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Immobilization of cellulase and yeast for the hydrolysis and fermentation of pre-treated bagasse for ethanol production

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Abstract

Lignocellulose ethanol promises to be the cheapest form of fuel, however, the drawback in the production is in the pretreatment process to remove lignin and the efficient hydrolysis of free cellulose. This research work is designed to delignify sugarcane bagasse, hydrolyze and ferment it with immobilized cellulase from the snail gut isolates and yeast respectively. The biomass were pretreated with $Ca(OH)_2$ and then placed in the water-bath with temperature of 20^{0}C, 40^{0}C, 60^{0}C, 80^{0}C,100^{0}C and 120^{0}C. The pretreated biomass was hydrolysed with free and immobilized cellulase at 50^{0}C for 5-48hrs. The activity, optimum pH, optimum temperature, substrate concentration profile and kinetic parameters, V_{max} and K_m of cellulase were also determined. The optimum pH for free and immobilized cellulase ranged from 4.0-5.5 and optimum temperature was recorded at 45^{0}C and 55^{0}C for free and immobilized cellulase respectively. The effect of temperature on both free and immobilized cellulases showed that immobilized cellulase has higher resistance to temperature than the free cellulase. Also the yield of glucose (40mg/ml) was higher with immobilized enzyme after 24hrs. The results obtained has also shown that immobilized cellulase has a higher Km when compared with free cellulase The maximum reaction rate (V_{max}) obtained from Michaelis Menten plots was lower for immobilized cellulase than for the free enzyme. Higher value of V_{max} for free enzyme indicated that the enzyme converted more substrate to product per unit time upon saturation with substrate. The biomass was fermented for 48hrs with immobilized *Saccharomyces cerevisiae* and the results showed the ethanol yield of 31.75% at 24hrs and 70.84% at 48hrs. The initial glucose concentration was 40mg/ml and this significantly reduced to 6.21mg/ml after 24hrs and 1.25mg/ml after 48hrs of the fermentation process. These results showed a proportional increase in ethanol yield against a depleting concentration of glucose which is being used up in the fermentation reaction revealing the maximum efficiency of the immobilized yeast cells. In this study, it has shown that the entrapped cellulase cells produced high levels of reducing sugars in hydrolysis compared with their native counterparts and immobilized yeast cells also gave a high yield of ethanol. The immobilization process therefore obtained more thermostable biocatalysts with increased productivity which is more economical for biofuel production.

Key words: *Immobilization, Cellulase, Saccharomyces cerevisiae,* biocatalysts, delignification

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Introduction

Bioethanol is becoming one of the main actors in the fuel market. The depletion of fossil fuel reserves and the unstable petrol price have led to intensive search for renewable energy sources such as solar energy, geothermal energy and also energy from biomass. One of the commonest sources of bioethanol production are materials such as sugar cane, corn and wheat which have high sugar and starch contents. Ethanol made from sugar cane biomass (bagasse & straw) as well as other lignocelluloses materials has one unprecedented feature as it could generate viable and sustainable energy sources, which are ecofriendly, safe and very clean**.** It may be a potential replacement to fossil fuels, therefore, lignocellulose biomass can act as a cheap substrate with constant supply as a substrate for bioconversion to fuel ethanol Cellulose, hemicellulose and lignin are the key biomass polymers found in sugarcane bagasse consisting about 50, 27.5 and 9.8% respectively (Girio et al., 2010). The rest 11.3 % are cell contents of sugarcane. Natural cellulose is a crystalline and linear polymer of thousands of D-glucose residues linked by β-1,4-glycosidic bonds, considered the most abundant and renewable biomass resource and a formidable reserve of raw material (Quiroz-Castañeda and Folch-Mallol,2013).Structural features of cellulose such as the degree of crystallinity, the degree of polymerization, the degree of water swelling, and the surface area, limit accessibility of substrate to enzyme and have been demonstrated to affect the rate of enzymatic hydrolysis of cellulose. Pretreating lignocellulosic biomass to disrupt the lignin-carbohydrate complex, to decrease native cellulose crystallinity (cellulose I), and to partially remove lignin and hemicellulose, has been shown to significantly enhance the subsequent hydrolysis of cellulose (Kumar et al., 2009). Immobilization allows one to re-use the enzyme for an extended period of time and enables easier separation of the catalyst from the product. Additionally, immobilization improves many properties of enzymes such as performance in organic solvents, pH tolerance, heat stability or the functional stability. Increasing the structural rigidity of the protein and stabilization of multimeric enzymes which prevents dissociation-related inactivation (Guzik et al., 2014). In this study cellulase enzyme from snail gut isolates and Saccharomyce cerevisea were immobilized on chitosan beads for the hydrolysis and production of ethanol from microwave-alkaline pretreated bagasse.

