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Research Article

Immobilization of Cellulase on Cobalt Oxide Nanoparticles for Efficient Bioethanol Production by Simultaneous Saccharification and Fermentation

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Abstract

Cellulose is a major biopolymer found in plants which can be used for many industrial applications. Major portion of cellulose is getting wasted along with forest and agricultural waste. This can be efficiently used for the production of many industrially important products. Availability of limited fuel, which is a major threat in the present scenario, can be solved upto an extent by the production of biofuel. In the present study cellulase produced by of *Aspergillus fumigatus* JCF was used for the bioconversion of agricultural wastes to bioethanol. Produced cellulase was immobilized on cobalt oxide nanoparticles for increased catalytic efficiency and utility. Immobilized cellulase was found to retain 75% of the activity even after 4th cycle. Pretreated *miscanthus* leaves were used as substrate for bioethanol production by simultaneous saccharification and fermentation using immobilized and free cellulose and baker's yeast. Action of immobilized cellulaseand baker's yeast released more bioethanol of about 21 g/l which was higher than free cellulase. Thus the immobilized cellulase was found more effective than free cellulase for hydrolysis of agricultural waste to produce of bioethanol.

Keywords: *Aspergillus fumigatus* JCF; Cellulase; Immobilization; Cobalt oxide nanoparticles; Bioethanol; Simultaneous Saccharification and fermentation.

Introduction

Cellulase is an industrial enzyme which helps in the conversion of lignocellulosic materials into simpler units. Cellulases are group of enzymes which include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC3.2.1.91) and β–glucosidases (EC3.2.1.21) [1, 2]. This group of multicomponent enzymes works synergistically for the hydrolysis of crystalline cellulose. These crystalline or amorphous celluloses are hydrolyzed first to small soluble fragments and then to glucose by cellulase group. The monomeric glucose can be further used for the production of different biochemical [3].

High cost of raw materials is the major problem in the enzyme production. It can be overcome by microbial fermentation using low cost substrates. In the present situation, large amount of agricultural cellulosic wastes have been deposited in the environment. These wastes are not used properly and are getting accumulated. The effective treatment and utilization of cellulosic wastes can improve the economic interest. The cellulase production was already carried out with substrates like wheat straw [4], rice straw [5] and fruit processing waste such as apple pomace , grape pomace and pineapple waste [6,7].

The inherent properties of native lignocellulosic materials make them resistant to enzymatic attack. Pre-treatment of biomass promotes disruption of the lignocellulosic matrix, thus preparing the substrates for enzymatic degradation. The use of cellulase for enzymatic degradation of cellulose requires large enzyme loadings. This increases the production cost and economic demand [8]. There are many methods for improving the efficiency of enzymatic activity. Immobilization of cellulase could be one of the effective methods for increasing the enzymatic stability and reusability. This could also reduce the cost of cellulose degradation. The immobilized enzyme is one which is attached to or enclosed by an insoluble support medium or one where the

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enzyme molecules have been cross-linked to each other, without loss of catalytic activity. Various materials such as chitin, chitosan and polyvinyl alcohol can be used as the supports for immobilizing enzymes. Presence of some metals also will improve the activity of cellulase enzyme. Many metal oxidenanoparticles like TiO_2, ZnO and FeO are commonly used for immobilizing enzymes when prepared [9]. In the current work, cobalt oxide nanoparticle was used for immobilizing cellulase. Nanopartcicles were characterized by Scanning Electron Microscopy (SEM) and the immobilization was validated by Fourier Transform InfraRed spectroscopy (FTIR).

In the present scenario, entire world is looking forward for an alternate energy source due to increased concern on environmental pollution and energy security [10]. Bioethanol produced through saccharification and fermentation is a good solution to the present situation [11]. Thus the present study also aimed at the production of bioethanol by simultaneous saccharification and fermentation of pretreated *miscanthus* leaves using cellulase immobilized on cobalt oxide nanoparticles.

