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Optimized Saccharification of Carboxymethyl Cellulose (CMC) using Free and Immobilized Cellulase from *Aspergillus niger*

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ABSTRACT

Cellulase immobilization on solid supports is a revolutionary approach towards making cellulose hydrolysis economically viable at the industrial scale. Immobilized cellulases have significant advantages in operation stability, reusability, and affinity to the substrate over free enzymes. These advantages significantly reduce the enzyme usage, product contamination and allow continuous or repeated-batch processing, which are critical to economic industrial biocatalysis. In this study, we applied sodium alginate as a solid support in the immobilization of *Aspergillus Niger* cellulase. The model substrate used was carboxymethyl cellulose (CMC). The influence of pH, temperature, and substrate concentration on the saccharification of CMC by free and immobilized cellulase from *A. niger* were determined. Re-useability of the immobilized cellulase in the saccharification of CMC was also examined. CMC hydrolysis by both free and immobilized *A. niger* cellulase recorded highest yield at pH 6.0 and 3.0 with 10.9% and 8.5%, respectively. Optimal temperature for CMC hydrolysis by free and immobilized *A. niger* cellulase was obtained at 50°C and 40°C with 9.2% and 7.20% saccharification, respectively. Optimal saccharification of CMC by free and immobilized cellulase from *A. niger* was obtained at 1.5% and 0.5% (w/v) substrate concentration with 23.32% and 11.39% saccharification, respectively. Overall, immobilized cellulase systems enable higher catalytic productivity, lower enzyme cost, and improved process robustness making cellulase immobilization a critical enabler in industrial-scale biomass valorization and biofuel production.

Keywords: *Aspergillus Niger*; Cellulase Immobilization, Sodium Alginate; Saccharification, Carboxymethyl cellulose (CMC)

Introduction

Cellulose is a linear polysaccharide composed of β-1,4-linked D-glucose units (Li *et al*., 2018), with polymer chains ranging from hundreds to thousands of glucose units (Jabeen and Atif, 2023). Due to its abundance, availability and accessibility, cellulose is often used in various industrial sectors as raw materials for bio-processes. Cellulose is used in pulp, paper, textiles, packaging and biofuel industries (Aziz *et al.,* 2022). In the biofuel industry, cellulose serves as limitless primary product that can be broken down into fermenTable sugars used in the production of biofuels. Pure cellulose exists in various forms such as carboxymethyl cellulose (CMC) which are linear polymers consisting of several glucose units held together in highly crystalline microfibrils (Nasatto *et al.,* 2015). Pure cellulose are used in biotechlogy as model substrates in the study of saccharification of

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cellulose to simple sugars for biofuel production. Various methods are used in breaking down cellulose into monomers of glucose; including acid or alkali hydrolysis, chemical hydrolysis and biological methods which involves the use of microbes such as fungi or bacteria and enzymes. Enzymatic hydrolysis has proven to be the most suiTable method among other methods due to its higher conversion efficiency, cost effectiveness and minimal by-product formation (Amandio *et al.,* 2023). Saccharification of cellulose with the use of hydrolytic enzymes is achieved by breaking the glycosidic bonds within the cellulose structure to release fermenTable sugars (Zhao *et al.,* 2022).

Saccharification of cellulose occurs via a synergistic cascade of three major cellulases namely: *Endoglucanase, exoglucanase and β-Glucosidases.* They work simultaneously by first cleaving the internal glycosidic bonds to make available new chain ends, thereafter removes glucose units from cellulose chains terminal, and finally breaks the cellobiose units into glucose monomers, mitigating product inhibition and completing the saccharification process (Contreras *et al.,* 2020).

