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Magnetic Nanoparticle Immobilized Cellulase Enzyme for Saccharification of Paddy Straw

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A B S T R A C T

The production of the bioethanol from the vast quantity of ligno-cellulosic (LC) biomass using cellulases and yeasts has been suggested as an alternate energy source and offers potential to reduce the burden on fast depleting fossil fuel reservoirs. The major bottlenecks in commercial production of second generation bioethanol are complex feed stocks, high cost of enzymes and non-availability of co-fermenting yeasts. Immobilization of commercial enzyme (Cellic CTec3) on five nanoparticle (Iron oxide, Silicon oxide, Magnesium oxide, Zinc oxide and Silver oxide) were studied by two different methods-physical adsorption and covalent coupling. For covalent coupling method, nanoparticles were activated with aminopropyltriethoxysilane (APTES). The enzyme-nanoparticle formulations were screened in the terms of protein binding and immobilization efficiency. Iron oxide-enzyme formulations were found to perform best with 60 to 80% immobilization efficiency. Besides better thermo-tolerance, the covalently immobilized enzyme showed better catalytic efficiency. The saccharification yields of the free and immobilized enzyme under optimized condition (60 ˚C temperature, 5.0 pH and 6% substrate loading) were compared. The commercial immobilized enzyme showed slightly lower sugar yield (314.77 mg/gds) as compared to the free enzyme (366.6 mg/gds) with 45 % enzyme activity recovery. Bioethanol production from the lignocellulosic biomass can be made economical feasible by using the covalently immobilized magnetic enzyme nanoparticle complex. Besides bioethanol, the prepared enzyme formulation can be used in textile, detergents and food industries.

Introduction

World's increasing interest toward non-petroleum energy sources especially lignocellulosic ethanol has revitalized the research on renewable energy sources. The major driving forces behind the renewed interests in biofuels are increased demand of energy for transportation and industries, fast depleting fossil fuels reservoirs and environmental concerns like global climate change. Therefore, it is important for both developing and developed countries to work towards sustainable fuels for energy security. Biofuels derived from food crops like corn, sugarcane, sugar beet (1st generation biofuel) have potential but they compete with food production because of limited land

availability. Lignocellulosic bioethanol (2nd generation biofuel) does not compete with food production because it uses the agriculture waste materials, urban organic waste material and that's why it is environmentally more sustainable (Naik *et al.,* 2010). Hence, bioethanol developed from the cheaper and more abundant lignocellulosic biomass is seen as the most feasible option especially as transport fuels for reducing petroleum consumption and as chemical feedstock for other industries.

India being one of the fastest growing economies of world would require additional energy sources to meet the demands of industries and transport sector. The national policy on biofuel approved by the Government of India (GOI) in 2009 encourages the use of renewable energy sources as alternate fuel for mixing in the transport fuels. This policy has planned of 20% biofuel blending (bio-diesel and bio-ethanol) by 2017. Currently, India is producing bioethanol from the sugar industry by product molasses which is neither economical nor sustainable. Due to the insufficient supply of the sugar molasses the government of India is not able to fulfill the compulsory blending of 5 percent ethanol in petrol (gasoline). Therefore, ethanol from lignocellulosic biomass is seen as an important viable option to meet the future energy demands (Sukumaran *et al.,* 2010). Lignocellulosic (LC) biomass contains glucose polymer cellulose (40-50%), a sugar heteropolymer hemicellulose (25-35%) and a non-fermentable monolingual polymer lignin (15-20%) (Holtzapple 1993).The lignocellulosic biomass composition varies from plant to plant and age of plant. The hydrolysis product of cellulosic polymers, glucose, can be readily fermented into ethanol or other high value added biochemicals. Success of LC bioethanol is dependent on the development of economically sustainable technologies by employing recent technologies and cheaper starting materials like agro-residues. However, the current bioethanol production process involves 3 major steps (pre-treatment, saccharification and fermentation) and is not competitive with crude at present. The major bottlenecks in commercial exploitation of LC bioethanol are recalcitrant nature of raw material, high cost of enzymes for saccharification and non-availability of co-fermenting yeasts. Many novel methods including physico-chemical and biological have been used successfully for removing lignin and improving the saccharification efficiency of various feedstocks (Alvira *et al.,* 2010; Saritha *et al.,* 2011). Cellulase that hydrolyses β-1, 4 glycosidic bonds of the crystalline complex of cellulosic crystals to produce glucose, is complex mixture of endoglucanase, exoglucanase and β glucosidase. The multicomponent cellulase enzymes is rapidly inactivated during saccharification due to denaturation and unspecific binding with lignin (Chundawat *et al.,* 2017). There are reports on the stabilization of cellulase enzymes by modulation of medium components, chemical crosslinking, protein engineering or enzyme immobilization (Khoshnevisan *et al.,* 2011). Many useful commercial cellulase cocktails namely Accelerase, Cellulocast, Cellic CTec 2&3, Novozyme 88 are available in market but their cost is still very high for an economically feasible process. Moreover, these enzymes are sensitive to end product inhibition. Immobilizing the enzyme on/in a solid matrix support can avoid deactivation of enzyme. Immobilization of the cellulase enzyme on different nano-materials like magnetic supports has been reported and is known to enhance temperature tolerance and activity besides creating an opportunity for recycling for future use (Jordan *et al.,* 2011). Enzymes can be immobilized with nanoparticles by many different ways like physical adsorption, entrapment and covalent binding (Chen and

