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Co-immobilization of cellulase extracted from *Schizophylum commenfr* and *Saccharomyces cerevisieae* in the bioconversion of sugar cane bagasse to ethanol.

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Abstract.

The production of ethanol from sugar cane bagasse using co-immobilized yeast cells (saccharomyces cerevisieae Y300) and crude cellulase enzyme extracted from mushroom, Schizophylum commenfr, was studied. The yeast cell and cellulase enzyme were immobilized on activated bone for simultaneous saccharification of sugar cane bagasse and ethanol production. The preliminary bagasse hydrolysis, cellulase enzyme extraction from mushroom, effect of bagasse concentration and immobilization on glucose yield and ethanol yield from co-immobilization of enzyme and yeast cells were determined. From the result, glucose yield from hydrolysis of bagasse with immobilized cellulase enzyme increased from 225.20g to 1531.36g within 2 to 10hrs, while glucose yield with free enzyme decreased from 4296.81g to 3319.44g within 6 to 10hrs. Immobilized enzyme and free yeast cells showed an increased ethanol yield from hydrolyzed bagasse 28% in 12hrs to 44% in 48hrs, but co-immobilized enzyme and yeast cells gave a higher ethanol yield of 38% in 12hrs to 48% in 48hrs. There was significant difference between the use of free yeast cells and immobilized enzyme and that of co-immobilized enzyme and yeast cells ($p<0.05$).

Key words: Co-immobilization, Cellulase, *Schizophylum commenfr, Saccharomyces cerevisieae,* Bagasse and Ethanol
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Introduction

Bio-ethanol is an important renewable fuel contributing to the reduction of negative environmental impact generated by the daily utilization of fossil fuels. As a result, production and use of biofuels have increased. Although, at the moment bio-ethanol is mainly used in blends with gasoline as E10 and E20, the demand has soared. For instance, consumption of bio-ethanol in most countries of the European Union is far greater than the quantity produced in those countries. According to a study by Hart's Global Biofuels Center (a division of Hart Energy Publishing LP, one of the world's largest energy industry publishers), the Global biofuel use may double from 2009 to 2015 (Johnson, 2009).

Bio-ethanol can be obtained from a variety of feedstocks using cellulosic, starchy and sugar sources. These feedstocks include sugar beet, corn, sugar cane, bagasse, sorghum, switch grass, barley, hemp, potatoes, wheat, wood, paper, straw, cotton and other biomass. When ethanol fuel is produced from lignocellulosic materials such as wood, herbaceous plants, wild tubers, agricultural and forestry wastes, its use provides new markets for depressed farm economies. Although each source of biomass represents a technological challenge, the diversity of raw materials will allow the decentralization of fuel production with geopolitical, economic and social benefits (Bon and Ferrara, 2011). A lot of agricultural raw materials rich in fermentable carbohydrates are being used worldwide for bio-ethanol production, but the costs of these raw materials have become a limiting factor for large scale production. Since the price of feedstock contributes more than 55% to the production cost, inexpensive feedstock such as lignocelluloses biomass and agric-food waste, are being considered to make bio ethanol competitive in the open market (Petersson, *et al.*, 2009). In addition, the use of food materials will put pressure on the cost with attendant food scarcity. Therefore there is the need for sourcing of ethanol from non-food materials. These lignocelluloses biomass and agric-food wastes can be used as potential feed stock for bio-ethanol production and could also be an attractive alternative for disposal of the polluting residues (Pramanik and Rao, 2005). However most of the wastes are not as viable as expected and therefore needs to be optimized for large production.

Processing of lignocellulosics to ethanol involves pretreatment, hydrolysis, fermentation and separation/purification. Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its sub–microscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved and more rapidly with a greater yield (Mosier, *etal.*, 2005). It is also necessary for degrading the lignin, decrease cellulose crystallinity, and

increase the surface area for enzymatic activity (Krishnaand Chowdar, 2000). There are several pre-treatment methods, using acid, steam and liquid hot water (LHW). However, LHW has the major advantage that the solubilized hemicellulose and lignin products are present in lower concentrations, when compared to steam pretreatment, due to higher water input. Due to these lower concentrations, the risk on degradation products like furfural and the condensation and precipitation of lignin compounds is reduced.