Saccharification of pure cellulosic substrates is a canonical procedure in biotechnology and bioengineering, both used as a model system to study cellulolytic processes and as a reference to test the effectiveness of cellulose to sugar conversion reactions (Sun *et al*., 2018). Although commercially available cellulase cocktails have been widely used, free enzymes present considerable drawbacks such as thermal and pH instability, product inhibition, recovery and reuse difficulties, and high costs when high turnover and enzyme recycling is necessary to make the process economical (Khoshnevisan *et al.,* 2019). It is therefore imperative to study immobilization of cellulase in order to overcome limitations encountered with the use of free enzymes (Seenuvan *et al*., 2020) and improve cellulose hydrolysis for biofuel production.

Enzyme immobilization is the confinement of enzymes onto solid supports offering numerous advantages such as reuseability, stability in the presence of inhibitors and extreme condition of pH and temperature (Bié *et al.,* 2022). Immobilization methods include entrapment, physical adsorption, covalent binding, cross-linking and encapsulation (Spasojevic *et al*., 2019). Immobilization by entrapment using sodium alginate as a solid support is considered a more accessible technique since it is rapid, cheap, easy, and can be conducted at mild conditions (Weng *et al.,* 2023). In this study, we evaluated the use of *Aspergillus Niger* cellulase in its free and immobilized form to hydrolyze CMC.

Materials and Methods

Commercial *A. niger* cellulase (0.8 U/mg), carboxymethyl cellulose (CMC), methyl cellulose, sodium alginate, calcium chloride, glucose, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, dinitrosalicyclic acid, sodium hydroxide, hydrochloric acid, sodium citrate, citric acid, potassium sodium tartarate, tris-(hydroxymethyl)-aminomethane, were purchased from Sigma-Aldrich labor chemikallen, EC label C.O.O. Germany. All other reagents used were of analytical grade.

Preparation of Pure Cellulase from *Aspergillus Niger*

Commercial cellulase (1.18u/mg) from *A.niger* was dissolved in 10 ml of 50 mM sodium citrate buffer (pH 4.8).

Cellulase Immobilization

The entrapment method of cellulase immobilization was carried out according to the method described by Niladevi and Prema (2008), using sodium alginate as the effective carrier material. The sodium alginate solution (2.5 % w/v) was measured and put into a 1:1 proportion with cellulase and mixed thoroughly by slight shaking in a rotary shaker. Beads were produced by adding the viscous mixture of alginate and cellulase dropwisely with the use of a 0.4-mm diameter syringe into 0.2 M calcium chloride solution with stirring. The beads were hardened in

4℃ for 2 h. The harvesting of the alginate beads was done through filtration and the beads were washed multiple times with distilled water until no cellulase activity was observed in the washed water.

Cellulase Activity

Cellulase activity was measured following the method described by Wood and Bhat (1998) with slight modifications. 150ul of cellulase was reacted with 450 ul of 1 % (w/v) CMC, (prepared in 50mM sodium citrate buffer (pH 4.8)) and incubated at 40℃ for 20 minutes. For the immobilized cellulase, the reaction consisted of 8 cellulase immobilized beads (equivalent to the volume and activity of 150 ul free cellulase) and 450 ul of substrate solution. Reaction was stopped by adding 400 ul of dinitrosalicylic acid (DNS) and reaction tubes were placed in water bath at 100℃ for 5 minutes. Absorbance was measured at 575 nm against the blank (50 mM sodium acetate buffer). A unit of CMCase activity was defined as 1 umole of liberated glucose released per minute under standard assay condition.

Enzymatic Saccharification of Carboxymethyl Cellulose (CMC)

Enzymatic hydrolysis of CMC was carried out following the methods of Holtzapple *et al*. (1994) with slight modification. CMC (2% w/v) was dissolved in 10 ml buffer and mixed with 5ml cell-free cellulase in a 50-ml Erlenmeyer flask. Sodium azide (0.3 g/L) was added to inhibit microbial contamination (Hendrix *et al*., 2019). The reaction mixture for immobilized cellulase comprised 9.15 g of beads of immobilized cellulase of equivalent volume and activity to 5 ml free cellulase and 2% substrate solution. The enzymatic hydrolysis was carried out for 2 h at 50°C in orbital incubator (150 rpm). After the saccharification period, the reaction mixture was filtered with the use of 0.22µm sterile syringe filters to remove unhydrolyzed substrate and glucose concentration of the filtrate was determined according to standard procedure.