Duan 2015). The cellulase immobilization has advantages of more hydrolytic activity and reusability of the enzyme. However, complete recovery of the bound enzyme from the nanoparticle is the main obstacle with the reusability. The immobilization also alters the temperature and pH optima of the enzyme and may be useful in processes like simultaneous saccharification and fermentation (SSF) which requires similar operating conditions for cellulases and yeasts.

Immobilization using various nanoparticles can be employed as a viable solution to conserve the enzyme activity under these unfavourable conditions. Immobilized enzymes have been used for developing sensors and in food industries but there is paucity of information on nanoparticle assisted saccharification for bioethanol production from lignocellulosic biomass.

Therefore, the main focus of research work is to immobilize the cellulase onto different nanoparticle to achieve better saccharification and to explore the enzyme reusability options. Further, enzymes are most expensive reagents in saccharification and are potentially recyclable but collecting the free enzyme from the treated bath is difficult. However, the iron oxide nanoparticles allows for the magnetic separation of the immobilized cellulase.

Materials and Methods

Enzyme and materials

Commercial enzyme (Cellic® CTec3) from Novozyme was used. Nanoparticles viz. Silicon dioxide (SiO_2, Zinc oxide (ZnO),Silver oxide (Ag_2O), Magnesium oxide (MgO), Iron oxide (Fe_2O_3) and chemicals like 3-aminopropyltriethoxysilane (APTES) glutaraldehyde and were purchased from Sigma Aldrich (USA). The Bradford reagent was purchased from G-Bioscience (USA).

Enzyme assays

Cellulolytic activities (CMCase, FPase, xylanases & β-glucosidase) were determined in original enzyme, bound enzyme and unbound fraction.

FPase (EC 3.2.1.91) activity

FPase activity or exo-β-glucanase activity of the enzyme was determined using the Whatman No.1 filter paper (50 mg) as a substrate by incubating in 0.5 ml citrate buffer (pH 4.8) and 0.5 ml enzyme sample at 50°C for 1h (Ghose 1987). After incubation, released sugars were measured by DNSA method (Miller 1959). One enzyme unit was expressed as release of the µmole of reducing sugar per minute during hydrolysis. One substrate blank and one enzyme blank run simultaneously to measure the sugar which was already present in the substrate and enzyme. In enzyme blank, 0.5 ml enzyme and 0.5 ml citrate buffer (0.05 M) was added. In substrate blank 50 mg filter paper and 1ml citrate buffer was added.

CMCase (EC 3.2.1.4) activity

CMCase activity of the enzymes was determined by adding in tube 0.5 ml CMC substrate (2%) and 0.5 ml enzyme and kept for the incubation at 50°C for 30 min. in water bath (Ghose 1987). Post incubation, tubes were kept at room temperature and reducing sugars were determined by using DNSA method as described above.

pNPGase (β-D-glucosidase; EC 3.2.1.21) activity

The β-glucosidase activity of enzyme was determined by incubating 0.5 ml enzyme and 0.5 ml of substrate (p-nitrophenyl β-D-glucopyranoside) at 50°C(Wood and Bhat 1988). Following incubation, the reaction was

stopped using Glycine buffer of pH 10.8 (1 ml) and the concentration was checked at 430 nm. The activity was expressed in terms of release of µmoles of p-nitrophenol produced per ml of enzyme per minute.

Xylanases activity

Xylanases activity of the enzyme was checked by mixing of enzyme and xylan (0.5 ml of 1% solution) followed by the incubation at 50°C for 30 min (Ghose and Bisaria, 1987). Reducing sugars were quantified by DNSA method. One enzyme unit was expressed in terms of µmole of product (xylose) formed per min under standard assay conditions.