Materials and Methods

Production of cellulase enzyme

Cellulase enzyme was produced by submerged fermentation with previously isolated *Aspergillus fumigatus* JCF and using cotton seed powder as substrate. Substrate was collected locally and dried properly in hot air oven. It was mill ground and sieved. The particles taken from 240 µm mesh was used as the substrate. Substrate was added to the modified Mandel's medium, sterilized, inoculated with *A. fumigatus* JCF and kept in orbital shaker for 4 days at 120 rpm for cellulase production. Conditions for cellulase production were previously optimized and cellulase activity obtained was 2.3 IU/ml.

Synthesis of cobalt oxide nanoparticle

Cobalt oxide nanoparticles were synthesized by co-precipitation method. 0.1 M cobalt chloride was dissolved in distilled water and 0.2 M NaOH was added drop by drop under constant stirring for 3 hrs. Precipitated particles were collected by centrifugation at 10,000 rpm for 15 min. The supernatant was discarded and the pellet was washed thoroughly with deionized water several times. The pellet was dried at 80°C for 12 hrs [12].

Immobilization of cellulase by covalent binding method

The cobalt oxide nanoparticles were suspended in deionized water at a concentration of 125 mg/ml. This suspension was sonicated for 1 hr, after which it was suspended in 1.5 ml of glutaraldehyde solution. Support activation was achieved by shaking cobalt oxide nanoparticles for 1 hr at 25°C and 180 rpm. After incubation, the activated cobalt nanoparticles were washed with deionized water and citrate buffer. The covalent binding of the enzyme to the nanoparticle was achieved by incubating the activated nanoparticles support with enzyme at a concentration of 5 mg/ml at 25°C for 2 hrs in a shaker at 180 rpm. The supernatant obtained after the immobilization was used for protein estimation. The immobilized enzyme and support complex was thoroughly washed with deionized water and buffer to remove any loosely bound protein.

Binding efficiency of enzyme was found by calculating the ratio of protein bound to nanoparticle (A) to the total protein available for binding (B) to the total protein available for immobilization. The concentration of protein was determined by the Bradford assay. The amount of bound protein (A) was calculated as (B-C), where C is the concentration of the protein in the supernatant (mg/mL) [13].

$$BindingEfficiency = \left({}^{A}/_{B}\right) \times 100 \quad \text{.....(1)}$$

Characterization of cobalt oxide nanoparticle with and without cellulase

Characterization of cobalt oxide nanoparticles with and without cellulase was done by SEM and FTIR. The size and morphology of the cobalt oxide nanoparticles with and without enzyme were determined using Field Emission Scanning Electron Microscope (FESEM) from CARL ZEISS, Germany. Synthesized nanoparticles were characterized with respect to attached functional groups by FTIR from 13-ELMER, US, with the scanning range of 4000-400 cm^{-1} and resolution of 4 cm^{-1}.

Kinetic study of free and immobilized cellulase

The kinetic study of the free and immobilized enzyme was done by varying the concentration of carboxyl methylcellulose

substrate from 5 mg/l to 50 mg/l). K_m (Michaelis-Menten Constant) and V_m (Maximum Velocity) values were calculated by Lineweaver Burk plot [14]. The reusability of immobilized enzyme was determined using immobilized cellulase for further cellulolysis on fresh substrate. After each cycle immobilized enzyme was recovered and washed with deinoized water. Cellulase activity in the first assay was taken as the control (100% activity).

Bioethanol production by simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation is a process where complex lignocellulosic materials were broken and simultaneously fermented to bioethanol by the action of cellulase enzyme and baker's yeast. *Miscanthu*s leaves were used as the substrate for bioethanol production in the current study. The substrate was collected locally. Substrate was dried, mill ground and sieved using mesh with pore size 240 µm and it was pretreated with 2.5 N sodium hydroxide. The mixture (substrate and chemical) was kept in water bath at 80°C for 1 hr. At the end, liquid fraction from hydrolysate was filtered using filter paper and residual part was dried overnight at 60°C for further analysis.

