame ionization detector (FID). Sample (2-3 µl) was injected from slit injector. The carrier gas was hydrogen at the flow rate of 1.0 ml/min. The fractionation was carried out in an isothermal temperature of 210°C. The injector and detector temperature were 250°C and 325°C respectively. Typical coefficient of correlation for standard curve was 0.95-0.99. Pecks were identified by comparison of retention times with those of standard glucose, xylose, arabinose, maltose, rhamnose, lactose, sucrose, ribose and fructose.

**Fermentation of hydrolysate**

The fermentation studies were carried out using *K. marxianus* (IMI 398399) and *P. carribica* (IMI 398400) in the hydrolysate obtained from pretreated, enzymatic hydrolyzed agro-waste. The yeast broths were separately added at the ratio of 1:50 (v/v). In order to study the effect of enzymatic saccharification on ethanol yield in the agro-waste, a separate set of fermentation experiment was carried out in a similar manner using the hydrolysates obtained from the pretreatment without natural acid saccharification. Fermentation was allowed for 72 h at 28-30ºC.

**Distillation and determination of quantity of ethanol**

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 78°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 78°C by the density of ethanol (0.8033 g/ml). g/L is equivalent to the yield of 100 g of dried substrate (Oyeleke and Jibrin, 2009).

**RESULTS**

**Cultural, morphological and biochemical characteristics of isolates**

The organisms used for fermentation were *K. marxianus* and *P. caribbica*. *P. caribbica* showed flat colonial growth with cloudy white, entire, smooth surface. Cells were ellipsoidal in clusters, measuring 1.0 – 3.5 µm in diameter. *K. marxianus* grew moderately slow in culture, without covering a 9 cm Petri dish after 72 h of growth at 28- 30°C. The colonies mature to form creamy-white clusters. The cells were short ovoid to elongate with some at the point of constriction measuring 2.0 - 5.0 µm in diameter. The cells were produced singly. Table 1 shows the phenotypic characteristics (Table 1).

**Enzyme assay of cashew nut shell extract**

The plant acid extracted from empty cashew nut shell used in the course of this study had a pH of 3.2. The

**Table 1.** Phenotypic characterization of organisms

| Biochemical Reaction | Organisms | |
|---|---|---|
| | ***K. marxianus*** | ***P. caribbica*** |
| Cell morphology | Elongated | Ellipsoids |
| Gram reaction | + | + |
| Sucrose | + | + |
| Glucose | + | + |
| Fructose | + | + |
| Dextrose | + | - |
| Maltose | - | + |
| Xylose | - | + |
| Galactose | + | - |
| Lactose | V | + |
| Urea | - | + |
| Resistance to chloramphenicol | + | + |

