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Immobilization of Cellulase Enzyme on Zinc Ferrite Nanoparticles in Increasing Enzymatic Hydrolysis on Ultrasound-Assisted Alkaline Pretreated *Crotalaria Juncea* Biomass

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Abstract

Objectives: To synthesize ferrite nano particles; to measure size of the particles by FTIR and XRD studies and enzyme saccharification on ultra sound assisted alkaline pretreated biomass using free and immobilized enzyme. **Methods/ Statistical Analysis**: In the present work, Zinc ferrite particles were synthesized using co-precipitation method. Cellulase enzymes were immobilized on covalently activated ferrite nanoparticles via glutaraldehyde as a crosslinker. Biochemical characterization of free and immobilized enzyme activity were performed on CMC as a substrate. The efficiency of immobilized enzyme was evaluated based on its binding efficiency on the nanoparticles, thermostability, and reusability. Enzymatic hydrolysis was performed on ultrasound-assisted alkaline pretreated sunn hemp biomass using free and immobilized enzymes. **Findings:** Around 74% of binding was achieved at 4mg/ml of ferrite loading to enzyme concentration of 20 units. Comparative study on effects of pH and temperature was done on both free and immobilized enzyme and it was observed that the immobilized enzyme has maximum activity at pH 5 and temperature 60˚C. Also, the immobilized enzyme was stable at 60˚C while retaining its activity up to 3 recycles. The immobilized enzyme showed 53% hydrolysis yield on pretreated sunn hemp biomass. **Application/Improvements:** The research on the interaction between zinc ferrite and cellulase in immobilization and also the recovery of enzymes can determine an efficient approach for bioethanol production in industrial scale. The lab scale can be scaled-up to use at pilot and industrial scales.

Keywords: Crotalaria Juncea Biomass, Enzymatic Hydrolysis, Immobilization, Nanoparticles, Ultrasound

1. Introduction

Lignocelluloses materials have shown prominent attention as a raw material for biofuel production due to the immense amount of supply compared to other biomass. Lignocellulosic materials contain many different polysaccharides, phenolic polymers, and proteins with distinctive physical and chemical characteristics. Lignocellulosic polysaccharide polymers are cross-linked via ester and ether linkages with strong bonds leads to the highly complex structure. Due to highly complex structure, economically feasible production of bioethanol from lignocelluloses is really a challenging one.

Enzymatic hydrolysis of lignocelluloses materials to fermentable reducing sugars offers the possibility for higher yields, higher performance at lower energy costs with minimal operating conditions than chemical methods[1]. Cellulose refers to a class of enzymes catalyzes the hydrolysis of cellulose into glucose by attacking the glycoside linkages. This glucose is further fermented using microorganism and converted to ethanol[2]. Due to the complex structure of lignocelluloses biomass, enzyme quantity required for hydrolysis is one fold higher than for starch[3]. Immobilizing cellulose on solid materials is a viable way to overcome this difficulty by increasing the

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efficiency, stability to unflavored conditions, biochemical properties and reusability of the enzyme[4]. It has been found that several enzymes immobilized by different immobilization techniques have shown higher activity than free enzymes[5-10].

The application of magnetic materials in enzyme immobilization are attracting significant interest due to their efficiency, easy and fast enzyme recovery with the addition of external magnetic fields[11]. The binding of enzymes onto a nanosized magnetic particle provides a higher surface area, robust cross-linking through covalent bonds and better separation from the reaction mixture. Ferrite based materials have been investigated for a long time based on their electromagnetic properties, controllability of super magnetic and magnetic behaviors. To date application of Zinc ferrite in cellulose, enzyme immobilization is comparatively limited. Zinc ferrite has long been the topic of study due to its distinctive properties like chemical and also the thermal stability and the particle size dependent magnetic properties[12-13]. Glutaraldehyde has been used as a cross linker as it possesses unique characteristics that provide it one of the most effective proteins cross linking reagents. Glutaraldehyde activated nanoparticles support provides covalent bonding between carbonyl groups and amino groups of enzymes[14].