Protein assay

Protein concentration in the culture filtrate was checked using Bradford method (1976). Enzyme (100 µl) was mixed with 1ml of Bradford reagent (G-Biosciences) and mixture was vortexed and incubated for 10 minutes at room temperature for blue colour development. The concentration of protein was determined by spectrophotometry at 595 nm and standard curve of the different concentrations (1-100 µg/ml) of bovine serum albumin (BSA).

Immobilization of enzyme

Physical adsorption

Immobilization of the commercial enzyme on the different nanoparticles by physical adsorption were performed by mixing 50mg different nanoparticle which were prepared in sodium citrate buffer and 3 ml of enzyme (2 mg/ml) solution (prepared in sodium citrate buffer, pH 4.8). The mixture was incubated at 25˚C with continuous shaking on revolving shaker for 4 h. Postincubation, the centrifugation of the samples were done at 10000 rpm for 5 min at 4˚C. Immobilized nanoparticles were washed with sodium citrate buffer to remove the loosely bound enzyme molecules. Total protein and enzyme activities were checked in both unbound and bound fraction to measure the immobilization efficiency and residual activities (Khoshnevisan *et al.,* 2011).

Covalent coupling of enzymes and nanoparticles

Preparation of ligand-nanoparticles

Initially the nanoparticles were modified by mixing 0.1 g nanoparticles in 12 ml ethanol and 0.5 ml deionized water. After that, 1 ml ammonium hydroxide (30%) and 0.25 ml of 3-aminopropyltriethoxysilane (APTES) were mixed in the sample. The solution was stirred continuously for 24 h at room temperature followed by centrifugation and washing with ethanol. The modified nanoparticles were treated with 2 ml of 25% glutaraldehyde and 3 ml deionized water. The solution was stirred again continuously for 24 h at room temperature. Finally the modified nanoparticles were separated and washed thrice with 0.05M sodium citrate buffer (pH 4.8) to remove the loosely bound reagents (Alahakoon *et al.,* 2012).

Immobilization of enzyme

Immobilization of cellulase (Cellic CTec3) on the modified nanoparticles was performed by mixing the 10 ml (1 mg/ml) of enzyme and 0.1 mg functionalized nanoparticles. The mixture was incubated for 18 h at room temperature. Following incubation, the centrifugation of the mixture was done to recover the ENC followed by washing thrice with sodium citrate buffer (pH 4.8) to remove loosely bound proteins molecules. Protein content and enzyme activities of both bound and unbound fraction was determined using standard assays as described earlier.

Determination of immobilization efficiency of nanoparticles

Immobilization efficiency for different nanoparticles was measured by measuring the enzyme activities and protein in the initial solution of enzyme, supernatant and washing (Alahakoon *et al.*, 2012).

$$\text{Immobilization efficiency} = \frac{Mi - Ms}{Mi} \times 100$$

Mi. = Initialenzyme activity/protein concentration

M_S = Enzyme activity/Protein concentration in supernatant and washing.

Determination of optimum incubation time and protein loading for preparation of magnetic enzyme nanoparticle complex (MENC)

The optimum incubation time and protein loadingwere measured by incubating the functionalized nanoparticles with varying amount of protein for different time intervals. Post incubation, protein loading (µg/mg NP) was checked using Bradford protein assay.

Determination of optimum temperature, pH optima and kinetic parameters for free and immobilized enzymes

The optimum temperature for both free and immobilized commercial enzyme were determined using the range of temperature (40-80°C).The substrate were prepared in buffer of different pH (3, 4, 5, 6 and 7) to determine the optimum pH of the free and immobilized enzyme and enzyme activity was determined using standard assay as described earlier. Kinetic parameters such as V_{max}, K_m and kinetic efficiency (V_{max}/K_m) were measured under steady state conditions using various concentration of CMC as substrate.

Alkali pretreatment of the paddy substrate

Paddy straw was pretreated using 1% NaOH with 10% substrate loading in Erlenmeyer flask and the flask was kept static for 1 h followed by 6-7 washing with distilled water in equal ratio until the pH of washed water reaches near neutral. Finally biomass was dried and stored in refrigerator at 4˚C as such till used.

Optimization of saccharification parameters

The optimization of the saccharification parameters of the best ENC like temperature, pH and substrate loading was done by using one factor at a time approach. The saccharification of the alkali pretreated paddy straw was performed using different temperature (30˚C- 60˚C), pH range (4.0- 6.0) and at different substrate loading (1, 2, 4, 5%). The reducing sugars released were determined using HPLC (discussed in next sections).