+ = Present; - = Absent; V = Variable

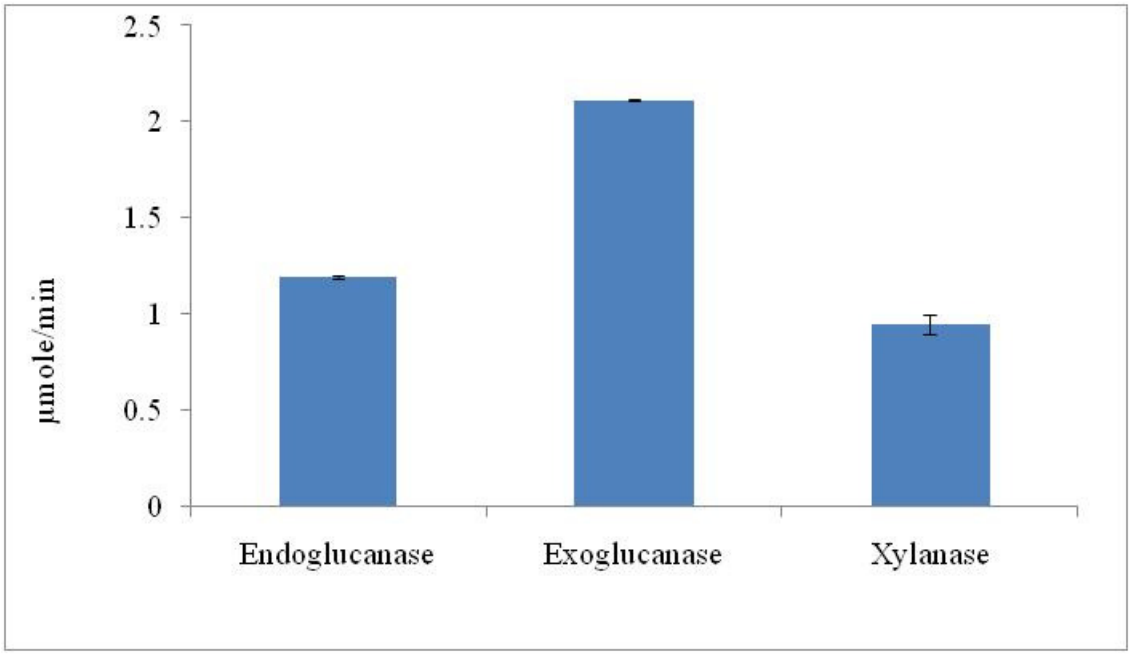


**Figure 1.** Amount of enzyme activity of plant extract

experiment for enzyme assay was performed in triplicates and the values are presented as Mean ± SEM of the triplicate results. The endoglucanase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per minute. Exoglucanase is also expressed in terms of units. One unit is the amount of enzyme releasing one µmole of reducing sugar from microcrystalline cellulose per minute and one unit of xylanase activity is the amount of enzyme liberating one µmole of xylose from xylan per minute. This is as displayed in Figure 1.

**Reducing sugar concentration in the hydrolysates**

Test for reducing sugar in the hydrolysates showed that sugarcane chaff contained the highest amount of reducing sugar of 491 mg/g while rice husk showed the lowest of 46 mg/g. This showed that the cellulosic and hemicellulosic substances in the substrates had been broken down into simple sugars which the fermenting