Estimation of Glucose Concentration during Enzymatic Hydrolysis

Dinitrosalicylic acid method of glucose estimation was used to determine the amount of glucose released during enzyme hydrolysis (Miller, 1959). The reaction mixture consist of 300 ul of the supernatant and 700 ul of the dinitrosalicylic acid (DNS) solution, these was boiled at 100℃ in 5 minutes. The reaction mixture was cooled on water and absorbance recorded at 575 nm. The quantity of glucose released in the hydrolysis was determined by the use of glucose standard curve and presented in mg/mL. The percentage saccharification was determined by following the equation of Mandels and Sternberg (1976) as shown below: The percentage saccharification was determined by converting polysaccharide to monosaccharide in the presence of water as shown by the following equation:

$$\% \text{ Saccharification} = \frac{\text{Reducing sugars } \left(\frac{\text{mg}}{\text{mL}}\right) \times 0.9 \times 100\%}{\text{Initial substrate concentration } \left(\frac{\text{mg}}{\text{mL}}\right)} \quad (1)$$

The factor 0.90 was used to convert polysaccharide to monosaccharide accounting for water uptake during hydrolysis.

Optimization of Enzymatic Saccharification of Carboxymethyl cellulose (CMC)

Effect of pH on Enzymatic Saccharification of CMC

The influence of varying (pH 3.0 Glycine-Hcl, pH 4-5 Sodium Citrate, pH 6-7 Pottasium Phosphate, pH 8-9 Tris-NaoH, and pH 10-11.0 Glycine NaoH) on the saccharification of CMC was studied using free and immobilized cellulase from *A. niger*. Carboxymethyl cellulose (2% w/v) was mixed with 5 ml of cell-free cellulase in a 50 ml Erlenmeyer flask containing 10 ml buffers of varying pH (3.0 to 11.0). In order to inhibit microbial contamination (0.3 g/L) sodium azide was added to the reaction mixture (Hendrix *et al*., 2019). The enzymatic saccharification was carried out for 2 h at 50°C in orbital incubator (150 rpm). For immobilized cellulase, the reaction comprised 9.15 g of immobilized cellulase beads equivalent to the volume and activity to 5 ml cell-free cellulase, and 2% substrate solution. After the saccharification process, the unhydrolyzed substrate was filtered with the use of 0.22 µm sterile syringe filters, and

glucose concentration of the filtrate was determined according to standard procedure.

Effect of Temperature on Enzymatic Saccharification of CMC

The influence of temperature (30 - 70°C) on saccharification of CMC was studied using free and immobilized cellulase from *A. niger*. CMC (2% w/v) was mixed with 5 ml of cell-free cellulase in a 50 ml Erlenmeyer flask containing 10 ml buffer and 0.3 g/L of sodium azide was added to eliminate contaminants by microbes (Hendrix *et al*., 2019). The enzymatic saccharification was carried out for 2 h at varying temperatures 30 to 70°C in orbital incubator (150 rpm). For immobilized cellulase, the reaction comprised 9.15 g of immobilized cellulase beads equivalent to the volume and activity to 5 ml cell-free cellulase, and 2% substrate solution. After the saccharification process, the unhydrolyzed substrate was filtered with the use of 0.22 µm sterile syringe filters, and glucose concentration of the filtrate was determined according to standard procedure.