Saccharification of alkali pretreated paddy straw under optimized conditions

Saccharification of alkali pre-treated paddy straw was done as per NREL LAP-009 method (Brown and Torget, 1996) using free enzyme and best enzyme nanoparticle complex (ENC).

Pretreated substrate (0.6g) and free or immobilized enzyme (20 FPU/g substrate) were mixed in screw capped bottles (50 ml) and final volume was made up to 10 ml by adding 0.05 M citrate buffer (pH 4.8).

Then incubation of the bottles were carried out in shaking water bath at 50°C for 72 h. Aliquots were taken out at different intervals of time from the bottles and the amount of reducing sugars released was determined by HPLC.

HPLC analysis for quantification of reducing sugars released

The reducing sugars released during saccharification process were determined using the high performance liquid chromatography (HPLC). The HPLC system consisted of auto sampler, refractive index detector (RID), Waters 515 binary pump, column oven and pump control module. The mobile phase was 5 mM H_2SO_4 with flow rate of 0.5 ml/min. The column Biorad's Aminex HPX-87H) was operated at 50°C temperature.

Recovery of ENC and calculation of residual activities

After the completion of the saccharification process the ENC were recovered by the centrifugation of the sample and these ENC were washed with citrate buffer. The residual activities were determined using the method described earlier for FPase. The recovered enzyme was used again for saccharification of alkali treated paddy straw. The data was analyzed statistically by using completely randomized design (CRD) as outlined in Panse and Sukhatme (1954) and the test of significance was done at the 5% level.

Results and Discussion

Screening of nanoparticles for their immobilization efficiency

In this study five different nanoparticles Iron oxide (Fe_2O_3), Silicon dioxide (SiO_2), Zinc oxide (ZnO), Silver oxide (Ag_2O) and Magnesium oxide (MgO) were used for the immobilization of the enzyme and the best combination was selected on the basis of protein loading and immobilization efficiency.

The immobilization efficiency of all the ENPs prepared via physical adsorption and covalent coupling are shown in Figure 1. The result clearly showed that magnetic enzyme nanoparticle (ENPs) retained highest enzyme activities among all the nanoparticles in both physically adsorption and covalent coupling methods. Although the SiO_2 showed higher protein binding than MNPs but the immobilization efficiency in the terms of enzyme activities were poorer. ZnO showed the least immobilization efficiency among all the nanoparticles. The immobilization efficiency of Iron oxide was found to be 50 to 60% for physically adsorbed enzyme but covalently bound enzyme showed 60 to 80 % immobilization efficiency.

In physical adsorption method, again magnetic nanoparticle-enzyme complex showed the highest immobilization efficiency in terms of enzyme activities and protein binding. The highest immobilization efficiency of 59.58% was shown by the enzyme FPase followed by CMCase (59.23%) and xylanase (59.12%).

In covalent coupling method also, MENP was found best in the terms of immobilization efficiency and protein binding. Among different enzymes, highest immobilization efficiency was shown by pNPGase.

Many scientists have shown that when enzymes immobilized on the solid support, immobilization efficiency ranges from 50-145% (Cheng and Chang, 2013; Samra *et al.*, 2012; Khoshnevisan *et al.*, 2011). Physical adsorption method of the immobilization is very simple and does not require any ligand chemical which makes this method cost effective. This method involves direct attachment between enzyme and nanoparticles with the help of the hydrogen bonding, hydrophobic interaction and van der Waals forces. Physical adsorption is reversible in nature by which NPs support can be recovered easily after the inactivation of the enzyme and can be reused or the immobilization. However, there are many drawbacks in the physical

adsorption method. Lower immobilization efficiency as reported in present study is the one of the commonly reported drawbacks of the physical adsorption. The leaching of the enzyme is also one of problems in physical adsorption method due to which the efficacy of this method reduce during the repeated uses (Liao *et al.,* 2010). Ahmad and Sardar (2015) have reported the lower enzyme activities of the silicon oxide in spite of the better protein binding than the magnetic nanoparticles which might be due to the blocking of the active sites of the enzyme by nanoparticles.

Magnetic nanoparticles have been the most studied and reported by the scientist because of their large surface area, environment compatibility, lower cost, high protein binding and ease of recovery from reaction mixtures (Netto *et al.,* 2013). The covalent coupling methods of the immobilization are more stable than physical adsorption due the strong crosslinking of the enzyme and nanoparticles by using the ligand. The covalently bound enzyme nanoparticle complex can be used repeatedly without any leaching of enzyme.