Materials and Methods

Drying and Pretreatment of Samples: The sugarcane bagasse was purchased from National Cereal Research Institute Bida, Niger State Minna. It was dried and milled to a size less than 1 mm. The milled samples were stored at room temperature. The biomass were pretreated with $Ca(OH)_2$ and then placed in the water-bath with temperature range of 20^0C, 40^0C, 60^0C, 80^0C, 100^0C and 120^0C**.** Giant snail (*Achatina Maginata*), from which cellulase was isolated, was bought from Kure Central Market Minna.

Microorganisms

Aspergillus niger isolated from snail gut was used for the production of cellulase enzyme. The microorganism was placed in a basal medium just like the modified medium of Deacon (1985) containing (in gram per liter), 2.0g/L yeast extract, 5.0g/L $NaNO_3$, 1.0g/L KH_3PO_4, 0.5g/L $MgSO_3.7H_2O$, and 0.001g/L $FeCl_3$. To the culture mixture, an addition of 1% Carboxyl methyl cellulose was made. The culture mixture was then allowed to grow for a week (seven days) at room temperature. By filtering the culture mixture with Whatman no 1 filter paper, the filtrate was then used as the enzyme solution (Singh et al., 1988).

Immobilization of cellulase and assay: The immobilization procedure was carried out according to the method of Andriani et al., (2012) with slight modifications. The chitosan beads were added to cellulase solution with a given enzyme/beads ratio (2 ml/g beads) and the solution stirred with magnetic stirrer at 25^0C, 150 rpm for 2 hours and then placed in refrigerator for 24 hours at 4^0C. The supernatant was removed and the chitosan beads with adsorbed enzyme were separated and washed thrice with 100 mM Phosphate Citrate buffer (pH 7.0) to remove the unbound enzyme.

Assay reaction for the immobilized cellulase was set up using DNSA method according to the protocols described Shoemaker and Brown 1998. For assay, 100 mg immobilized cellulase was added into 3 ml of 1% Carboxyl methyl cellulose (CMC) dissolved in a citrate buffer (pH 4.0). The mixture was incubated at 40^0C for 20 mins and filtered. The reaction was terminated by adding 3ml of 3,5-dinitrosalicylic acid (DNSA) reagent, heated at a temperature 100^0C for 15 minutes and cooled to room temperature. The activities of the immobilized enzyme were determined using spectrophotometer and the absorbance was read at 540nm The enzyme activity was measured in

mmol glucose that was released in 1min per 1ml of the filtrate. The immobilized enzyme was characterized for optimum temperature, optimum pH and the enzyme loading time.

Optimum Temperature and pH:

The effect of temperature was studied by incubating the free cellulase enzyme and the immobilized enzyme (Im) each in 3ml of 1% CMC (dissolved in 0.05M citrate phosphate buffer pH 4.0).The mixtures were placed in a water bath at different temperature 30^{0}C, 40^{0}C, 50^{0}C, 60^{0}C and 70^{0}C for 20mins by the method of Akinsoye et al. (1995) and by following assay conditions according Dey et al. (2003) with minor modifications. The activities were measured as above.

The optimum pH for free enzyme was determined by incubating 1 ml in 3 ml 1% CMC at different pH values of the buffer ranging from 3 - 7. The solutions were incubated at 50^{0}C for 20 mins and the activities determined as above.

Kinectics of the immobilized as well as free enzyme was analyzed using Lineweaver-Burke and Michaelis- Menten plots. The enzyme kinetics experiment was performed by measuring the initial enzyme reaction velocity at different substrate concentration of starch in 0.05 M citrate buffer. The Lineweaver-Burke plot was used to establish the Michaelis constant (K_m) and maximum velocity (V_{max}) of the enzyme reaction.

Hydrolysis of Pretreated Bagasse Using Free and immobilized Cellulase

Hydrolysis was carried out by the free and immobilized enzyme separately in a 250 mL shake flask. Cellulase loading of 25ml/g pretreated bagasse was mixed and incubated at 50^{0}C at pH 4.7 in 0.05M Sodium citrate buffer. Samples were taken at 4 hours interval for 36hour to determine the reducing sugar content of the hydrolysis mixture using DNSA method. The absorbance was read at 540nm.