Effect of Substrate Concentration on Enzymatic Saccharification of Celluloses

The influence of varying substrate concentration (0.5% to 2.0% (w/v)) on saccharification of CMC was studied using free and immobilized cellulase from *A. niger*. CMC (0.5% - 2% w/v) was mixed with 5 ml of cell-free cellulase in a 50 ml Erlenmeyer flask containing 25 ml buffers of optimum saccharification pH of CMC, and sodium azide (0.3 g/L) was added to eliminate microbial contamination (Hendrix *et al*., 2019). The enzymatic saccharification was carried out for 24 h at optimal temperature of CMC hydrolysis, in orbital incubator (150 rpm). For immobilized cellulase, the reaction comprised 9.15 g of immobilized cellulase beads equivalent to the volume and activity to 5 ml cell-free cellulase, and 2% substrate solution. Samples were collected and filtered using 0.22 µm sterile syringe filters to remove unhydrolyzed substrate and the glucose concentration of filtrates were determined according to standard procedure.

Reuseability of Immobilized cellulase

The reuseability study of the immobilized cellulase from *A. niger* cellulase was achieved through the hydrolysis of CMC. The enzymatic hydrolysis of CMC 0.5 % (w/v) in 50 mM Glycine-Hcl buffer pH 3, was carried out for 4 h at 40°C in orbital incubator (150 rpm). After each cycle of CMC hydrolysis, the alginate beads were collected, washed with distilled water and applied into a fresh solution of carboxymethyl cellulose. The steps were repeated 3 times, and the activity estimated from the solution after each cycle.

Statistical Analysis

All studies were done in triplicates and statistical analysis (Mean ± Standard Deviation) was evaluated and plotted using Microsoft Excel (2007).

Results and Discussion

Optimization of Cellulose Saccharification by Free and Calcium Alginate Entrapped Cellulase from *A. niger*

Effect of pH on Saccharification of CMC

The effect of pH (3.0-11.0) on saccharification of carboxymethyl cellulose (CMC) by both free and immobilized cellulase from *A. niger* was evaluated. Carboxymethyl cellulose had optimal saccharification at pH 6.0 and 3.0 with (10.91%) and (8.500%) percentage saccharification, respectively. Percentage saccharification of carboxymethyl cellulose at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 had 10.5 and 8.50%, 10.50 and 8.10%, 10.60 and 8.10%, 10.90 and 7.60%, 10.50 and 8.30%, 10.40 and 5.20%, 10.10 and 4.20%, 10.60 and 4.50%, 9.50 and 4.30%, respectively (Figure 1).

Saccharification of CMC by *A. niger* cellulase (free and immobilized) was higher in the acidic region with optimal saccharification values at pH 6 and 3, respectively. It was observed that, the yield of CMC hydrolysis by the immobilized cellulase stabilized from the acidic pH 3.0 towards the neutral pH 7.0

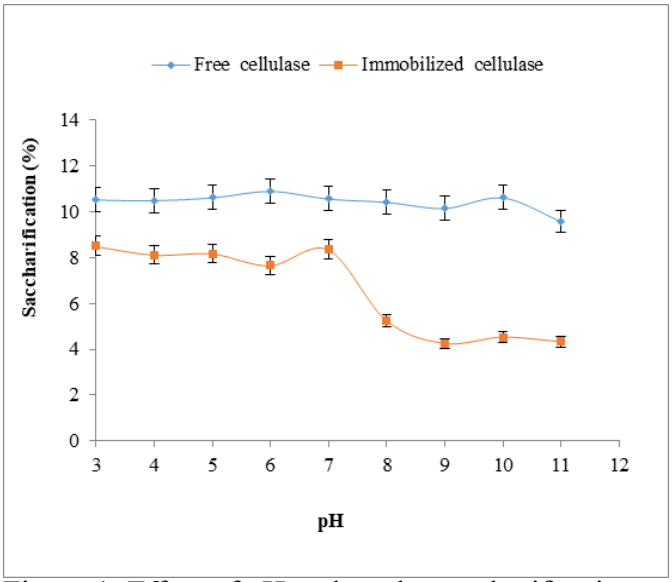

Figure 1: Effect of pH and on the saccharification of CMC by free and immobilized cellulase from *A. niger.*