The immobilization of the enzyme on the nanoparticle was confirmed by the Fourier transform infrared spectroscopy (FTIR) analysis (data not shown). The FTIR spectra clearly showed that the binding of the enzyme on the nanoparticle is confirmed by the band at 2100 $cm^{-}1$. The confirmation of binding of enzyme on nanoparticle has been reported by many scientists using the Fourier transform infrared spectroscopy (FTIR) (Cheng and Chang, 2013; Khoshnevisan *et al.,* 2011).

Determination of optimum incubation time and protein loading for preparation of MENC

The efficient and cost effective ENP complex should show high protein loading in shortest incubation time. The protein loading of 63.6µg protein/mg NPs was achieved within short incubation period of 15 h. Further, the optimization of the incubation time was carried out by incubating for different time. The maximum protein binding was found after15 h of incubation of the enzyme and functionalized nanoparticles (Figure 2). Only few studies have reported the optimization of protein loading and incubation time. Khoshnevisan *et al.,* (2011) reported cellulase loading to magnetic NP ratio of 0.033 which was very low. However, some other researchers like Cheng and Chang (2013) and Samra *et al.,* (2012) have reported a higher protein loading of130 mg/g magnetic NP and 117 mg/g silica NP for mannosidase and cellulase respectively. The enzyme loading on the nanoparticles depend upon the different factors like properties of enzyme, size and shape of NPs and ligand properties (Mohamad *et al.,* 2015).

Determination of temperature and pH optima and kinetic parameters for free and immobilized enzyme

The enzyme activity of the free and immobilized enzyme was measured in the range of temperature from 40 to 80°C by using CMC as a substrate. The relative activity of free and immobilized enzyme on magnetic NPs is depicted in Figure 3.

The result clearly indicated that the optimum temperature for the free and immobilized enzyme was 50°C and 60°C respectively. This report shows that the immobilization of the enzyme on the solid support increased the thermostability of the enzyme which can tolerate higher temperature whereas the free enzyme denature at this temperature. The similar result has been reported where immobilized cellulase enzyme was more stable than the free cellulase enzyme at the temperature higher than 60˚C (Chen and Duan, 2015).

Fig.1 Immobilization efficiency (%) of nanoparticles by physical adsorption (A) and covalent coupling (B)