Fermentation Process to Produce Ethanol from the Pre-treated Bagasse

Separate Hydrolysis and Fermentation (SHF) was adopted. The hydrolysate of the pre-treated bagasse (500ml) obtained after 24hrs enzymatic hydrolysis was filtered and the supernatant was used as a sole carbon source in the fermentation medium. Immobilized yeast nutrients were added to have a basal medium composition of 5.0g/L yeast, extract 5.0g/L peptone, 5.0g/L NH_4 PO_4, 0.2g/L $MgSO_4$ and $7H_2O$. Suspension (2% V/V) of *S. Cerevisiae* (10 cells/ ml) was then inoculated to the broth to initiate the fermentation process and incubated at 30for 48hrs.

Results and Discussion

Pretreatment of bagasse: The results of the pretreatment of bagasse using alkaline method at different calcium hydroxide concentration and temperature presented in figure 4.1 showed that maximum lignin removal was obtained at 2% calcium hydroxide treatment at all the temperature of study. Highest lignin removal was recorded at 120^{0}C Temperature. It was found out that degree of temperature was inversely proportional to time in lignin removal. The efficiency of lignin removed by this alkaline pretreatment method was examined by estimating the amount of lignin present in the solution after pretreatment. Highest percent (96%) of lignin was removed from the sugar cane bagasse when treated with 2% $Ca(OH)_2$ at 120^{0}C given a total yield of 40mg/ml of reducing sugar. This result is in agreement with that obtained by Sharma et al. (2012) who demonstrated that the mild reaction of $Ca(OH)_2$ at low concentration prevent condensation of lignin leading to its high solubility and greater removal. Also, in the study carried out by Egwim et al. (2015), it was discovered that the highest biomass reduction (lignin removal) was observed with 8% NaOH at 80°C .As the pretreatment temperature is lowered, time required for efficient lignin removal also increased. The results showed that biomass pretreatment using alkaline assisted with moderate heat and humidity has great and beneficial future prospects. The cost of the alkali has been the hurdle in alkaline pretreatment; however, considering the efficiency, it is cost effective (Verma et al. 2011; Feng and Chen, 2008).

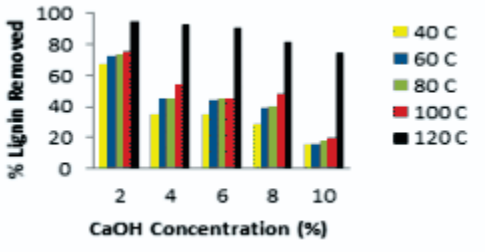

Figure 4.1 Pretreatment of bagasse using alkaline method

Optimum pH of Free and Immobilized Cellulase Enzyme: The result of relative activities of free and immobilized enzymes at different pH is presented in figure 4.2. The result showed that, the optimum pH for free cellulase is 4.5 and immobilized cellulase is 5.5. This result is in agreement with that obtained by Viet et al. (2013). They demonstrated that the optimum pH for free cellulase is 4.5 while the immobilized cellulase pH range from 5.0 to 7. Sheila et al. (2014) also demonstrated that for commercial cellulase immobilized on chitosan, both for free and immobilized cellulase proved stable at acidic pH medium. He reported that immobilized cellulase retained 50 to 60 % of its initial activity. These differences in the behaviors of free and immobilized enzyme processes could be explained by the poly-cationic nature of the chitosan used for enzyme support. They attract more OH- ions around the immobilized enzyme, thus making the pH of the enzyme's micro-environment more than the bulk solution which eventually leads to a shift in pH towards alkalinity. Immobilized enzyme therefore requires a higher pH for optimal activity than free enzyme (Feng et al., 2009).