after which it declined (Figure 1). This result shows that cellulase is highly dependent on pH for maximum output, due to the influence of pH on both enzyme structure and substrate interactions **(**Røjel *et al*., 2020)**.** This result agrees with a report by El-Gendi *et al.* (2022) that, enzyme efficiency depends on the structure, conformation and the correct ionization states of key catalytic residues. As the pH shifts away from the enzyme's optimum, essential amino acids in the active site may become improperly protonated or deprotonated, disrupting catalysis and reducing efficiency (Iyengar *et al*., 2022). Previous study by Kakde and Aithal (2019), reported that *A. niger* derived cellulase exhibited its highest yield of filter-paper degradation at **pH 5.5**. As the pH becomes more alkaline, the enzyme tertiary structure might be destabilized as a result of dissociated electrostatic interactions and hydrogen bonds. Therefore, denaturation or loss of binding affinity takes place, and the efficiency of saccharification is decreased. The stability of immobilized cellulase in the acidic area during the hydrolysis of CMC indicates that it can be used in industries to hydrolyze cellulosic substrates that need acidic pH. A report by Ashkan *et al.* (2023) revealed the ability of immobilized enzyme to withstand higher acidic pH, and more temperatures compared to free enzyme.

Effect of Temperature on Saccharification of CMC

The influence of temperature (30℃-70℃) on saccharification of CMC by free and immobilized cellulase of A. niger was determined. The best saccharification of CMC was achieved at a temperature of 50℃ and 40℃ with a saccharification of 9.26% and 7.25% respectively. Percentage saccharification of carboxymethyl cellulose at 30°C, 40°C, 50°C, 60°C, 70°C was 1.20 and 6.50%, 7.10 and 7.20%, 9.20 and 7.20%, 3.70 and 5.20%, 2.40 and 5.00%, respectively (Figure 2). In the free or immobilized form, temperature had a great impact on the saccharification of CMC using the *A. niger* cellulase. The immobilized cellulase of *A. niger* showed maximum hydrolysis of CMC at 40°C and minimum at 50 °C after which it decreased steadily with rise in temperature. Interestingly, immobilized *A. niger* cellulase still showed 69.06% of the saccharification activity at 70°C as opposed to the free cellulase which only showed 26.90% at the same temperature. One can explain this behavior by the effect of calcium alginate support on stabilization (Kalita and Sit, 2023). The support matrix containing the calcium alginate may be what gives the enzyme its native state protection against the effects of heat or loss of its activity due to heat, and thus the enzyme increases its catalytic ability. According to Li *et al.* (2019), their research revealed that alginate beads confer a rigid external framework to the cellulase molecules. The properties of the immobilization matrix provide improved thermal stability, limiting conformational flexibility of the enzyme and assist in preserving the active structure and retaining enzymatic activity at different temperatures (Shah *et al.,* 2008). A major consideration that is relevant to the use of enzymes is the capacity to maintain catalytic activity despite the increased temperature, which is challenging when using the free enzymes. The free enzyme's active site is easily exposed to denaturing effect such as heat which drastically reduces its catalytic efficiency unlike immobilized enzyme that is shielded by the matrix (Zhang *et al.,* 2016). Findings from this study has revealed the benefits of immobilized cellulase

over free cellulase, in its application across varying temperatures.

Effect of Substrate Concentration on Enzymatic Saccharification of CMC

CMC hydrolysis using free and immobilized *A. niger* cellulase was optimum at 1.50 and 0.5% concentration with 13.71% and 25.79% saccharification, respectively