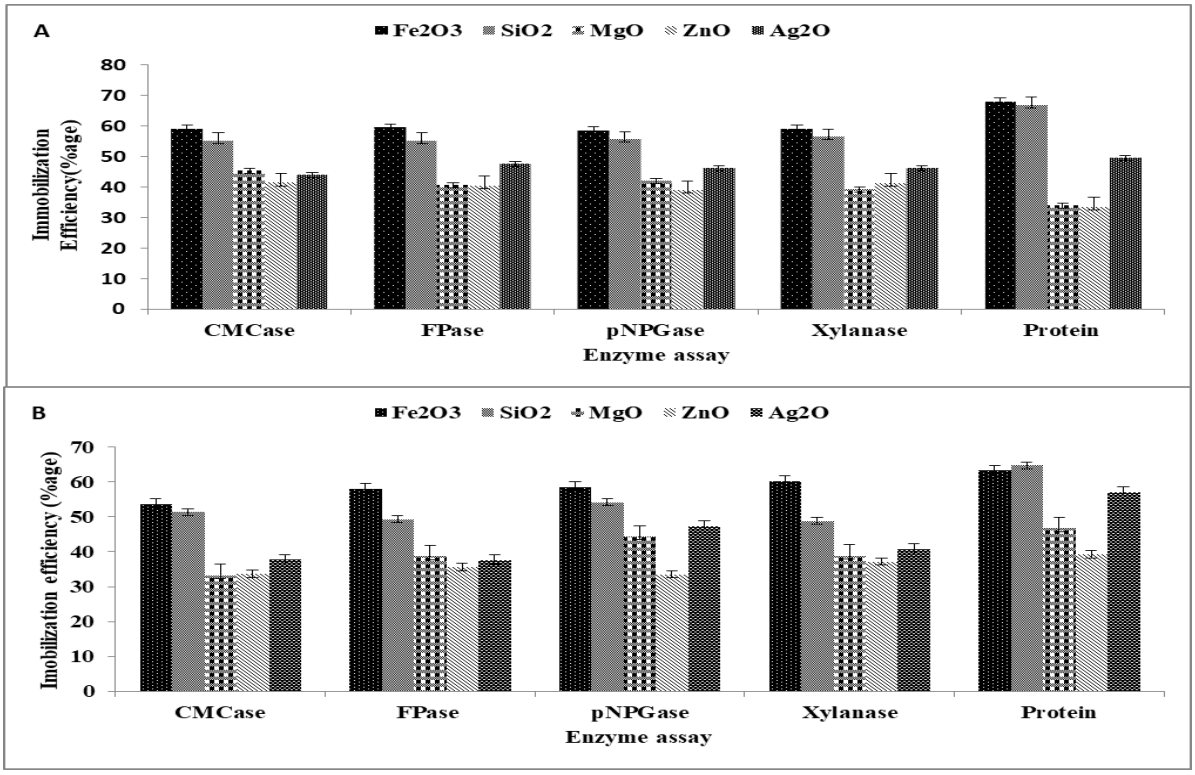


Fig.2 Optimization of protein loading (A) and incubation time (B) for preparing covalently bound MNC for commercial enzyme

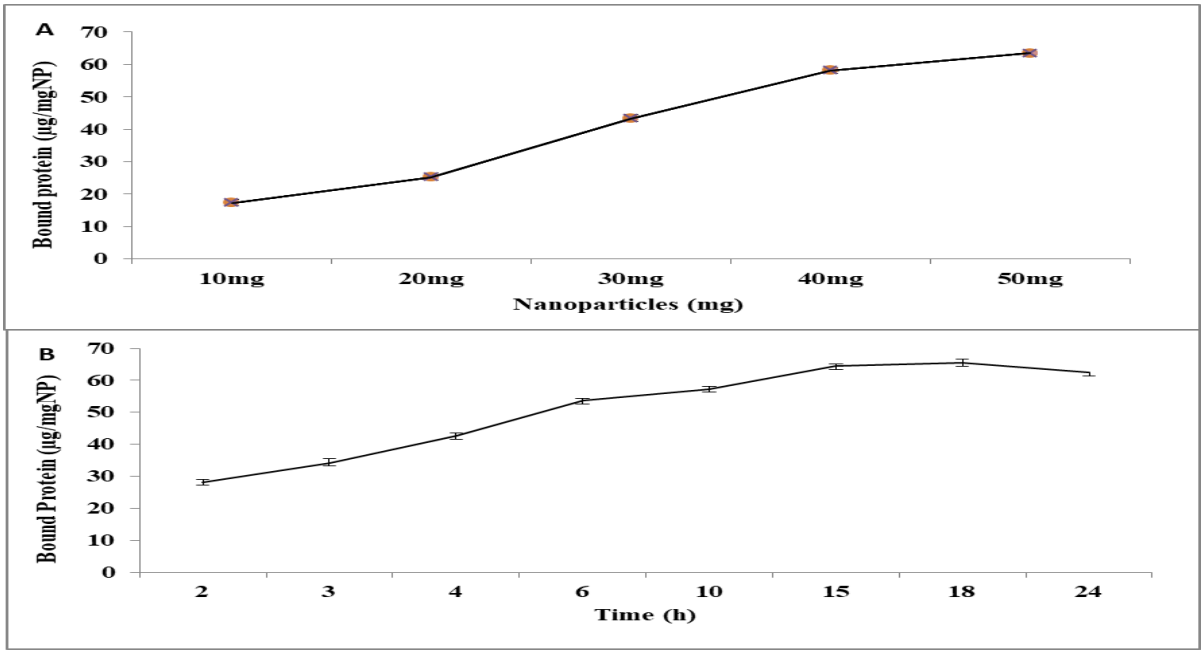


Fig.3 The comparisons of free and immobilized commercial cellulase for optimum temperature (A) and pH (B)