Sugar cane is starchy food crops and is expected to have high sugar content. However, it is not the case with its wastes such as the sugar cane bagasse. Hence, the present study was carried out to evaluate the production of ethanol from sugar cane bagasse using co-immobilized yeast cells (*Saccharomyces cerevisieae* Y300) and crude cellulase enzyme extracted from mushroom, *Schizophylum commenfr*.

Materials and Method

Collection of Samples

The various mushrooms *spp.* used in this work are *Agrocybebroadwayi, Schizophylum commenfr spp.* and *termitomyces* and Sugar cane bagasse used in this work were collected from Science Laboratory Technology garden, Federal Polytechnic, Bida and identified according to their habitat at the Biology Unit. Bones were obtained from New Market Area Bida, Niger State and activated in the Biochemistry Laboratory, Federal Polytechnic, Bida.

Pre-treatment of Bagasse

The pretreatment was done according to John Sendelius (2005). The bagasse was soaked in warm water for 20minutes and thoroughly washed with 0.1M NaOH after which it was finally rinsed with distilled water to remove any microorganism impurities, hemicelluloses and traces of cane juice that may be present in the bagasse. The water collected from final rinsing of the bagasse was tested using a refractometer to confirm any sugar in the bagasse. The bagasse was sundried, milled and sieved using a mesh size of 300μm in an automatic Tyler Shaker. It was then packed and stored in an air tight container.

Extraction of Cellulase from Mushroom

The mushrooms collected were blended with mortar and pestle in citrate buffer of pH 5. The mixture was centrifuged for 10 minutes and the clear supernatant was collected and stored below a temperature of 0^0C for further use.

Cellulase Enzyme Assay

Cellulase enzyme assay was done according to the method described by Resse and Mendels (1963). To determine the cellulase enzyme activity extracted from the three mushroom *spp.*, 1ml of appropriately diluted enzyme was incubated with 9ml of 5mg/ml Carboxymethyl-cellulose (CMC) for 60 minutes at 45^0C. After incubation, 3ml of 3, 5-dinitrosalicylic acid reagent (DNSA), was added. It was then boiled for 3 minutes and the absorbance was read using the UV spectrophotometer at wavelength of 550nm. The mushroom with highest cellulase enzyme activity was kept for further applications.

Hydrolysis of Sugar cane Bagasse with Cellulase Enzyme

To determine the concentration of sugar cane bagasse that produce the highest glucose yield, a serial concentration 20, 30, 40, 50 and60mg/ml of bagasse in citrate buffer solution (pH 5.0) was prepared. To 2ml of the slurry was added 1ml of cellulase enzyme solution, mixed thoroughly and placed in a water bath at 45^0C for 15minutes. Take 1ml of the aliquot and add 3ml of DNSA solution to stop the reaction in boiling water for 3minutes. Allow cooling and take absorbance at 550nm using UV Spectrophotometer.

Culturing and Inoculation of Yeast Cells

The yeast cells (*Saccharomyces cerevisiae* Y300) were cultured by preparing a yeast extract. Two 5g of yeast extract agar was measured separately and each was dissolved in 250ml distilled water contained in a conical flask and 1.25g of glucose was dissolved in the solution. The solution was then autoclaved for 1 hour at 121^0C to sterilize the solution which served as the media for the growth of the cells. Ten petri-dishes were arranged in the canister of the autoclave and sterilized at 121^0C for 1hour.

After cooling, the petri-dishes were removed from the canister and the extracts were dispensed into two different bijou bottles in an aseptic environment. A wire loop was used to inoculate the yeast in each bijou bottle. After inoculation, they were kept in the incubator at 27-30^0C for 5days.