Crotalaria Juncea i.e., Sunn hemp a fast growing green manure fibrous legume crop with high cellulose, low lignin content and ability to produce high biomass in shorter time duration has shown huge application in the industrial sector. Due to the rigid structure of lignocelluloses, it makes the enzymes accessibility to cellulose difficult. The bioconversion of lignocelluloses to ethanol requires economical pretreatment and hydrolysis method for the production of fermentable sugars that ought to be free from inhibitors for the economical fermentation[15]. In the previous work, studies were conducted on the effect of alkaline and ultrasound assisted alkaline pretreatment at 121˚C on the chemical composition of sun hemp biomass, it was observed that combination Sonolysis with alkaline pretreatment at 121˚C showed an increase in cellulose yield then alkaline pretreatment.

2. Materials and Methods

Ethanol, Cellulase from Trichoderma reesei ATCC 26921 aqueous solution, ≥700 units/g from Sigma-Aldrich. Iron(III) chloride hexahydrate, iron(II) sulfate heptahydrate, zinc chloride, sodium hydroxide, carboxymethyl cellulose, 3,5-dinitrosalicylic acid, Potassium sodium tartrate were procured from Himedia, sodium acetate and Orthophosphoric acid from Finar, Glutaraldehyde (25 wt% solution in water) from Merck, Comassive brilliant blue G-250 from S.D fine chem. Limited, Mumbai, India

2.1 Synthesis of Ferrite Particles

Ferrite particles were synthesized using co-precipitation method using alkaline solution under hydrothermal conditions. $FeCl_3.6H_2O$, $FeSO_4.7H_2O$ and $ZnCl_2$ were mixed thoroughly on continuous stirring in a molar ratio of 2.0:0.6:0.4 at 80˚C[16]. An aqueous solution of sodium hydroxide was added to neutralize the pH under constant stirring. The precipitates were filtered and subjected to repeated washing with deionised water to remove accumulated sodium chloride impurity salts and ions. The precipitate was further washed with ethanol to remove traces of water. The precipitate was dried at 150˚C for 12hrs and stored for future work[17].

2.2 Immobilization of Cellulase on the Activated Ferrite Nanoparticle

The ferrite particles at 4mg/ml concentration were suspended in deionised water and sonicated for 1hr. After sonication, the suspension was centrifuged. To achieve support activation, the pellets were suspended in 1M glutaraldehyde for 1hr at 25˚C, 200 rpm. The activated support particles were subjected to washing with deionised water and 0.1M sodium acetate buffer (pH 5) to remove unbound glutaraldehyde[18]. The covalent binding between the enzymes and activated support particles was observed by incubating activated support with the enzyme at a concentration (20 units) of 4mg/ml for 2hrs at 25˚C, 200 rpm. The enzyme support mixture was centrifuged and the supernatant was used for protein quantification. The immobilized enzyme on the ferrite particle support was washed with buffer to eliminate unbound protein. Immobilized enzyme binding efficiency was estimated by Bradford assay at 595nmn using **Coomassie Brilliant Blue** G250[19] by calculating the ratio of total protein bound to total amount of protein added for immobilization.

$$\text{Efficiency of binding (\%)} = \frac{\text{Amount of protein taken} - \text{amount of protein available in supernatent}}{\text{Amount of protein taken}}$$

2.3 Characterization

2.3.1 XRD Analysis

The crystalline structure of the ferrite particles was characterized and recorded using Bruker D8 Advanced X-ray

Diffractometer. The x-rays were produced using Cu K-alpha radiation having a wavelength of 0.154 nm and recorded on the 2θ scale, ranging from 10 to 80° with a step size of 0.019°.