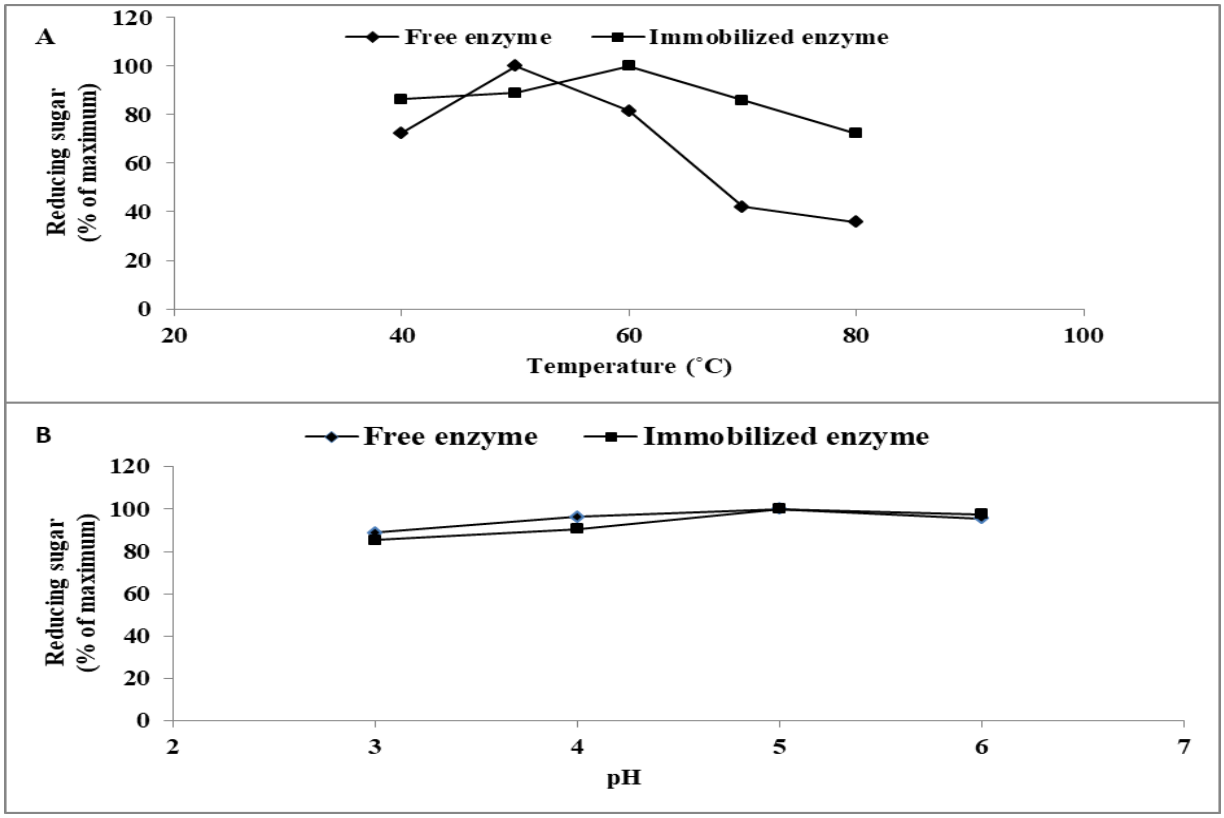
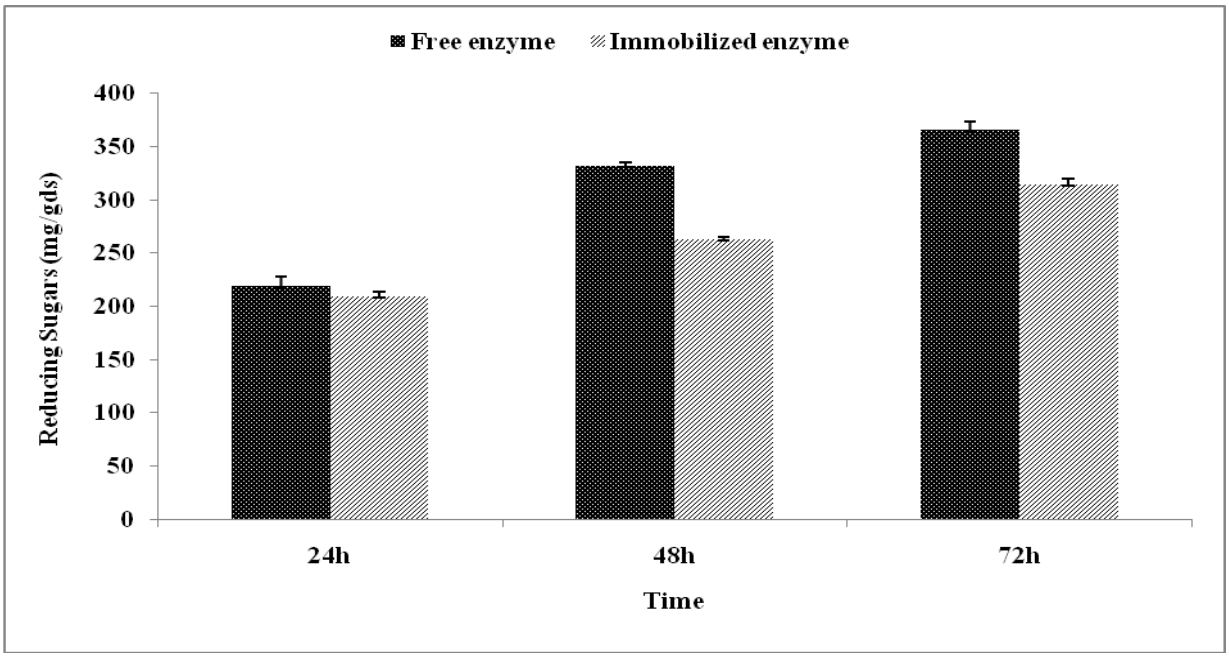