Fermentation media was prepared by mixing pretreated substrate with peptone and yeast extract in 100 ml citrate buffer. Media prepared was sterilized and inoculated with baker's yeast and 5 ml of free and immobilized enzyme respectively. The fermentation was carried out at 120 rpm and 30ºC. The sample was collected at regular intervals of 24 h for the determination of bioethanol. Bioethanol production was further optimized by varying substrate concentration (0.5-2.5 %), Yeast Extract (0.05-0.25%), pH (4 -8) and time (1-6 days).

Enzyme activity assay

The CMCase activity was determined by using a reaction mixture containing 1.5 ml of filtrate and 1.5 ml of 1% CMC. This mixture was incubated at 50ºC for 10 min. 3 ml of DNS reagent was added and heated for 15 min in boiling water bath. After heating contents were allowed to cool at room temperature. After cooling, 1 ml of 40% sodium potassium tartarate solution was added. The absorbance was measured at 575 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min [15].

Determination of Bioethanol

The amount of ethanol produced in the fermentation media was estimated by using dichromate method. 1 ml of cell free extract was diluted four times and 1 ml of potassium dichromate was added, after keeping all tubes containing the above mixture in ice water, 5 ml of concentrated sulfuric acid was added gently through the walls and then the optical density was measured on spectrophotometer at 660 nm [16].

Results and Discussion

Characterization of Cobalt Oxide nanoparticles before and after immobilizing cellulase

The cellulase produced from *Aspergillus fumigatus* JCF, with an activity of 2.3 IU/ml was immobilized on cobalt oxide nanoparticle by covalent bonding. The binding efficiency was found to be 85%. The SEM analysis was done to study the morphological features of the nanoparticles with and without enzyme bound to it. SEM image shown in Fig. 1a, displays the individual cobalt oxide nanoparticle as well as a number of aggregates and Fig. 1b represent cobalt oxide nanoparticle with cellulase. The average size of the nanoparticle was found to be 35-50 nm without enzyme and 1 µm with immobilized cellulase. EdX analysis was also done to confirm the presence of cobalt oxide nanoparticles. The strong signal of cobalt and oxygen confirms the presence of cobalt Oxide nanoparticles (Fig. 2).

The binding of cellulase on cobalt oxide nanoparticle was further confirmed by FTIR analysis. FTIR transmittance peaks of the cobalt oxide nanoparticles with and without cellulase enzyme is shown in Fig. 3. The bond stretching at 1591.0 cm^{-1} clearly indicated the presence of cobalt oxide and at 1399.8 cm^{-1} was due to NO_3 stretching. The NH_3 bond stretching was obtained at 3434.4 cm^{-1}. The characteristic band of protein at 1399 cm^{-1} and 1261 cm^{-1} respectively indicate that the cellulase was successfully immobilized on onto cobalt oxide nanoparticles.

Free and immobilized cellulase enzyme kinetics

The kinetics of free and immobilized cellulase was studied by varying the

concentration of Carboxy Methyl Cellulose (CMC) as substrate. The Michaelis-Menten Constant (K_m) and maximum velocity (V_m) for free and immobilized enzyme were evaluated. K_m was found to be 6.567 and 3.694 mg/ml for free and immobilized enzyme respectively. The change in the K_m value may be due to the alteration in the substrate binding ability of the enzyme due to immobilization. V_{max} for free and immobilized enzyme was found to be 2.109 and 3.104 mg/ml respectively. V_{max} / K_m value for free and immobilized enzyme was of 0.32 and 0.84 respectively. This clearly indicates that the catalytic efficiency of immobilized enzyme is greater than the free enzyme. Similar results were obtained in many other studies [3].