2.3.2 Particle Size Analyzer

The Particle Size analysis was performed using Malvern Zetasizer (Nano S90 version 7.02) by dissolving the solid particles/sample in solvents like water, ethanol, isopropanol etc. and characterized using dynamic light scattering method.

2.3.3 Fourier Transform Infrared Spectroscopy and SEM Analysis

Cellulase enzyme binding onto the ferrite nanoparticle support was recorded using Perkin Elmer 100S FTIR spectrophotometer. The samples pellet was obtained by adding nanocomposite to KBr. The scanning range was from 4,000 to 450 cm^{-1} wavenumber. Tescan Vega-3 LMU electron microscope was used to check the morphology of ferrite particles.

2.3.4 Enzyme Assay

The enzyme activity was determined by measuring glucose realize after a reaction of free and immobilized enzymes with CMC as substrate. Free enzyme and immobilised enzyme assay was performed at 50°C and 60˚C with a sample mixture containing 0.5 mL enzyme (20 units) and 0.5 mL of 2% substrate dissolved in 0.1 M sodium acetate buffer (pH 4 for free enzyme and pH 5 for immobilized enzyme) and incubated for 30 min[20]. DNS reagent was added to stop the enzyme substrate reaction and kept in boiling water bath for 10 min to allow color formation. The reducing sugar concentrations released during enzymatic hydrolysis were measured at 540 nm.

2.3.5 Thermo Stability Study

The thermostability of immobilized enzyme was studied with different temperature (30˚, 40˚, 50˚, 60˚, 70˚, 80˚C). Using DNS method, reducing sugar concentration was determined.

2.3.6 Reusability Assay

The reusability test of the immobilized enzyme was calculated by enzyme activity assay, subjecting immobilized enzyme to hydrolysis reaction with substrate solution at 60˚C for 30min using 0.1M sodium acetate buffer (pH 5). After every cycle of the assay, the immobilized enzyme particles were resuspended in buffer and substrate solution till the activity has fallen below 10%.

2.3.7 Sunn Hemp Pretreatment

Sunn hemp plant used in this study was cultivated near the Department of Biotechnology, National Institute of Technology Warangal, Telangana, and harvested between 9 to 12 weeks after plantation. The biomass was washed to remove soil residues and air dried to obtain a constant weight. In the previous study, we have observed that combination of sonication with alkaline pretreatment showed greater efficiency is biodegradability of lignocellulose material in lignin removal and increase in cellulose yield. In this present study for enzymatic hydrolysis, we have taken 0.25N NaOH treated biomass subjected to 5min and 10min sonication. Sonicated NaOH treated biomass were autoclaved at 121˚C for 20min. Pretreated biomass was recovered by filtration and washed with deionized water to take off excess alkali and dissolved byproducts and dried till it attains constant weight[21].

2.3.8 Enzymatic Hydrolysis of Biomass

Enzymatic hydrolysis was performed on raw biomass and pretreated sunn hemp biomass with free and immobilized enzymes. Hydrolysis experiments were conducted for 48hrs in 0.1M sodium acetate buffer pH 4 and pH 5 at 50˚C and 60˚C, respectively. Every 12hrs interval the samples were taken and estimated for reducing sugars using DNS method. Cellulose hydrolysis yield was calculated by using equation. The value 0.9 was used in the equation as a correction factor for hydration.

$$\text{Cellulose hydrolysis \%} = \frac{\text{reducing sugar concentration X total reaction volum}}{\text{Amount of cellulose added}}$$

3. Results and Discussion

3.1 Characterization of Ferrite Particles

Figure 1 shows the results of the X-ray diffraction (XRD) analysis of ferrite particles. The XRD patterns were characterized on the 2θ scale over the 10˚– 80° range. It was observed that the peaks appeared at 35.0°, 42.8°, 56.6 and 62° having lattice structures at (3 1 1), (4 0 0), (3 3 3) and (4 4 0) respectively showed the presence of formation

of zinc ferrite. All the peaks matched with the standard (JCPDS #82-1049) XRD patterns of zinc ferrite nanoparticles. The average ferrite particles size is shown in Figure 2. It was observed that the particle size after 1hr sonication was 121nm (0.1µm) with the size distribution in the range of 100-130nm. The size of the prepared ferrite particles was characterized by using dynamic light scattering method.