Fig.4 Comparisons of the saccharification yield of the free and immobilized commercial enzyme at different time interval



Many other reports are also available which showed that immobilized enzyme has more thermostability as compared to the free enzyme (Huang *et al.,* 2015; Jordan *et al.,* 2011). The reason for higher thermostability of the immobilized enzyme as compared to the free enzyme may be the less flexibility of the enzyme on the solid support that increased the tolerance against denaturation and unfolding of the enzyme due to the conformational changes. The increase in the thermostability of the immobilized enzyme is directly related to the covalent bonding of the enzyme and solid support (Jaroslava, 1978).

The optimum pH for the immobilized enzyme was same as free enzyme but immobilized enzyme showed more stability over the broader pH range as compared to the free enzyme (Figure 3). The reason for stability over broad range may be the interactions between charge on the carrier molecule and charged group present on the enzyme molecules which were used for the covalent coupling of the immobilization. It is documented that the optimum pH for the immobilized and free enzyme showed no difference but the immobilized enzyme showed the broader pH stability as compared to the free enzyme (Abraham *et al.,* 2014; Tao *et al.,* 2016).

Kinetic parameters

The kinetic parameters of both the free and immobilized cellulase enzyme were measured using different concentrations of CMC. The result indicated that the immobilized enzyme has higher K_{cat} value (4.73) and lower K_m (20.11 g/l) value as compared to the free enzyme. The similar result has been reported that K_m value of the free and immobilized enzyme were 2.6 and 0.87 respectively (Abraham *et al.,* 2014). The decrease in the K_m value and increase in the K_{cat} value makes the rate of reaction faster. The catalytic efficiency can be assessed by the kinetic parameters (K_m, V_{max} and $V_{max/Km}$). The affinity of the enzyme for the substrate changes with the changes in kinetic parameters. The reason for the variation in the kinetic parameters might be due to the several factors like protein conformational changes induced by the steric hindrance, attachment to the support, and diffusional effects.

Enzymatic saccharification of alkali-pre-treated paddy straw using MENC

Saccharification of alkali pre-treated paddy straw with commercial immobilized enzyme under optimized conditions (pH 5.0, 60 °C temperature, and 6% substrate loading) resulted in release of more reducing sugars (366.43mg/gds) than immobilized enzyme (314.77 mg/gds) reducing sugars (Figure 4). Kumar *et al.,* (2017) used MENP prepared from indigenous enzyme from *Aspergillus niger* SH3 for saccharification of alkali pre-treated paddy straw and observed that immobilized enzyme released 11% more sugar than free enzyme. However, the reverse trend observed in present study might be due to blocking of active enzyme site by the sugars present in enzymes formulation used for preparing ENPC. Various sugars are added to preserve the activity of enzymes during storage (Gonçalves *et al.,* 2013).

Recovery of ENC and calculation of residual activities

After the saccharification of the paddy straw, ENP complex were recovered and enzyme activity (FPase) was measured to determine the residual activity. It was found that 70 and 45 % enzyme activity is left after the first and second saccharification cycle respectively. The recovery and reuse of the immobilized enzyme may help to reduce the overall cost of process during bioethanol production. Magnetic nanoparticles are most studied and

used for the immobilization of the enzyme because of the easy recovery by centrifugation or using magnates. However, such studies are more common for the high value enzymes used in diagnostic industries and pharmaceutical. Only few reports are available on the use of immobilized enzyme for saccharification of lignocellulosic biomass (Alftrén and Hobley, 2014).

The magnetic nanoparticle was found best among all the nanoparticles in terms of the immobilization efficiency. The magnetic ENC was used twice for the saccharification of the pre-treated paddy straw which can help to bring down the overall bioethanol production cost.

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