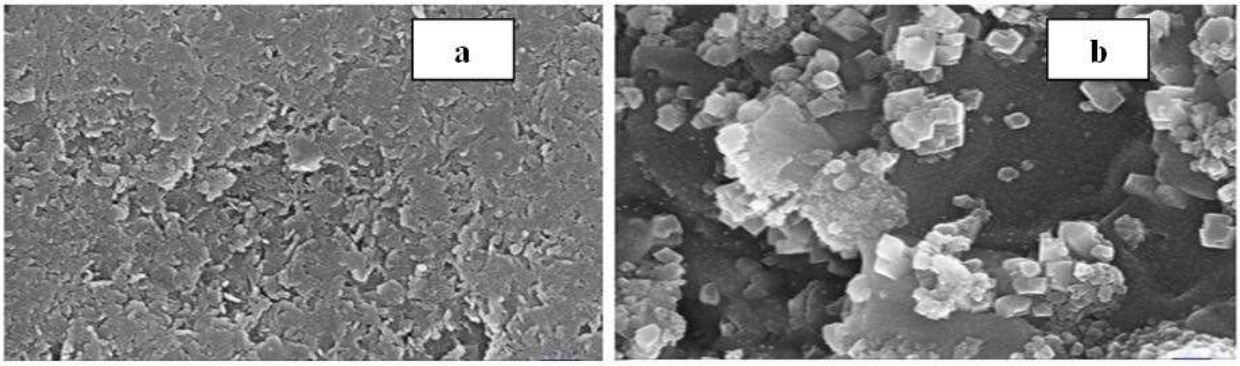

Fig. 1. SEM image of (a) cobalt oxide nanoparticle (b) cellulase immobilized on cobalt oxide nanoparticle