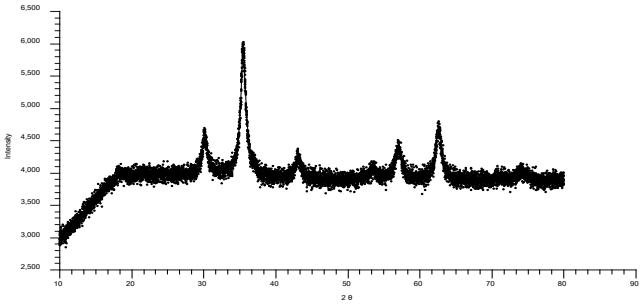


Figure 1. XRD diffraction pattern of zinc ferrite.

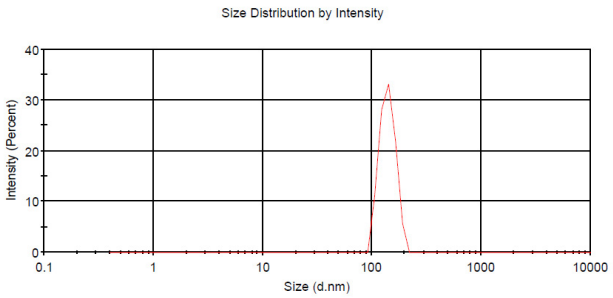


Figure 2. Particles size analysis of ferrite particles.

3.2 Binding Confirmation Studies

The binding of Cellulase enzyme on ferrite nanoparticles was validated by FTIR spectroscopy analysis method. Figure 3 shows the FTIR spectra of cellulase enzyme, ferrite nanoparticles, and enzyme immobilized on ferrite nanoparticles. The characteristic FTIR spectra frequencies at 1546 and 1649 on cellulase enzyme were also present on immobilized ferrite cellulase, therefore it indicates the confirmation of binding of cellulase onto magnetic nanoparticles. SEM analysis was performed to study the surface morphology of ferrite nanoparticles before and after cross-linking with cellulase. Dried Ferrite particles and immobilized enzyme samples were mounted on stubs and sputter coated with gold for 15min in a high vacuum and analyzed at a voltage acceleration 10 kV Figure 4 optimization for enzyme binding.

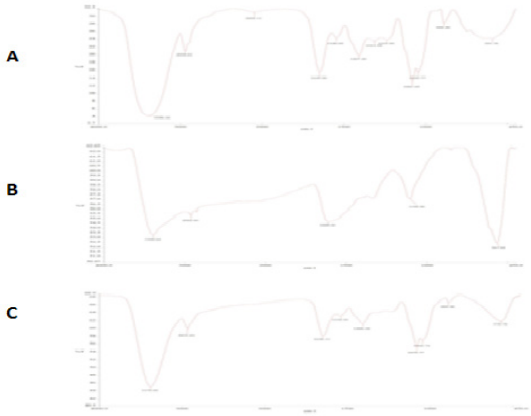


Figure 3. FTIR spectra (A) free enzyme, (B) ferrite particles (C) immobilized enzyme.

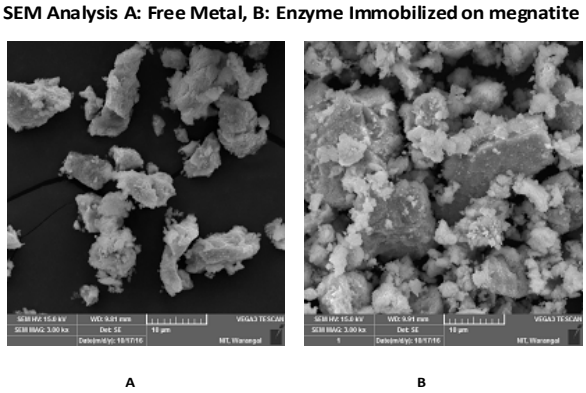


Figure 4. SEM analysis.