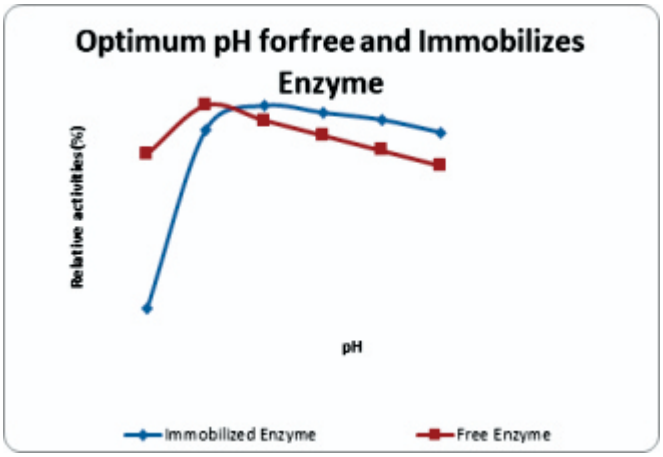

Figure 4.2 Optimum pH for free and immobilized cellulase enzyme from snail gut isolates

Optimum Temperature for Free and Immobilized Cellulase

The optimum temperature for free and immobilized cellulase is shown in figure 4.3. In this study, the optimum temperatures for free and immobilized cellulase were 45^{0}C and 55^{0}C respectively. This result however agrees with that observed by Viet et al. (2013). They reported that the activity of free enzyme decrease over a wider range of temperature when compared with the immobilized enzyme which has its optimum temperature at 55^{0}C. Similar findings are reported by Sheila et al. (2014) that for immobilized cellulase on chitosan beads, enhanced thermal stability was observed with results significantly better than those of the native enzyme at temperatures over 75^{0}C

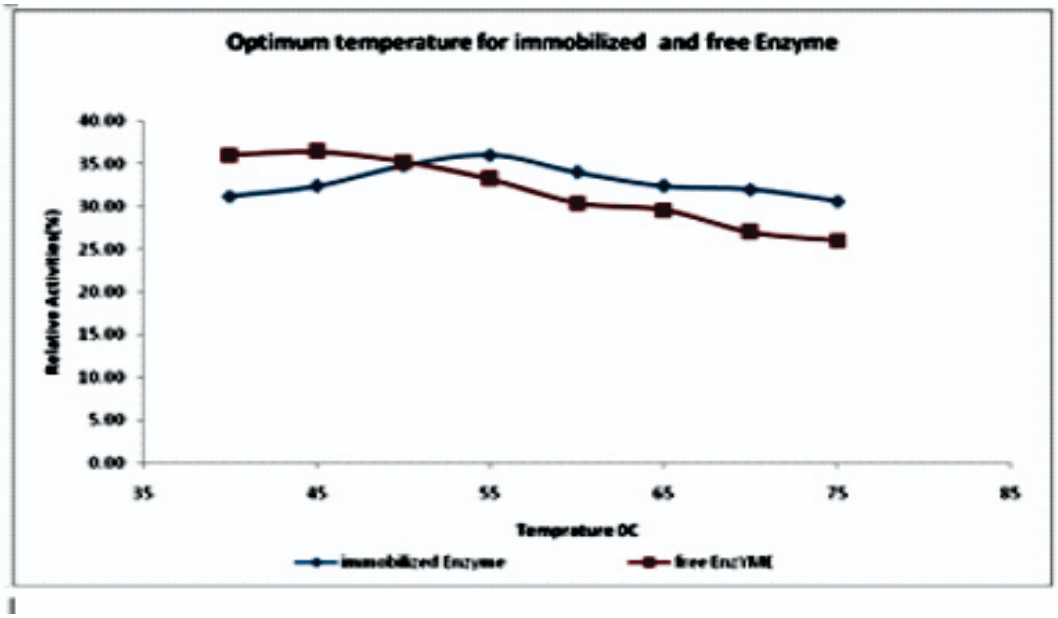

Figure 4.3 Optimum temperature for immobilized and free enzyme from snail gut isolates.

Cellulase Loading Efficiency on Chitosan Beads: The loading efficiency of enzyme on Chitosan support as shown in figure 4.4 indicated the highest efficiency was at a loading of 2 ml of enzyme/g of support. Increased loadings beyond 2 ml of enzyme/g of support led to progressive decrease in efficiency of the enzyme. This result is in accordance with those obtained in the study conducted by Pereira et al., (2003). They found out that the hydrolytic activity of immobilized enzyme increased as more enzyme was loaded onto the support until it reaches a particular threshold where reduction in activity was observed. The results suggested that instead of obtaining the desired crowded upright adsorption of enzyme onto the support surface multilayer adsorption occurred, possibly blocking access to enzyme active sites. Some previous experimental works that have been done were carried out using immobilized preparation at cellulase loadings of 2 ml enzyme g^{-1} of dry chitosan beads.

