IMMOBILIZATION OF CELLULASE ENZYME IN CALCIUM ALGINATE GEL AND ITS IMMOBILIZED STABILITY

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ABSTRACT

In this study, cellulase enzyme is immobilized in calcium alginate bead by entrapment method. Sodium alginate concentration used for alginate bead forming is 2%. Immobilized enzyme activity is evaluated on carboxymethyl cellulose (CMC). The maximum efficiency of enzyme immobilization is 83.645% with immobilized time 30 minute and bead diameter 3mm. The optimum pH value of immobilized enzyme and free enzyme is 4.5. The optimum temperature value of immobilized enzyme is higher than free enzyme, 60^{o}C and 55^{o}C respectively. Immobilized enzyme is more stable versus the change of pH and temperature of environment than free enzyme. Immobilized enzyme can stand in higher acidity and temperature than free enzyme. Immobilized cellulase could be reused many times. Immobilized enzyme activity remains 69.2% after 5 recycles and still 20.3% after 8 recycles.

Keywords: *cellulose, calcium alginate, sodium alginate, entrapment, immobilization, enzyme activity, recycle*

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1. INTRODUCTION

1.1 Cellulose

Cellulose is a long chain of linked sugar molecules that gives wood its remarkable strength. It is the main component of plant cell walls, and the basic building block for many textiles and for paper. Cotton is the purest natural form of cellulose. In the laboratory, ashless filter paper is a source of nearly pure cellulose [6, 21].

1.2 Cellulase

Cellulase, a multicomponent enzyme, consisting of three different enzymes (endocellulase, cellobiohydrolase and ß-glucosidase) is responsible for bioconversion of cellulose into soluble

sugar. Generally, cellulases are used in various applications, including food, brewery and wine, agriculture, textile, detergent, animal feed, pulp and paper industry, as well as in research development [1, 5, 8, 9, 12, 13, 14, 15, 18, 22]. The technique of protein cross-linking by the reaction of glutaraldehyde with reactive Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities. Cross-linked enzyme aggregates (CLEAs) are selected to be a prominent route of enzyme immobilization technique, without the necessity of a solid support. Moreover, the cross-linked cellulase aggregates represent a suitable form of immobilized enzymeto be used in largescale production processes and biotransformations, even on industrial scale. The cross-linked cellulase aggregates is carrier-free immobilized enzyme, in virtuallypure cellulase and the negative effects of carriers can thus be avoided In general practice, the procedure to prepare CLEAs includes two major steps that involve precipitation of soluble enzyme with suitable precipitant and crosslinking with an appropriate cross-linker, during which the particle size increases [7].

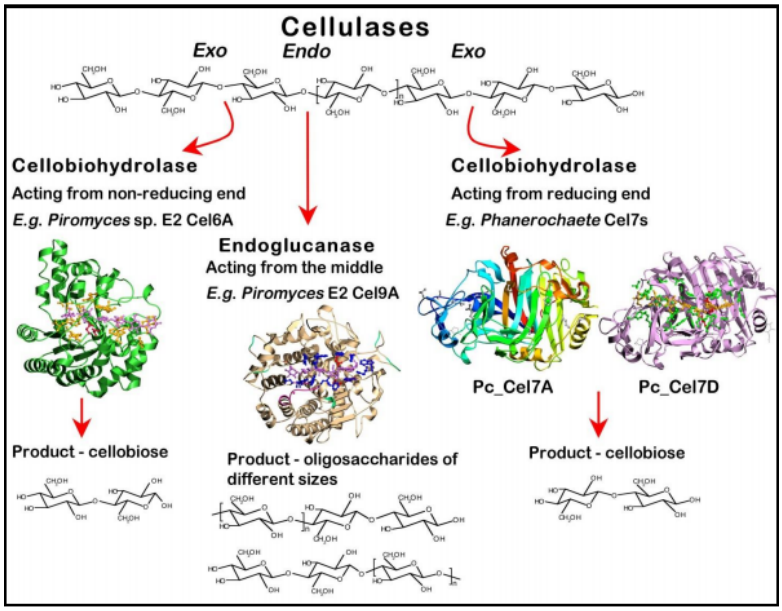


Figure 1. Synergistic action of cellulose.