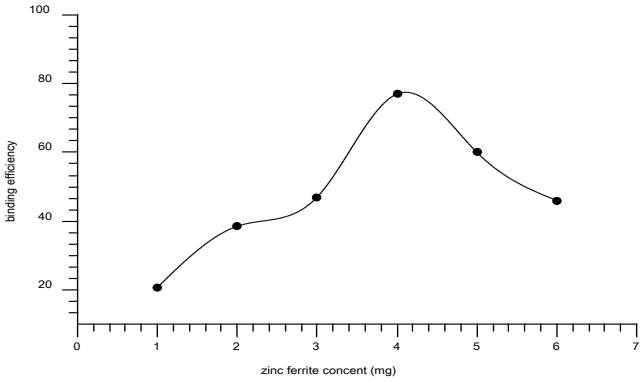


Figure 5. Effect of ferrite concentration on immobilization.

3.3 Effect of Ferrite Concentration

Figure 5 shows that the effect of ferrite concentration on immobilization was performed by varying the concentration of ferrite particles on the enzyme to be immobilized.

The optimum amount of 4mg/ml of ferrite particle loading showed 74% binding efficiency. It was observed that as the ferrite concentration increased the enzyme immobilization decreases due to a decrease in the covalent attachment of the enzyme to the activated support particles. At a lower concentration, there were not enough support particles at the medium for enzyme binding.

3.3. 1 Effect of Enzyme Concentration

Figure 6 shows the result of enzyme concentration in binding onto the surface of ferrite particles. It was observed that optimum binding enzyme concentration was 20 units with 74% binding efficiency. As the concentration of enzyme exceeds, the amount of enzyme immobilized per gram of support particles reaches saturation limit and the binding activity decreased due to the ferrite particles and cross linker concentration were not enough for immobilization. Enzyme molecules may interact by themselves causing steric hindrance by blocking active site of enzyme molecules and also the active side of enzyme molecules possibly restricted by random bonding of the support particles[22].

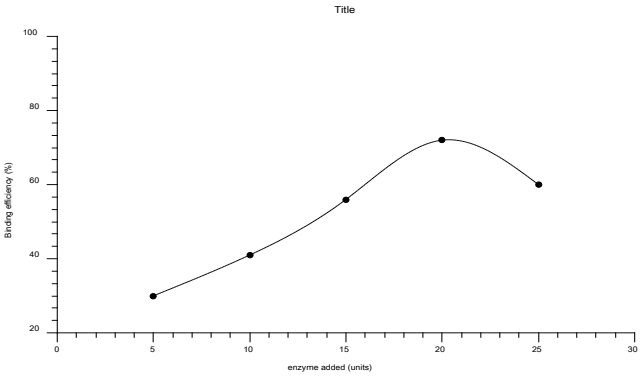


Figure 6. Effect of enzyme concentration.

3.3.2 Effect of Incubation Time and Temperature on Covalent Binding

Figure 7 shows the effect of time and temperature on covalent binding between enzyme and activated support particles. It was observed that after 1.5 to 2 hrs of incubation time at 25˚C showed favorable cross linking as compared to 35˚C. As the incubation temperature increased above 25˚C, the enzyme binding started leaching from activated support particle and resulted in poor binding.