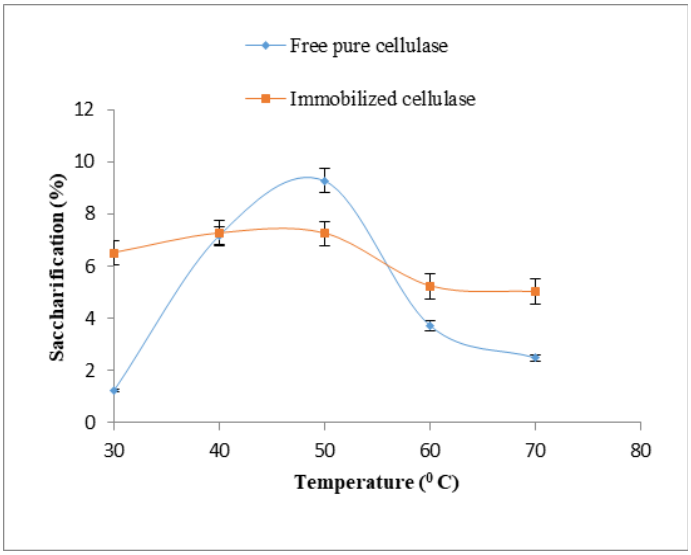

Figure 2: Effect of Temperature on the saccharification of CMC by free and immobilized cellulase from *A. niger.*

(Figure 3). It was observed in this study that the rate of CMC saccharification by immobilized cellulase gradually decreased as the substrate concentration increased. Contrary to this report, a study by Kalita and Sit (2023) reported an increase in calcium alginate immobilized cellulase activity at increased substrate concentration during CMC hydrolysis. Another report by Abraham *et al.* (2019) reported a rise CMC hydrolysis at higher substrate concentration by both form of cellulases. The lesser yield observed with an increasing substrate concentration might be due to steric hinderance, reduced enzyme flexibility and diffusion. In addition, saturation of the immobilized cellulase active site might hinder its accessibility to higher substrate concentration (Homaei *et al.,* 2013). Also, the bead size, pore size, and enzyme loading per bead might contribute to the limit in diffusion of the substrate into the enzyme's active site (Andriani *et al.,* 2012).

CMC hydrolysis by free cellulase showed minimal increase in saccharication yield from 0.5% to 1.5% substrate concentration after which it declined. It is interesting to note that higher yield of CMC hydrolysis by immobilized cellulase from *A. niger* was achieved across the varying substrate concentration evaluated, outscoring the result obtained by free cellulase. This result might be attributed to the interaction and the stabilizing effect of calcium alginate on cellulase catalytic site (Weng *et al.,* 2023). The enhanced catalytic efficiency of immobilized cellulase over free cellulase observed in CMC hydrolysis shows its ability to overcome the rigorous challenges of enzymatic hydrolysis such as high substrate loading. This result ensures its robustness in industrial applications where conversion of larger volume of cellulose is required.

Reuseability Study on Immobilized Cellulase from A. *niger*

The *A. niger* immobilized cellulase was tested for its residual activity for three consecutive cycles. The immobilized cellulase activity in the first cycle

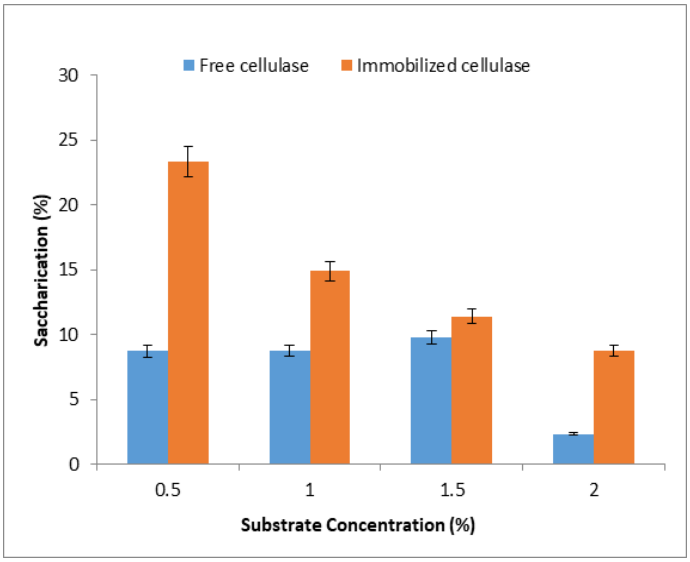

Figure 3: Effect of substrate concentration on the saccharification of CMC by free and immobilized cellulase from A. *niger.*

of CMC hydrolysis was considered 100% activity (Figure 4). In this study, it was observed that the residual activity of *A. niger* immobilized cellulase upon CMC hydrolysis rapidly declined after the first

cycle to about 57% in the second reused cycle, and finally retained approximately 48% residual activity.