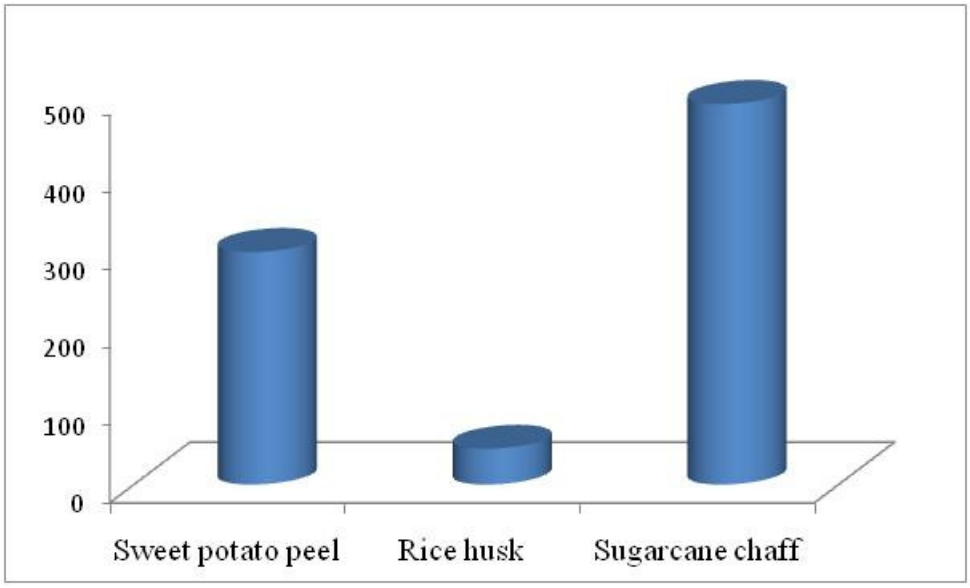


**Figure 2.** Amount of reducing sugar in hydrolysed agricultural waste

**Table 2.** Concentration of different sugar components

| Sugar component (mg/g) | Sample | | |
|---|---|---|---|
| | Sugarcane chaff | Rice husk | Sweet potato peel |
| Ribose | 2.06 x$10^{-4}$ | 5.19 x$10^{-1}$ | 2.06 x$10^{-4}$ |
| Xylose | 5.17x $10^{-5}$ | 2.57x $10^{-1}$ | 1.28x $10^{-4}$ |
| Arabinose | 3.80 | 1.17 | 2.07x $10^{-4}$ |
| Rhamnose | 3.55x $10^{-5}$ | 3.55x $10^{-5}$ | 5.55 x $10^{-5}$ |
| Fructose | 11.42 | 4.95 | 12.68 |
| Glucose | 10.67 | 11.60 | 15.70 |
| Maltose | 9.85 | 5.68x$10^{-5}$ | 2.07x$10^{-5}$ |
| Lactose | 2.73x $10^{-4}$ | 5.17x $10^{-5}$ | 1.67x $10^{-4}$ |
| Sucrose | 32.84 | 6.67x $10^{-1}$ | 23.76 |

organisms can utilize. Result is shown in Figure 2.

### Types and concentration of sugars present in plant acid hydrolyzed agro-waste

The hydrolysates were subjected to Gas Chromatographic analysis in order to obtain the type and amount of sugars present. The standards used include glucose, fructose, sucrose, arabinose, xylose among others. The correlation co-efficient of each the selected standards is 0.99 g/L. The results showed the presence of the test sugars in varying concentrations. The highest concentration of glucose was obtained in sweet potato peel while sugarcane chaff showed the highest concentration of sucrose. Rice husk gave the lowest concentration of fructose as shown in Table 2.

### Determination of quantity of ethanol produced

The fermentation efficiencies and volume of ethanol production by the isolates in the hydrolysates of the agro-waste are presented in Table 3. Ethanol yield varied significantly between the microorganisms and the highest yield was 20.70±1.40 g/L with *Pichia caribbica*, followed by 16.47±1.21 g/L with *K. marxianus* from sweet potato peel while the lowest yield was 03.22±3.22 g/L by *K. marxianus* in the rice husk.

## DISCUSSION

As in most ethanol production using various lignocellulosic biomasses, the main process of pre-treatment involves alkaline or acid hydrolysis and enzyme

**Table 3.** Amount of bioethanol from agro-waste

| Microorganism | Amount of bioethanol (g/L) | | | |
|---|---|---|---|---|
| | Sugarcane chaff | Sweet potato peel | Rice husk | Control |
| *K. marxianus* | 11.65±1.21 | 16.47±1.21 | 03.22±3.22 | 0.00±0.00 |
| *Pichia caribbica* | 20.70±1.40 | 09.04±0.61 | 11.25±0.81 | 0.00±0.00 |