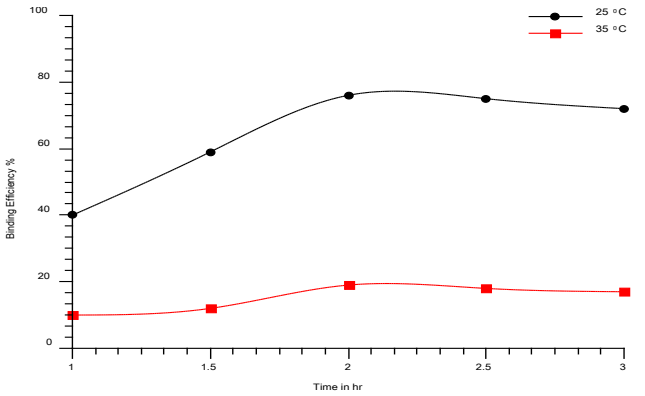


Figure 7. Effect of incubation time and temperature on covalent binding.

3.3.3 Effect of pH

Figure 8 shows the maximal activity of the immobilized enzyme at pH 5 where as for free enzyme it was pH 4. It was observed that as pH increased the activity of the both enzyme gradually decreased. A study conducted[23] by also reported that increase in net negative charge of the immobilized enzyme compared to optimum pH 4 for free enzyme. Changes in pH can affect active site of amino acid residues causing poor substrate binding.

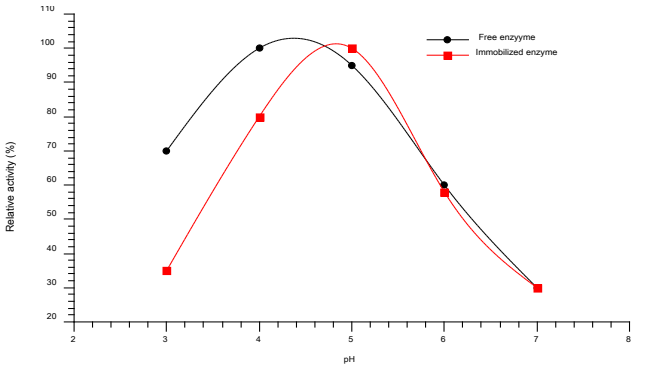


Figure 8. Effect of pH on free and immobilized enzyme.

3.3.4 Effect of Temperature

According to Figure 9, immobilized enzyme showed higher activity at 60˚C and retained its activity till 70˚C. When the temperature increased beyond the tolerable range, rate of the reaction increases and internal energy of the molecules will increase resulting in structural change and also undergoes physical modification on protein active site making them less well suited to bind substrate resulting in enzyme denaturation. Simultaneously free enzyme showed optimum activity at 50˚C.

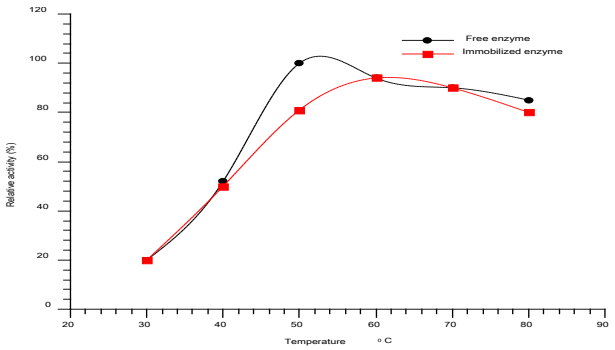


Figure 9. Effect of temperature.

3.3.5 Recyclability Test

Figure 10 shows that the recyclability of immobilized enzyme at 60˚C on CMC for 30min. It was observed that immobilized enzyme showed stable relative activity up to 3 times recycle before residual activity fallen to approximately 10%. It was observed that the loss in the residual activity may be due to structural modification of the enzyme structure, end product inhibition and also due to protein denaturation[24].