Preparation of Activated Bone (Carrier) for immobilization

The activated bone was prepared according to Findlay, (1991). The bones obtained were rinsed with distilled water, and then dried in an oven for 4 hours. The bones were milled coarsely, washed with 1% NaOH, and then charged in a furnace at 800^{0}C until it became whitish. The whitish residue was sieved in a shaker of 300µm to obtain fine particles. The bone particles were washed again with concentrated NaOH for 20minutes after which the bone particles was rinsed several times with distilled water. The water finally used in rinsing the bone particle was tested with litmus paper to confirm the neutrality. The bone particle was then activated in a furnace at 1000^{0}C for 30minutes and was preserved in a desiccator.

Immobilization and Application Techniques

Enzyme Immobilization with hydrazine

10g of bone was put in a conical flask containing 15ml of 0.05% hydrazine and stir. It was washed with 0.1mM of NaCl solution and placed at 0^{0}C in a beaker containing 10ml of 0.6N HCl and 1M sodium nitrile mixture for 5 minutes. The bone was then rinsed again with 250ml of 0.1M NaCl and was suspended in 15ml of acetate buffer (pH 5.0) containing the crude enzyme at 0^{0}C for 3hours. Immobilized enzyme activity was afterward determined.

Enzyme Immobilization without hydrazine

50g of activated bone was put into a beaker containing 20ml of the crude cellulase enzyme and mix thoroughly. It was allowed to stand for 4hours after which the immobilized enzyme was removed from the mixture. The activity of the crude cellulase enzyme solution after the immobilized enzyme has been harvested was determined in order to know the amount of enzyme entrapped. Activity of the immobilized cellulase enzyme was also determined.

Glucose Yield with Immobilized and free Enzyme

50ml of (30mg/ml) Sugar cane bagasse was put into a petri dish containing 5g of immobilized enzyme. The reaction was allowed to proceed for 2,4,6,8 and 10 hours and glucose yield was determined at each time interval. Repeat for free enzyme.

Different amounts of immobilized and free enzyme (1, 2, 3, 4 and 5g) were weighed separately into 50ml of 30mg/ml sugar cane bagasse respectively and were allowed to react for 1hour and glucose yield was determined. Different concentrations of sugar cane bagasse (10, 20, 30, 40 and 50mg/ml) were prepared respectively and 5g of immobilized and free enzyme were added separately to each. The reaction was allowed for 1hour and the glucose yield was determined.

Simultaneous Hydrolysis and fermentation

5g of immobilized cellulase enzyme was added to 5g of immobilized yeast and 50ml of 30mg/ml sugar cane bagasse was introduced into reaction mixture. Bio-ethanol yield was monitored by taking samples at 12 hours interval for 48hours (4 times). This was repeated for free yeast cell and immobilized enzyme.

Calibration of Glucose Standard Curve

An aliquot of 2mg/ml of glucose was used to prepare serial dilution of five samples with different concentrations of 0.2, 0.4, 0.6, 0.8 and 1mg/ml. Then 3ml of DNSA was added to each sample and allowed to boil for 15 minutes. The samples were read using UV Spectrophotometer at 550nm wavelength. A glucose standard curve obtained was used to determine glucose yield of sugar cane bagasse.

Calibration of Ethanol Standard Curve

A standard ethanol curve was used in determining the concentration of ethanol yield. The standard ethanol curve was obtained using the dichlorate colorimetric method reported by Williams and Reese (1950). Potassium dichlorate reagent solution was prepared by weighing 1g of potassium dichromate and make up to 100ml by adding concentrated 6N sulphuric acid and shaken to dissolve. Ethanol of various concentrations (0, 1,2,3,4 and 5ml) was pipette into 3ml of glucose solution and covered with paraffin film to avoid loss of liquid due to evaporation. An aliquot of 3ml dichlorate reagent was added to each and the mixture was heated at 90^{0}C for 5-15minutes to develop the red-brown coloration, after which 1M of 40% potassium sodium tartarate (Rochelle salt) solution was added to stabilize the color. It was then cooled to room temperature in a cold water bath and the absorbance was read at 575nm wavelength.