Several studies mentioned to cellulose immobilization. Ajoy C. Chakrabarti et al. (1988) conducted the immobilization of cellulase using polyurethane foam [2]. Albert Garcia et al. (1989) surveyed the cellulase immobilization on Fe_3O_4 [4]. Primoz Plahuta, Peter Raspor (1996) showed cellulase Immobilization on Ca-alginate Beads [16]. Sandy Budi Hartono et al. (2010) functionalized mesoporous silica with very large pores for cellulase immobilization [20]. Zhou J. (2010) investigated the immobilization of cellulase on a reversibly soluble-insoluble support [24]. Mandali, Pavani (2010) demonstrated the immobilization of cellulase and hemicellulases on porous glass beads [11]. Kamyar Khoshnevisan, et al. (2011) carried out the immobilization of cellulase enzyme on superparamagnetic nanoparticles and determination of its activity and stability [10]. S. Anuradha Jabasingh et al. (2011) investigated the optimization and immobilization kinetics of Aspergillus nidulans cellulase

onto modified chitin by response surface approach. The study dealed with the immobilization of Aspergillus nidulans cellulase onto modified chitin (MC) [19]. Al-Khatib et al. (2012) established *a* statistical modelling optimisation of cellulase enzyme immobilisation on functionalised multi-walled carbon nanotubes for empty fruit bunches degradation [3]. Zhongliang Su et al. (2012) determined the cellulase immobilization properties and their catalytic effect on cellulose hydrolysis in ionic liquid. Cellulase was immobilized on chitosan by the methodof covalent binding [23]. Rasha Mohammed Abd et al. (2012) optimized the immobilised cellulase onto carbon nanotubes using response surface methodology [17]. In our research, cellulose will be directly entrapped in calcium alginate by capture, then the immobilized enzyme activity will also be investigated with CMC substrate. Moreover we compare the characteristics of the immobilized enzyme and free enzyme on the same substrate CMC and reaction parameters to see the correlation between two enzymes.

2. MATERIAL AND METHODS

2.1 Raw material

2.1.1 Enzyme source

Celluclast 1.5L from *Trichoderma reesei* is provided by Novozymes (Denmark), kept at 4^0C

2.1.2 Cellulose substrate: CMC and filter

CMC used in our experiments is CMC CEKOL 4000 (powder, 99.5% purity). CEKOL 4000 soution 1% has viscosity 300- 700 mPas at room temperature 25^{o}C. CEKOL 4000 has the replacement degree 0.75-0.85. Filter Whatman No.1 is made from insoluble cellulose.

2.1.3 Calcium alginate carrier

Calcium alginate beads are made by external gel formation. Sodium alginate drips directly into $CaCl_2$ solution containing cellulase.