Values are expressed in mean ±SEM

saccharification to produce glucose and xylose followed by fermentation with yeast (Demirbas, 2005). Plant based (natural) acid was successfully used in this study implying that this consist of hydrolytic properties. Mineral acids ($H_2SO_4$ and HCl) being used in various studies have been reported by Palmqvist and Hahn-Hagerdal (2000) to produce substantial amounts of impurities like furfural, acetate and other phenolic compounds. It is also economical to produce plant based extract for the purpose of hydrolyzing agro-waste rather than relying on importation of mineral acids with scarce foreign exchange. *Anacardium occidentale* (cashew) nut shell is known for its production of acidic substances and consisting of various amounts of bioactive compounds (Trox *et al.,* 2010). The enzyme assay of the acidic extract showed that it contains β-endoglucanase, β-exoglucanase and xylanase which could break the β-1,4, linkage of the cellulosic and hemicellulosic materials in the substances used in this study. Therefore, confirming the presence of cellulases involved in lignocellulose hydrolysis in the cashew nut shell extract. Cellulases are complex of cellulolytic enzymes whose synergetic activities probably led to the breakdown of the substrates into their monomeric sugars. Najafpour *et al*. (2007), Taherzadeh and Karimi (2007) had earlier reported that proton in acid could catalyze and scissor the β-1,4, linkages of glucose and xylose monomer, acetyl groups and other products in cellulose and hemicelluloses biomass. Interestingly, Ohmiya *et al*. (1995) earlier extracted cellulase from poplar cell. According to Coughlan and Ljungdahl (1998), at least three major groups of cellulases are involved in the hydrolysis process; these are a) endoglucanase which attacks regions of crystalinity in the cellulose, creating free chain-ends, b) cellobiohydrolase (exoglucanase) which degrades the molecule further by removing cellobiose units from free chain-ends and c) β-glucosidase which hydrolyzes cellobiose to glucose. This further confirms that some plants contain hydrolytic compounds that can be useful in the biodegradation of agro-wastes to economically produce valuable organic compounds. Hammond and Ayernor (2000) were able to use malts extracted from various cereals in the hydrolysis of starch and they observed that rice malt gave the highest yield of sugars. The pH of the natural acid used was 3.2, thus showing that the extract is acidic. Though, the acidity could not be potent enough for the purpose of hydrolysis. Nevertheless, this could aid in the hydrolysis of the agro-waste into monomeric units. Muhammad *et al*. (2010) reported that cellulase from *Trichoderma viride* showed optimum activity at pH 5.5. Workers like Ahmad *et al*. (2009) have stated that the initial medium pH in the range of 4.5-5.5 is the optimum for carboxymethyl cellulase (endoglucanase) production.

The highest amount of reducing sugar was from sugarcane chaff hydrolysate (491 mg/g). Sun and Cheng (2002) reported that generally, during enzymatic hydrolysis, cellulose is degraded by cellulases to reducing sugars that can be fermented by yeast to ethanol. While Arumugam and Manikandan (2011), reported that an initial pretreatment of fibrous peel residues breakdown its structure to make it more susceptible to enzymatic reactions. This can be the reason for the valuable amount of reducing sugars librated from the hydrolysates. Likewise, based on the enzymatic saccharification study, it was observed that the sugar concentration varied in the different agro-waste. Sucrose was the dominant monomer sugar obtained from the process. Substantial concentration of glucose (16.58±1.90g/L) was also obtained by Mohd *et al*. (2011) from empty fruit bunches of oil palm through enzymatic hydrolysis. As observed from the ethanol activities of the yeast isolates *Pichia caribbica* and *K. marxianus* could be better fermenters of the $C_6$ and $C_5$ substrates. Lignocellulosic ethanol can be produced from various feedstocks such as lignocellulosic biomass, starchy materials, sucrose containing feedstock and microalgae (Mathiyazhagan *et al*., 2011; Templer and Murphy, 2012). Consequently, it was observed that the highest amount of bioethanol from the agro-waste using the natural acid was 28.12±1.61 g/l at 72 h of incubation. Previously, Oyeleke and Jibrin (2009), Kathiresan and Saravanakumar (2011), had respectively obtained 26.31± 1.41 g/l of bioethanol from guinea corn husk and 12.3 g/l of bioethanol from sawdust at 120 h of fermentation. Mohd *et al*. (2011) as well gave a corresponding yield of bioethanol from bio-waste where they obtained a yield between 9.55-10.32 g/l of ethanol from empty fruit bunches of palm oil fermented with *S. cerevisiae*. This further indicates that bioethanol production from agro-waste hydrolysates can significantly be comparable with bioethanol production from other feedstock like crop

plants that include sugarcane, sweet potato and others. Thewaste generated from processing the crops can be used for biofuel (bioethanol) production. This correlates the works of Giampietro *et al.* (1997); Mohd *et al*. (2011); Templer and Murphy (2012) who reported that bioethanol in high quantity can be derived from cellulosic biomass through acid or enzymatic hydrolysis followed by fermentation.

## CONCLUSION

This study has shown that, the hydrolysis of agro-waste can be achieved through enzymatic hydrolysis from a plant source. This could help in a more effective and economic way of hydrolysis, also to a larger extent in waste management and economic empowerment.

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