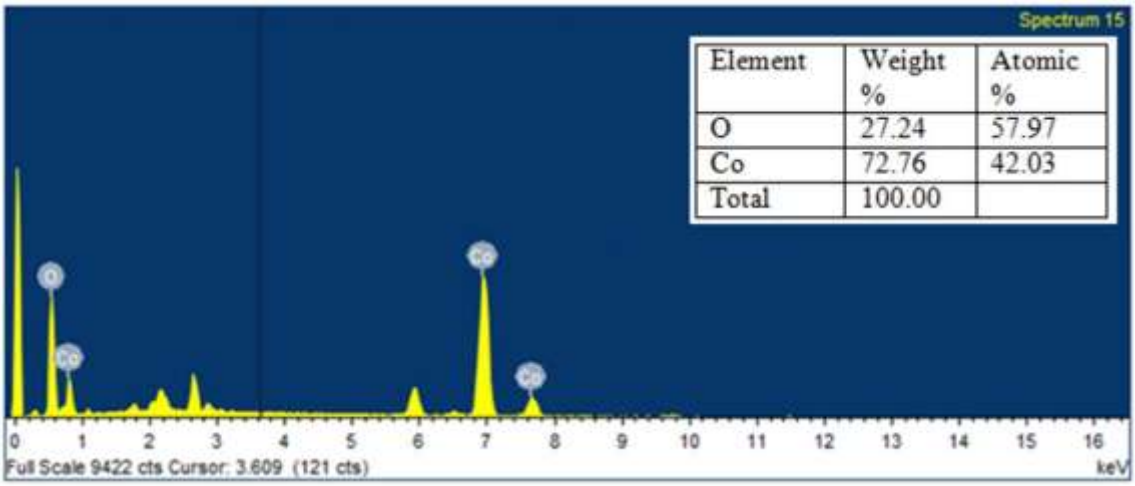

Fig. 2. EDX analysis of cobalt oxide nanoparticles

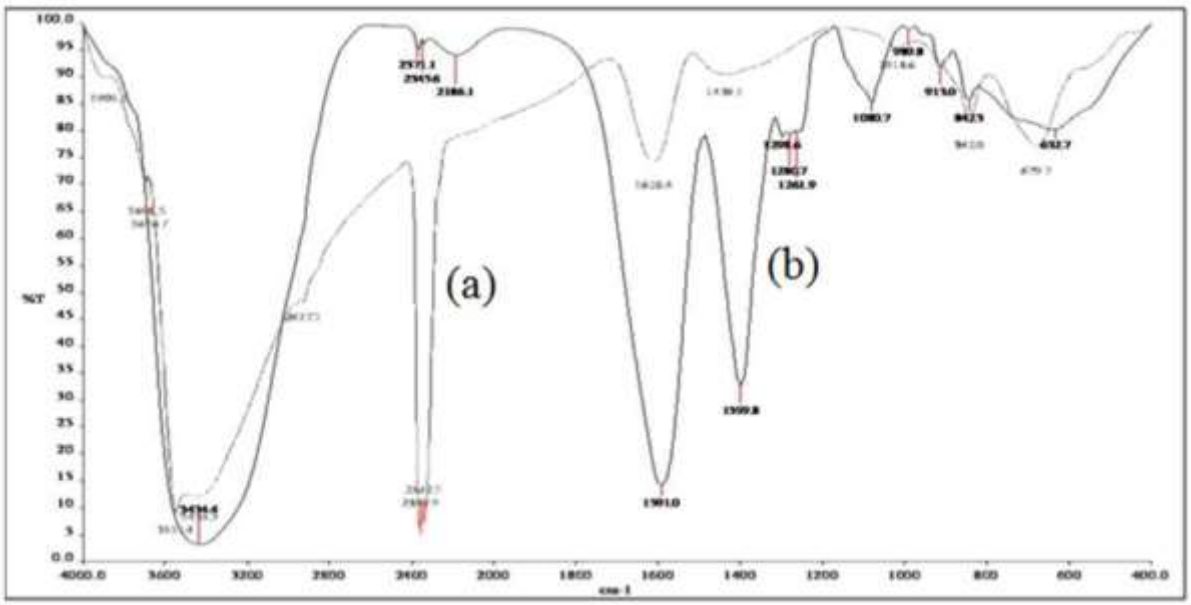

Fig. 3 FTIR Spectra of (a) cobalt oxide nanoparticles (b) cellulase immobilized on cobalt oxide nanoparticle

Bioethanol production by simultaneous saccharification and fermentation

Pretreated *Miscanthus* leaves were used as the substrate for bioethanol production by simultaneous saccharification and fermentation. The *S. cerevisiae* was proved to be very effective in converting glucose to ethanol in SSF. In the present work, conditions for the bioethanol production were optimized for maximizing the bioethanol yield. Since carbon is considered to be one of the primary nutrients for the action of cellulase and yeast, concentration of miscanthus biomass was optimized by varying the amount from 0.5 % to 3% (w/v). Bioethanol yield of 19 g/l was obtained for 2.5 % of substrate when immobilized enzyme was used (Fig. 4(a)). Further yeast extract concentration was also optimized and ethanol production was found to increase to 20 g/l (Fig. 4(b)). The pH also play an important role in the production of bioproducts. Maximum ethanol production was found to be at pH 5 (Fig. 4(c)). Under optimized conditions a maximum ethanol concentration of 21 g/l was obtained on 3rd day of fermentation (Fig. 4(d)). After that bioethanol production was found to decrease. In all the cases it is observed that bioethanol production was higher in the presence of immobilized enzyme than free enzyme. This can due to the presence of metal ions which increased the activity of cellulase enzyme. Thus immobilization improved the activity of enzyme thereby bioethanol production. Also cost of bioethanol production can be reduced extensively by using lignocellulosic wastes as substrates.