2.2 Method

2.2.1 Immobilisation protocol

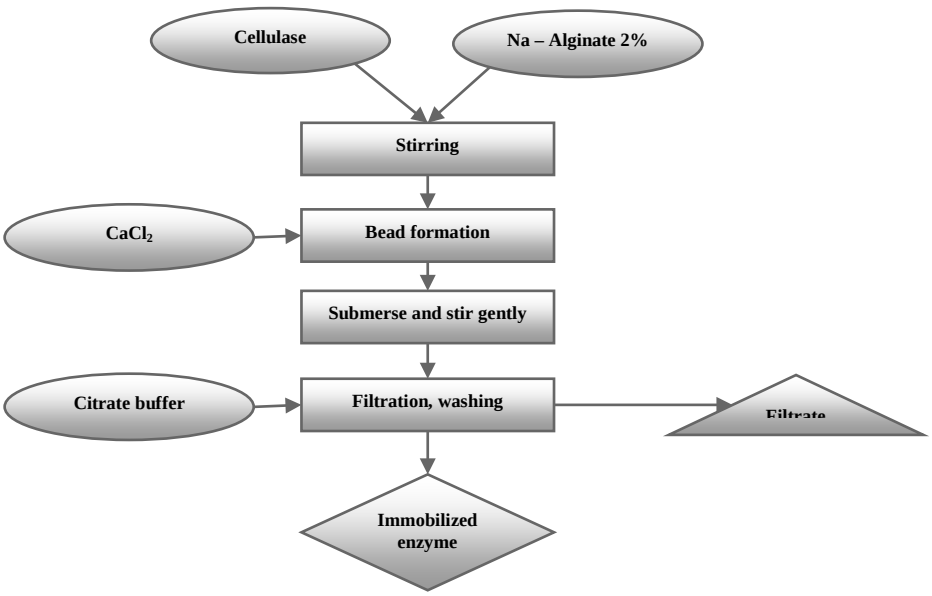


Figure 2. Cellulase immobilization protocol.

2.2.2 Research protocol

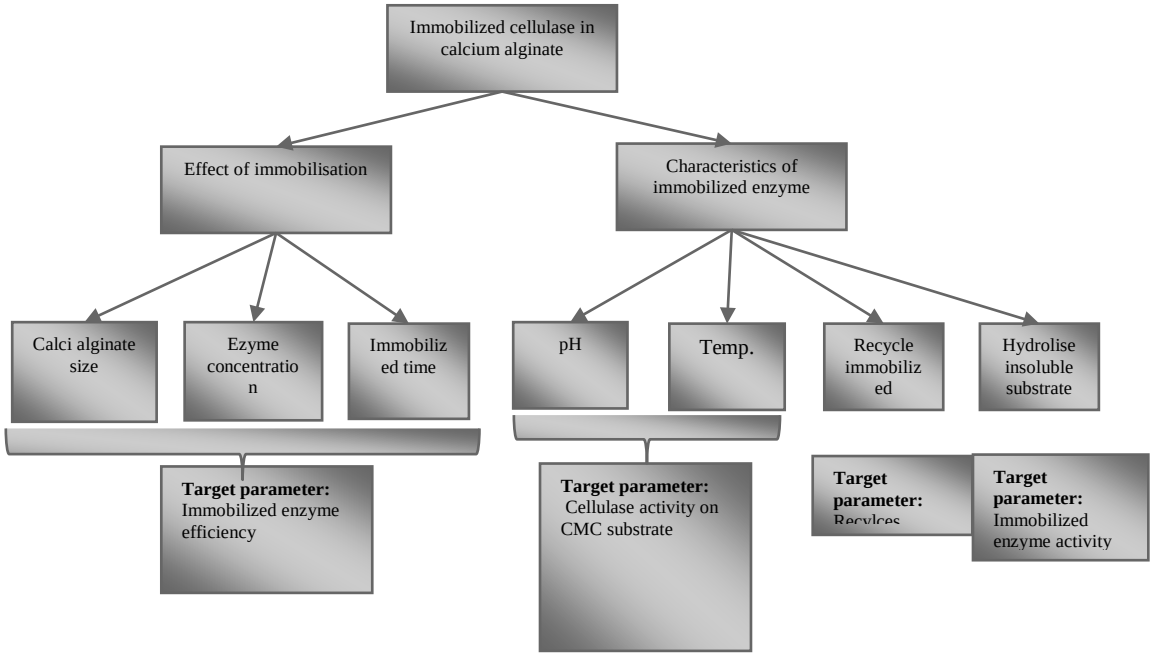


Figure 3. Research protocol.