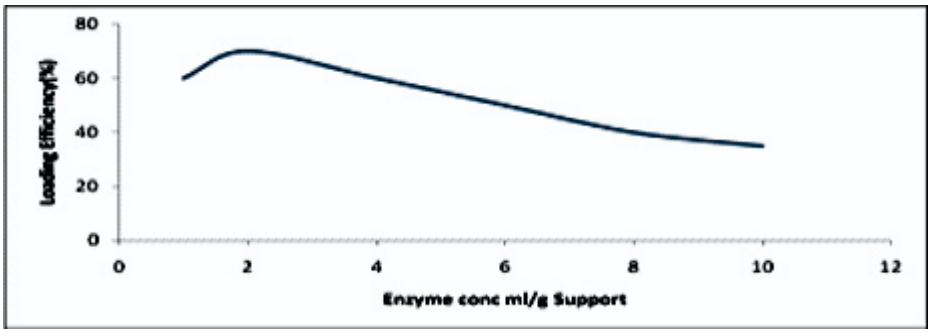

Fig. 4.4 Effect of cellulase loading on loading efficiency

Substrate Effect and Enzyme Kinetics (V_{max} and K_m): Kinectics of immobilized cellulase was determined at pH and temperature of 5.5 and 55^{0}C while for free enzyme at 4.0 and 45^{0}C respectively. The kinetic constant (K_m) and the maximum reaction rate (V_{max}) were obtained from Lineweaverburk plots as shown in figure 4.5. With regard to these parameters, immobilization prompted an increase in the value of K_m, which might be due to changes in the accessibility of the substrate to the active sites of the enzyme caused by diffusional limitations, steric effects and enzyme structural changes following immobilization (Monier et al.,2010). The results of this study showed a decrease in K_m for free cellulose which indicates faster reaction rate, whereas an increase of the K_m suggest the requirement of higher substrate concentration to achieve same reaction rate observed for the free enzyme Luz et al., (2014). These findings agree with that obtained by El-Masry et al. (2001). They stated that the immobilized enzyme decreased its affinity for substrate K_m= 8.1 and 1.84 mg/L for the immobilized enzyme and free enzyme respectively). This result was also in accordance with that reported by Romo-Sánchez et al. (2014). Maximum velocity, V_{max} which refers to the substrate decomposition rate (μmol·min−1·mg−1) unlike K_m values was smaller for immobilized cellulase and this was also reported by Kumar et al. (2011) and Romo-Sánchez et al. (2014). Higher value of V_{max} for free enzyme indicated that the enzyme converted more substrate to product per unit time upon saturation Kumal et al. (2012).

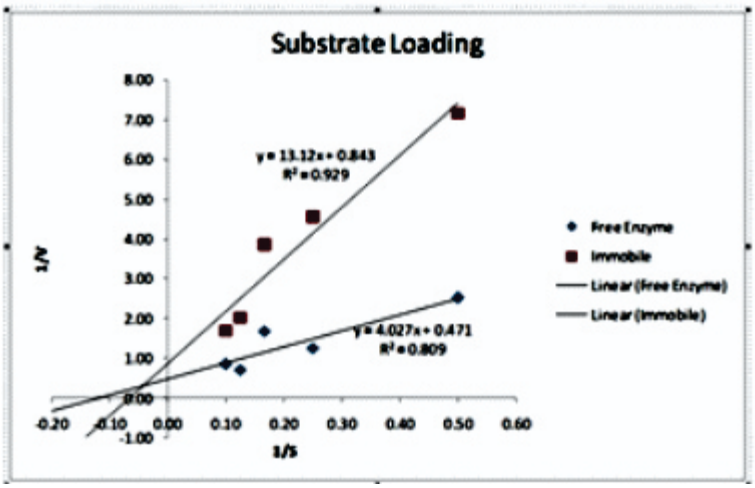

Fig 4.5 Lineweaver Burke plot to determine apparent K_m for immobilized and free enzyme

Hydrolysis of Bagasse Using Immobilized and Free Cellulase Enzyme: The result obtained showed that increase in cellulase loading time resulted in increase in the yield of glucose (mg/ml) in both immobilized and free enzymes (figure 4.6). After hydrolysis with immobilized cellulase, the reducing sugars yield reached more than 27 mg/ml in 20 hrs and up to 40 mg/ml within 24 hrs while only about 7mg/ml of glucose yield was obtained after 24hrs for its free counterpart. Increase in enzyme loading time beyond these hours for both immobilized and free cellulase enzymes had no further effect on percent glucose yield. The above result indicated that the available space on the immobilization carrier became saturated with prolonged loading time; hence the immobilized enzymes attained highest activity within 24 hrs of hydrolysis process.