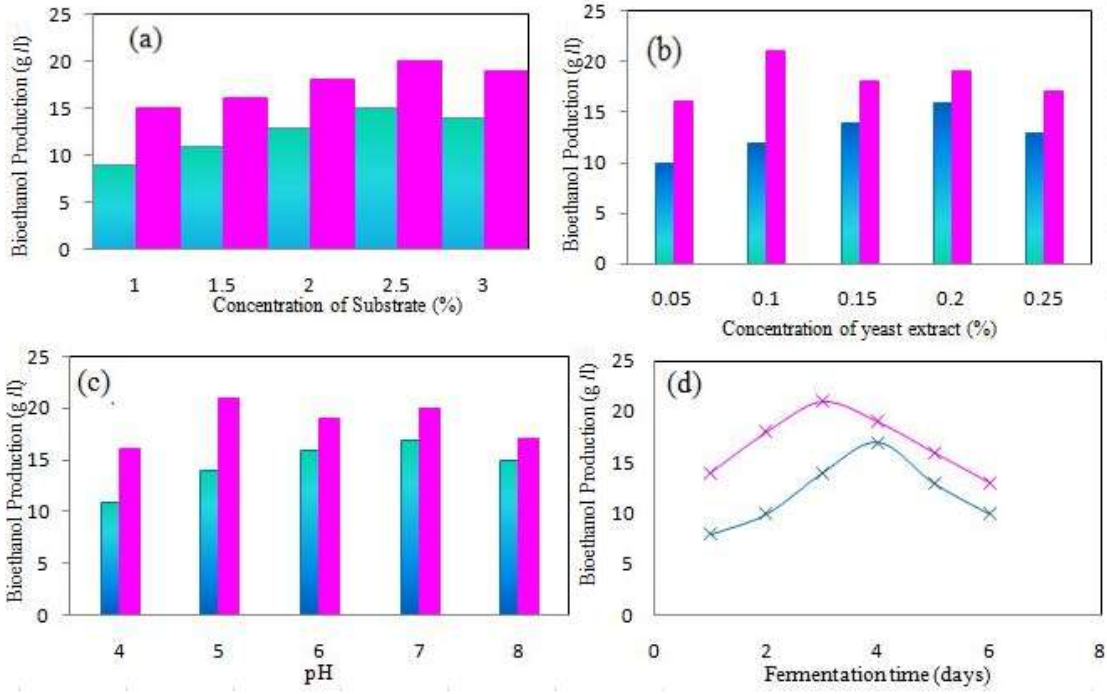

Fig. 4. Optimization of various process parameters for bioethanol production

Reusability of the immobilized enzyme

The reusability of enzyme is one of the main advantages of enzyme immobilization. It will also reduce the cost of the enzyme. The immobilized enzyme on cobalt oxide nanoparticle was used number of times to hydrolyze cellulose. After each use, the immobilized enzyme was washed properly with deionised water. It was observed that immobilized enzyme showed 75 % of activity even after 4th cycle (Fig. 5). This decrease can be due structural modification or protein denaturation due to repeated usage [17, 18].

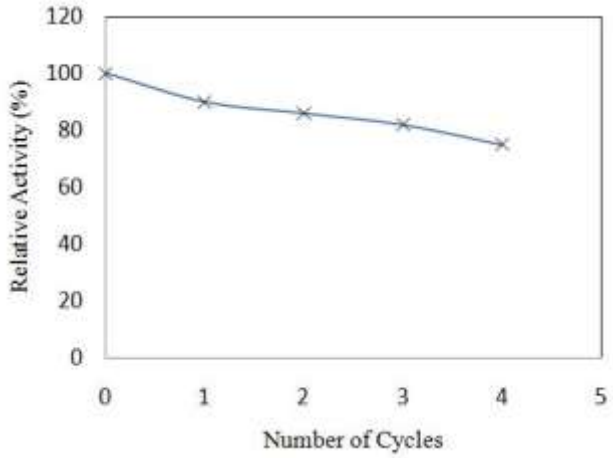

Fig. 5. Reusability of cellulose immobilized on cobalt oxide nanoparticles

Conclusion

Cellulase is one of the important industrial enzymes with wide applications. Due to the high cost of cellulase researchers are concentrating on the efficient usage of this enzyme by immobilizing on a support material. Immobilizing cellulase onto a nanoparticle can be a better option for increased stability and reusability. Fungal cellulase was immobilized on cobalt oxide nanoparticle by covalent method was efficiently used for production of bioethanol from lignocellulosic agricultural waste. The kinetics of immobilized was studied for both free and immobilized cellulase. The immobilized cellulase showed better hydrolytic activity than to free enzyme. Thus the cellulase immobilized on cobalt oxide nanoparticle can be effectively used for production of bioethanol.

Conflicts of Interest

The authors hereby declare that they have no conflict of interest.

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