2.2.3 Research description

a) Factors affect to immobilization

- **Effect of calcium alginate bead size**
- Experimental parameters: gel sizes 2.0, 2.5, 3.0 mm
- Fixed parameters:
  $CaCl_2$ concentration: 0.15M
  Sodium alginate concentration: 2%
  Submerse calcium alginate gel with enzyme in $CaCl_2$ solution within 30 minutes
  Immobilizing solution: 10%
- Target parameter: immobilized enzyme efficiency
- **Effect of immobilizing enzyme concentration:**
- Experimental parameters: enzyme concentration in immobilizing solution 5%, 10%, 15%, 20%, 25%, 30%.
- Fixed parameters:
  $CaCl_2$ concentration: 0.15M
  Sodium alginate concentration: 2%
  Submerse calcium alginate gel with enzyme in CaCl2 solution within 30 minutes
  Gel bead size (3 mm)

- Target parameter: immobilized enzyme efficiency

❖ **Effect of immobilizing time:**

- Experimental parameters: Gel beads are immersed in $CaCl_2$ (with stirring) in 5 minutes, 15 minutes, 30 minutes, 45 minutes and 60 minutes. Then washing beads to collect immobilized enzyme and determine protein in immersed soultion.
- Fixed parameters:
  $CaCl_2$ concentration: 0.15M
  Sodium alginate concentration: 2%, enzyme concentration 10%
  Gel bead size gel (3 mm)
- Target parameter: immobilized enzyme efficiency

b) Charactesistics of immobilized enzyme

❖ **Effect of pH to immobilized enzyme activity**

Immobilized enzyme reacts to CMC in different pH values within 30 minutes, at 55^oC. After reaction, we determine the immobilized enzyme activity in these pH values.

- Experimental parameters: pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0
- Fixed parameters:
- Reaction temperature: 55^oC
- Reaction time: 30 minutes
- Target parameter: Immobilized enzyme activity on CMC substrate.

❖ **Effect of temperature to immobilized enzyme activity**

Immobilized enzyme reacts to CMC in different temperature values within 30 minutes, at determined pH. After reaction, we determine immobilized enzyme activity in these temperature values.

- Experimental parameters: Reaction temperature 30, 40, 50, 55, 60,70, 80, 90°C
- Fixed parameters:
- pH: choose the optimized pH value as above.
- Reaction time: 30 minutes
- Target parameter: Immobilized enzyme activity on CMC substrate.

❖ **Recylce availability:**

Immobilized enzyme reacts to CMC in determined temperature, pH and replicate many times on the same enzyme. Collect reacted enzyme, then determine the immobilized enzyme activity after recycles.

- Experimental parameters: immobilized enzyme actitity after 5-10 recycles.
- Fixed parameters:
- pH: choose the optimized pH as above
- Reaction temperature: choose the optimized temperature as above.
- Reaction time: 30 minutes
- Target parameter: Recycles of the immobilized enzyme.

❖ **Hydrolise insoluble substrate:**

Hydrolize Whatman filter paper by the immobilized enzyme in the optimal temperature, pH and reaction time. Collect sample and determine enzyme activity on filter.

- Fixed parameters:
- pH: choose the optimized pH as above
- Reaction temperature: choose the optimized temperature as above
- Reaction time: 30 minutes
- Target parameter: enzyme activity on Whatman filter

2.2.4 Analytical and calculating methods

Determine cellulase activity (T. K. Ghose, 1987)
Determine protein soluble (M. M. Bradford, 1976)
Statistical analysis: Statgraphic *plus* software

3. RESULTS AND DISCUSSION

3.1 Effect of bead size to immobilized efficiency

In this experiment, free zyme is mixed in sodium alginate solution with ratio 10% (V enzyme/V enzyme –sodium alginate). Gel bead formation has diameter 2.0, 2.5, 3.0 mm.

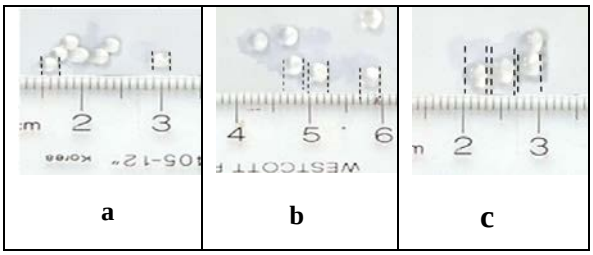


Figure 4. Immobilized enzyme a) Size 2 mm, b) Size 2.5 mm, c) Size 3mm.