Statistical Analysis

The data generated were analyzed with student's t test at p=0.05 significant level.

Results and Discussion

Cellulase enzyme activity and glucose yield

Result of cellulase enzyme activity and the corresponding glucose yield is presented in Table 1. Glucose yield was highest and lowest in *Schizophylum communefr spp.* (88%) and *Agrocybebroadwayi* (58.9%) respectively.

Table 1: Cellulase enzyme activity and glucose yield

Mushroom species	Absorbance (nm)	Glucose (%)
Agrocybebroadwayi	0.232	58.9
Schizophylum commenfr spp.	0.347	88.0
Termitomyces	0.307	78.0

Hydrolysis of Sugar cane Bagasse with Cellulase Enzyme

Table 3 show the result of hydrolysis of sugar cane bagasse in the presence of cellulose. It was observed that glucose yield increased with increase in sugar cane bagasse concentration, as the lowest bagasse concentration 20mg/mlyielded17.60mg/ml of glucose and the highest bagasse concentration 60mg/ml produced 44.60mg/ml.

Table 3: Enzymatic hydrolysis of bagasse

Bagasse concentration (mg/ml)	Absorbance (nm)	Glucose Yield (mg/ml)
20	0.116	17.60
30	0.138	22.60
40	0.178	31.70
50	0.192	34.95
60	0.196	44.60

Immobilization efficiency of activated bone

Immobilization of cellulase enzyme with activated bone with or without hydrazine is presented in Table 2. The result showed that, immobilization without hydrazine (25.45%) retained higher enzyme activity and efficiency than with hydrazine (17.5%).

Table 2: Immobilization efficiency of Activated bone

Activated bone	Efficiency (%)
Without hydrazine	25.45
With hydrazine	17.50

Hydrolysis of bagasse using free and immobilized enzyme

Figure 1 represents glucose yield from bagasse hydrolyzed with free and immobilized cellulase enzyme. It showed that there was higher bagasse hydrolysis and glucose yield with free enzyme compared to the immobilized. However, immobilized enzyme was still consistent at the tenth hour than the free enzyme.

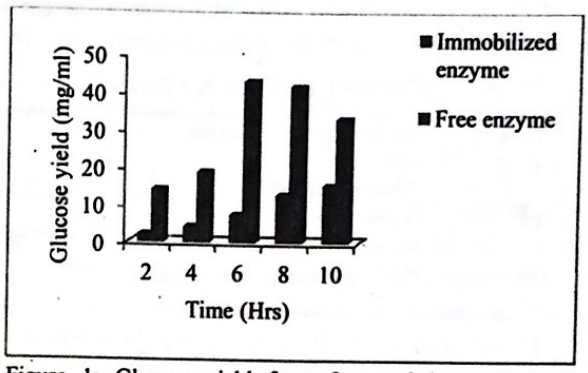

Figure 1: Glucose yield from free and immobilized enzyme

Glucose yield at different amount of immobilized enzyme

Glucose yield at different concentration of immobilized cellulase enzyme is presented in Figure 2. The result showed that there was steady increase in glucose yield as higher concentration of immobilized enzyme was introduced into the reaction mixture.

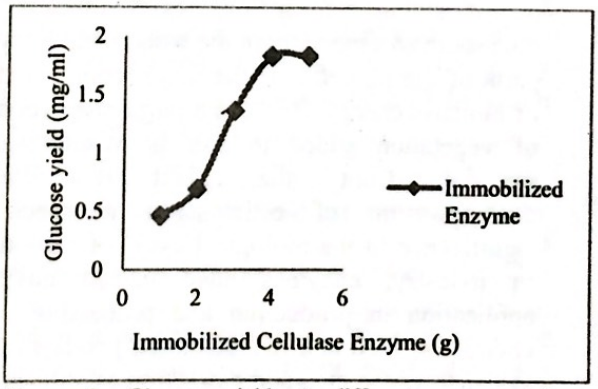

Figure 2: Glucose yield at different amount of immobilized enzyme

Glucose yield at different bagasse concentrations

The result of glucose yield at higher concentration of bagasse is shown in Figure 3. From the result, there was an increase in glucose yield as more sugar cane bagasse was supplied into the reaction medium.