These results showed that the hydrolysis of bagasse was highly effective within 24 hrs with immobilized enzyme when compared with the reducing sugar yield with free enzyme within the same hours. It is evident by these results that maximum glucose yield could be obtained within short time with immobilized cellulase which ultimately implies more ethanol yield from the available reducing sugars. This also was reported by Roth et al., (2013) after application of immobilized cellulase and yeast cells for ethanol production.

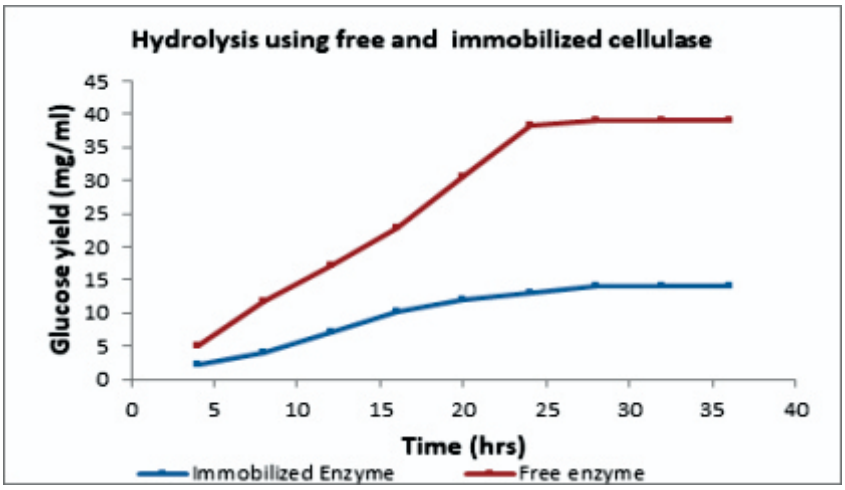

Figure 4.6 Effect of Cellulase Loading Time on Glucose Yield

Analysis of Ethanol Distillate.

Production of ethanol with immobilized *Saccharomyce cerevisiae* (yeast cell) was performed for 48 hrs. At zero hour, the initial reducing sugar concentration was 40 mg/ml and this significantly decreased to 6.21 mg/ml and 1.25 mg/ml after 24 hrs and 48 hrs of the fermentation process respectively as presented in figure 4.7. The ethanol yields reached 31.75% and 70.84% respectively at 24 hrs and 48 hrs of fermentation process with immobilized yeast cells as shown in figure 4.8. These results showed a proportional increase in ethanol yield against a depleting concentration of glucose which is been used up in the fermentation reaction (figure 4.9). The results presented at Figure 4.7 suggest that immobilized cells consume almost all available sugar during the first 24 hrs of fermentation. This was also in accordance with Janiszyn et al. (2007) who reported 38 g/L ethanol at 24 hrs. In another study it was observed by Rabelo et al., (2011) that after producing 83.20g/l and 132.39g/L ethanol concentration from molasses and thick juice using immobilized cells of *S. cerevisiae,* the immobilized yeast showed an important operational stability without any decrease of its activity. Maurice (2011) reported 61.48% and 62.77% ethanol yields at 24 hrs and 48 hrs respectively for fermentation process with free S. cerevisea with same concentration of reducing sugars (40 mg/ml). From the results of this study, it can be concluded that immobilized cellulase and yeast cells for hydrolysis and fermentation processes could attain maximum activity and ethanol yield within 24 hrs and 48 hrs of the whole process. Low fermentation times indicated that no period was needed for adaptation of biocatalyst in the fermentation environment considering the established alkaline nature of the chitosan carrier.