Similarly, Kalita and Sit (2023) reported a sharp decline in CMC hydrolysis by alginate entrapped cellulase between the first and the second cycle; and concluded that the immobilized cellulase might have experienced a sudden heat shock that caused the matrix pores to loosen up resulting in activity loss. Previously, Li *et al.* (2019) reported 60.90% of sodium alginate immobilized cellulase activity after three cycles of CMC hydrolysis. Noticeably, the cellulase entrapped calcium alginate beads gradually melted and loosed shape after the third cycle of CMC hydrolysis. This could be explained by the fact that every cycle is followed by repeated washes of the immobilized beads, which leads to the leakage of cellulase and changes in the enzyme structure, as well as the subsequent loss of residual activity (Zhou *et al.,* 2019; Rehman *et al*., 2013 *et al.,* 2013). This decrease in activity could also be attributed to the frequent exposure of the substrate to the immobilized cellulase active site leading to alterations in the conformation of the active site (Agrawal *et al.,* 2020). Previous study by Abraham *et al.* 2014 reported that, CMC hydrolysis was sustained to approximately 70 % of its activity by magnetic nano-particle immobilized cellulase until the third cycle. Another study by Zhang *et al.* (2016) stated that after the third cycle of CMC hydrolysis, the activity of the silica gel immobilized cellulase had reduced drastically to 58 % as compared to the first cycle. A remarkable characteristic of enzyme immobilization is its reuseability which limits operational cost in processes that require enzyme application. This study has revealed the potentials of calcium alginate immobilized cellulase by *A. niger* as a useful technique for industrial applications in cellulose bioconversion.

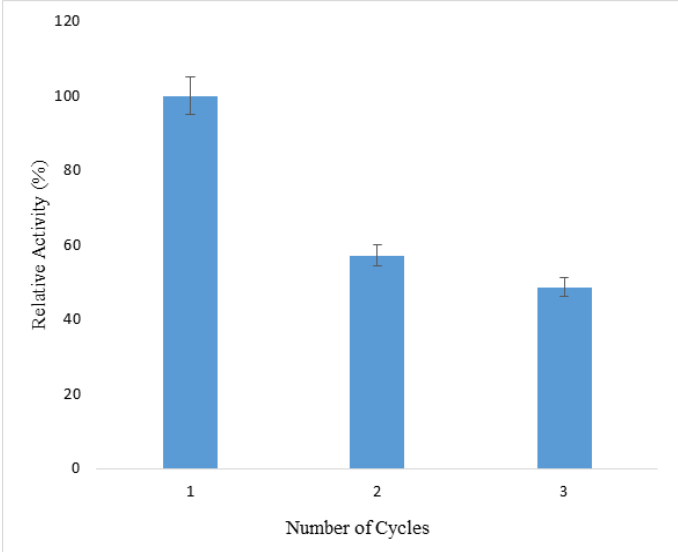

Figure 4: Reuseability of *A. niger* cellulase immobilized on sodium alginate and its relative activity on CMC hydrolysis

Conclusion

This study examined the effects of some parameters on the saccharification efficiency of both free and immobilized *Aspergillus niger* cellulase during the saccharification of CMC. Findings from this research highlighted the importance of these parameters in improving the process of cellulose conversion. Remarkably, *A. niger* cellulase in its immobilized state performed better than free cellulase with respect to catalytic efficiency on substrate concentration study. The results reveal the possibilities of cellulase immobilization as a promising and effective method of cellulose hydrolysis in industries.

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