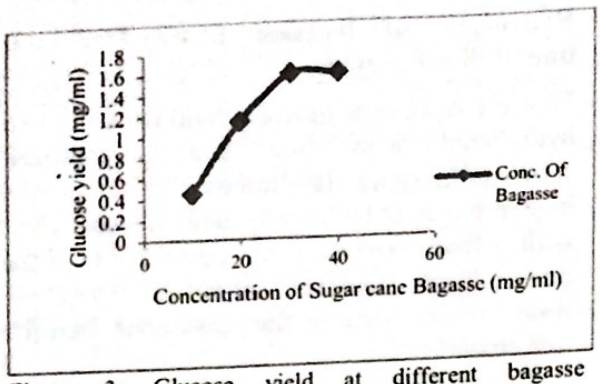

Figure 3: Glucose yield at different bagasse concentrations

Simultaneous hydrolysis and fermentation (SHF)

The result of ethanol yield from hydrolyzed bagasse with co-immobilized enzyme and yeast cells and immobilized enzyme and free yeast is shown in Table 4. From the result, there was an increase in ethanol yield within the time interval of 12-48 hours. However, ethanol yield with co-immobilization of enzyme and yeast was higher than immobilized enzyme and free yeast cells.

Table 4: Ethanol yield with co-immobilized enzyme and yeast cells and free yeast

Time (hrs)	Co-immobilized enzyme and yeast		Immobilized enzyme and free yeast	
	Absorbance (nm)	Ethanol yield (%)	Absorbance (nm)	Ethanol yield (%)
12	0.250	38	0.183	28
24	0.290	44	0.207	31
36	0.305	46	0.286	43
48	0.319	48	0.290	44

Discussion

This study was designed to investigate the possibility of producing ethanol from sugar cane bagasse using co-immobilized yeast cells (*Saccharomyces cerevisieae* Y300) and crude cellulase enzyme extracted from mushroom, *Schizophylum commenfr*. Sugar cane bagasse is a very useful feedstock for bioethanol production because it's rich in cellulose up to about 55%. Cellulose is a very abundant polysaccharide in the world and exists as a major storage form of photosynthesized glucose most especially in the woody and leafy parts of the plants. It is the major component of biomass energy. Because a large proportion of vegetation added to soil is ofcellulosic material from the plant therefore, decomposition of cellulosehas a special significance in the biological cycle of carbon. In industry, enzymes have gained novel application in production and processing of chemicals, food and manufactured goods such as paper, rayon etc., and extraction of valuable components from plants and improvement of nutritional values of animal feed (Sadaf *et. al.*, 2005).

The process of cellulose decomposition in the soil has been significantly aided by fungi of all genus and specie as described by Lynd *et. al.*, (2002). Among the majority of cellulase producing fungi, *Aspergillus, Fusarium, Alternaria, Rhizopus, Penicilluma* nd *Trichoderma* isolates were found to possess cellulytic activity in a respective order (Sadaf *et. al.*, 2005). *Aspergillus* produces wide range of enzymes capable of degrading plant cell wall polysaccharide. A wide range of *Aspergillus* species have been identified to possess all component of cellulase enzyme system (Vries and Visser, 2001). The most common and most potent cellulase producers are *Trichodermaressei, T. koningii, Fusarium sp., Aspergillus and Penicillium sp.*, (Yalpani, 1987). However, this study reported results obtained for three mushrooms which are *Agrocybebroadwayi, Schizophylum commenfr spp.* and *termitomyces*. Mushrooms are higher fungi capable of producing cellulase like the lower ones which have been studied extensively.In this study cellulase activity of *Schizophylumcommenfrspp* was found relatively high and *Agrocybebroadwayi*was of moderate range while *termitomyces* showed low cellulase activity. The cellulase enzyme activity had a corresponding glucose yield as sugar cane bagasse was hydrolyzed.