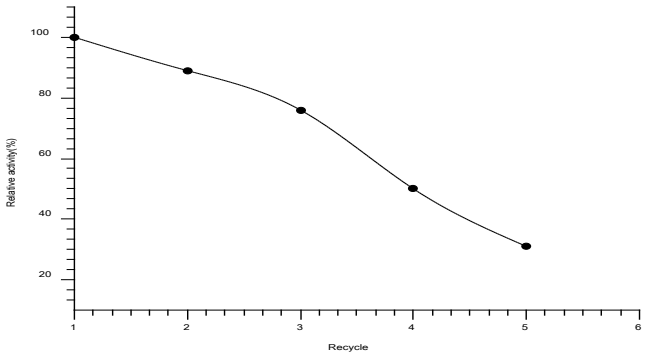
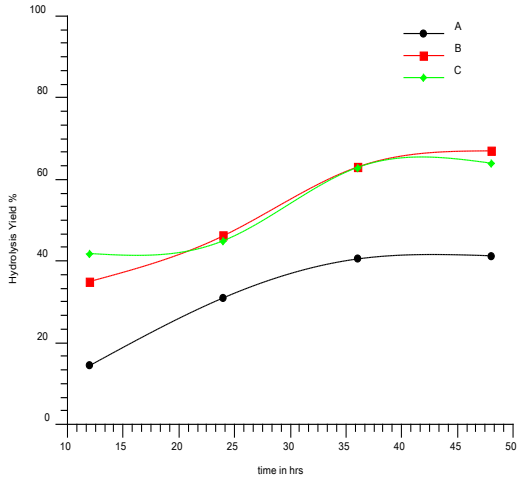
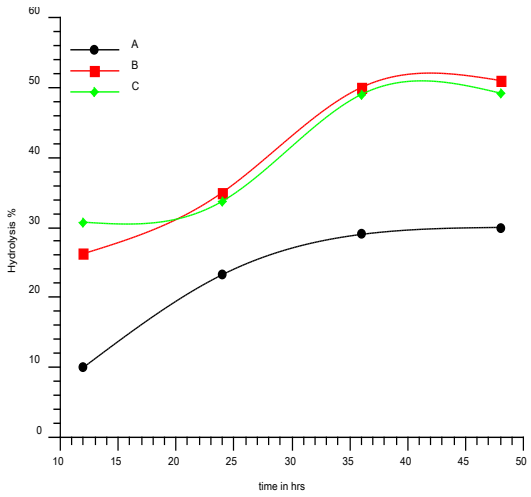


Figure 10. Recyclability Test.





A: Unpretreated biomass, B: NaOH treated with 5min Sonication and autoclaved Biomass, C: NaOH treated with 10min Sonication and autoclaved Biomass.

Figure 11. Reducing sugar hydrolysis yield of free enzyme and immobilized enzyme.

3.3.6 Enzymatic Hydrolysis

Enzymatic hydrolysis on raw and ultrasound assisted alkaline pretreatment sunn hemp biomass was performed with both free and immobilized enzyme up to 48hrs at 50˚C and 60˚C. It was observed that 5 min sonication assisted alkaline pretreated biomass showed 72% hydrolysis yield with free enzyme and 53% with immobilized enzyme. Whereas raw biomass hydrolysis was observed to be 30% with free and 22% with immobilized enzyme. Figure 11 shows the reducing sugar hydrolysis yield of free enzyme and immobilized enzyme.

4. Conclusion

The free cellulase enzyme was successfully immobilized on ferrite nanoparticle via covalent bonding and validated using FTIR spectrometer. Around 74% of binding was achieved at 4mg/ml of ferrite loading to enzyme concentration of 20 units. Comparative study on effects of pH and temperature was done on both free and immobilized enzyme and it was observed that the immobilized enzyme has maximum activity at pH 5 and temperature 60˚C. Also, the immobilized enzyme was stable at 60˚C while retaining its activity up to 3 recycles. The immobilized enzyme showed 53% hydrolysis yield on pretreated sunn hemp biomass. Further research on the interaction between zinc ferrite and cellulase in immobilization and

also the recovery of enzymes could determine an efficient approach for bioethanol production in industrial scale.

5. Acknowledgments

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