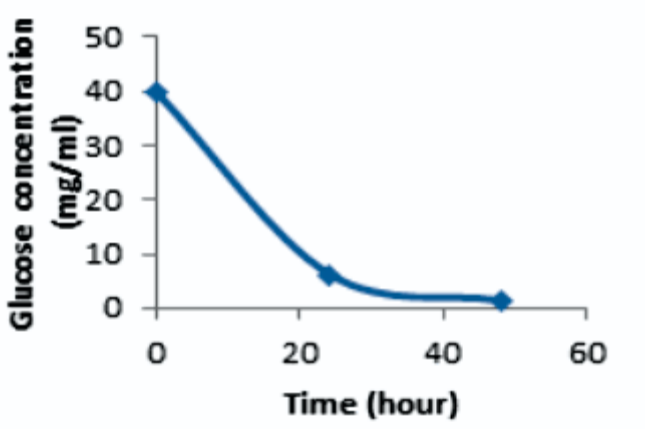

Fig 4.7 Effect of time on glucose concentration in fermentation process

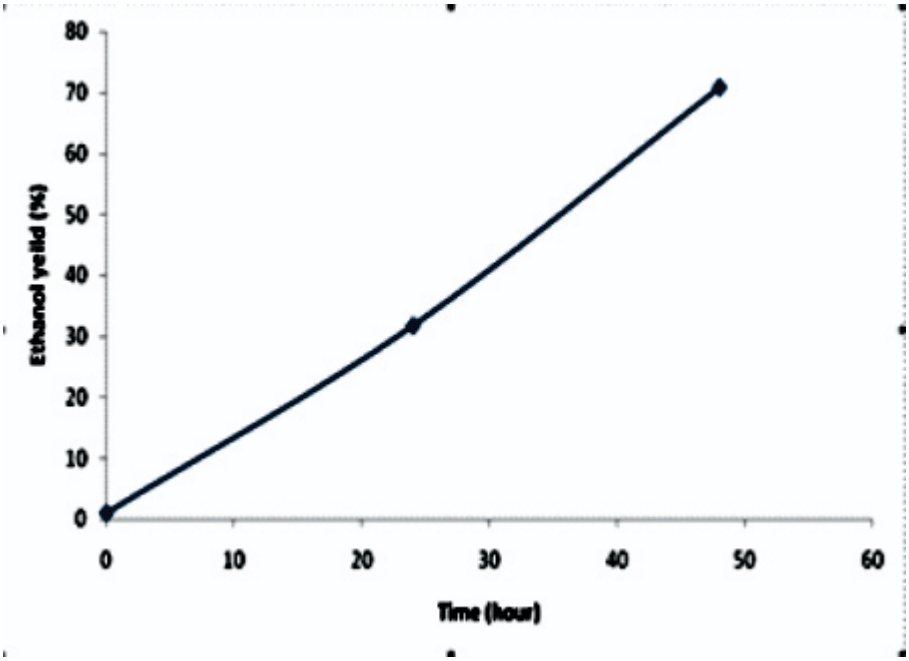

Fig 4.8 Effect of time on percentage ethanol yield in fermentation process

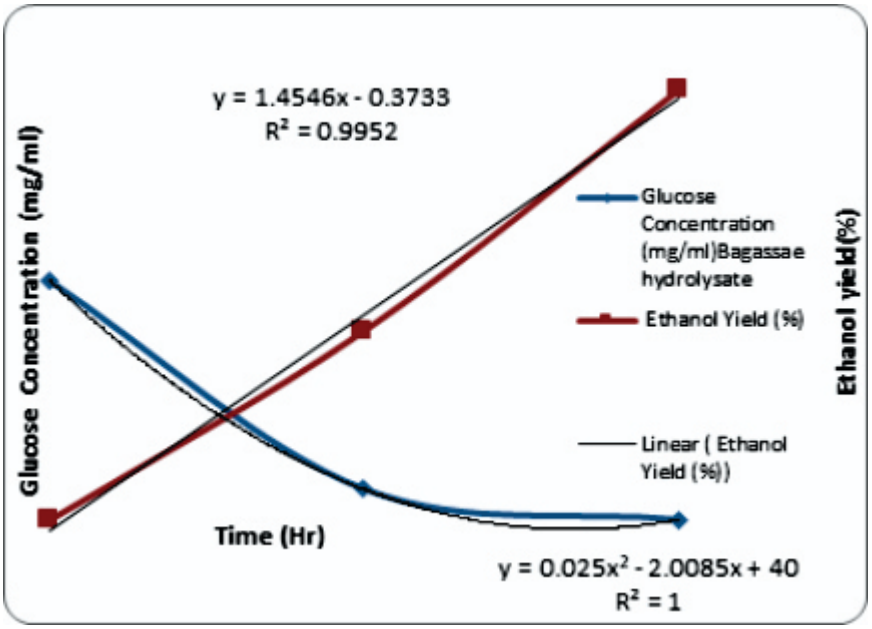

Fig. 4.9 Percentage yield of ethanol from bagasse hydrolysate

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