Result of bagasse hydrolysis in Table 3 described the fact that cellulase enzyme is not saturated at 60mg/ml. If the kinetic study of the enzyme wasconsidered, the result clearly showed that the enzyme would require high amount of bagasse at the optimum and it can therefore be said to have a high *Km*. However, further purification could remove impurities and higher sensitivity to substrate would be observed.

Principles of immobilization using natural carrier is based on physical adsorption or adhesion (Yu *et. al.*, 2007). Enzyme Immobilization process seems to offer mainly

the economic advantages.Many commercial carriers such as chitosan, alginate, silica gel etc are available for enzyme immobilization. In this work, activated bone was used with or without hydrazine. From the result, the presence of hydrazine reduced enzyme activity with an indication that hydrazine might have reduced attachment of enzyme molecules to the carrier. The difference in bagasse hydrolysis and the glucose yield using immobilized enzyme and free enzyme as observed in this study is not farfetched. Generally, free enzyme possesses higher activity than immobilized. This is owing to the fact that, when free enzyme interacts with substrate, all the enzyme molecules are involved. In the case of immobilized enzyme, series of conditions reduce enzyme activity. Among these are; not all enzyme molecules gets attached during immobilization, there are steric hindrances created by carriers and they usually prevent the substrate from having contact with the enzyme active site of and the flow back of the product,after catalysis, into the reaction medium(Anita, *et al.*, 2010). These and many other reasons reduce immobilized enzyme activity. However, with immobilized enzyme there is room for reuse which is not possible with free enzyme as they are discarded with spent reaction.

Besides, as amount of immobilized enzyme increased higher hydrolysis was observed. This was due to enzyme to substrate ratio. The principle of enzyme-catalyzed reaction according to Michaelis–Menten states that; the mechanism of enzyme-catalyzed reaction changes in response to changes in concentration of substrate in the reaction medium.So, introduction of higher amount of cellulase per sugar cane bagasse (vice versa) will lead to higher hydrolysis and glucose yield.

From the result of ethanol production with co-immobilization of cellulase and yeast, higher ethanol production was achieved. Amutha and Gunasekaran (2001) investigated the use of co-immobilized yeast cells to ferment cassava starch to ethanol. It was shown that co-immobilized yeast cells of *Zymomonasmobilis* and *Saccharomycesdiastaticus* could retain their activity during a continuous fermentation cycle of cassava and a final ethanol yield of approximately 0.3 $g.g^{-1}$could be obtained.Additionally, the production of ethanol byco-immobilization is much better than free cells because the immobilization system protects the cells from inhibition by ethanol when biofilmsare formed, enzyme maintains higher optimum temperature and pH when immobilized, half-life of an enzyme is increased with immobilization and immobilized enzyme can be reused several times before the enzyme activity is brought low (Yao *et. al.*, 2011).Studies carried out on co-immobilization have also shown that there was increase in the yield of ethanol as a result of co-immobilization (Uma *et al.*, 2010; Bretheuer*et et. al.*, 2010; Ivonova*et et. al.*, 2011; Evrim and Filiz, 2012).

Conclusion

In this study, simultaneous hydrolysis and fermentation of sugar cane bagasse to ethanol with co-immobilized crude cellulase enzyme extracted from *Schizophylum commenf rspp.* (mushroom) and yeast cells was carried out.It was observed that production of ethanol from hydrolyzed sugarcane bagasse by co-immobilized cellulase enzymeand yeast cells effectively increased ethanol yield. Therefore, sugarcane bagasse can be regarded as an alternative feedstock for bioethanol production and co-immobilization method could in no mean way meet industrial application.Notwithstanding, immobilization with physical adsorption does not really provide necessary anchorage for enzyme molecules and therefore may not meet industrial standard of immobilization as many enzyme molecules may not be able to attach thereby reducing activity.

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