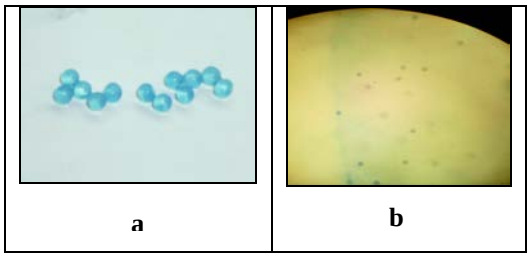


Figure 5. Immobilized enzyme a) Beads dyed Bradford, b) Enzyme inside alginate gel bead.

Dyed with Bradford reagent, sliced and observed under microscope x40, we can see dyed enzyme distributed inside small calcium alginate bead, and outer layer. Immobilized enzyme efficiency with different bead sizes is depicted as follow.

Table 1. Immobilized enzyme efficiency at different bead sizes

Bead diameter (mm)	Efficiency (%)
2.0	83.59 [a]
2.5	86.16 [b]
3.0	90.93 [c]

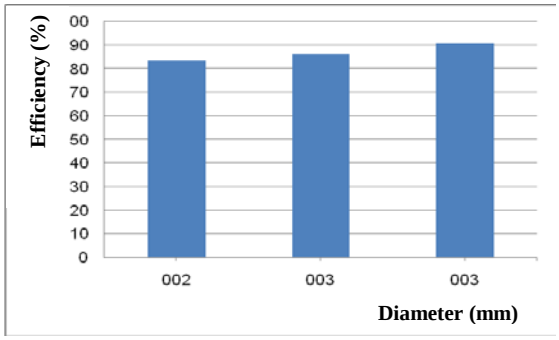


Figure 6. Immobilized enzyme efficiency at different bead sizes

We choose enzyme immobilisation bead size 3mm for further experiments.

3.2 Effect of enzyme concentration to immobilization efficiency

In this experiment, we use free enzyme immobilized in alginate gel 2% with various concentration 5, 10, 15, 20, 25, 30 % (V enzyme/V enzyme - alginate). Solution enzyme – alginate forms beads. Submerse them into $CaCl_2$ in 30 minutes. Immobilized enzyme efficiency at different enzyme concentration is shown as follow:

Table 2. Immobilized enzyme efficiency at different enzyme concentration

Enzyme concentration % (v/v)	Efficiency (%)
5	85,478[a]
10	85,372[a]
15	84,900[b]
20	84,808[b]
25	84,462[b]
30	83,668[c]

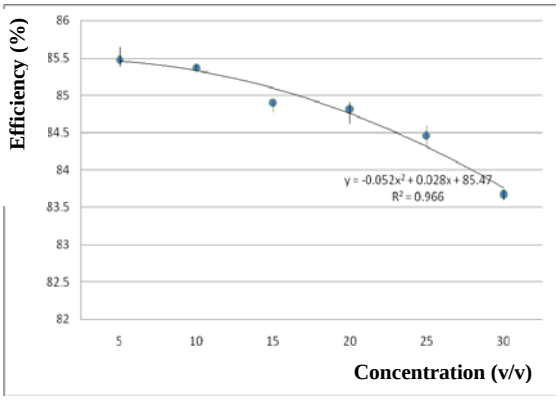


Figure 7. Immobilized enzyme efficiency at different enzyme concentration.

We select immobilized enzyme concentration 10% for further experiments.

3.3 Effect of immobilizing time to efficiency

In this experiment, we use free enzyme immobilized in sodium alginate gel 2% with various concentration 10% (V enzyme/V enzyme - alginate). Solution enzyme – alginate forms beads. Submerse them into $CaCl_2$ in 5, 15, 30, 45, 60 minutes. Immobilized enzyme efficiency at different submersed periods is shown as follow:

Table 3. Immobilized enzyme efficiency at different submersed periods

Time (minutes)	Efficiency (%)	Broken ratio (%)
5	96.087[a]	5
15	88.756[b]	7
30	83.645[c]	8
45	83.554[c]	8
60	83.504[c]	8

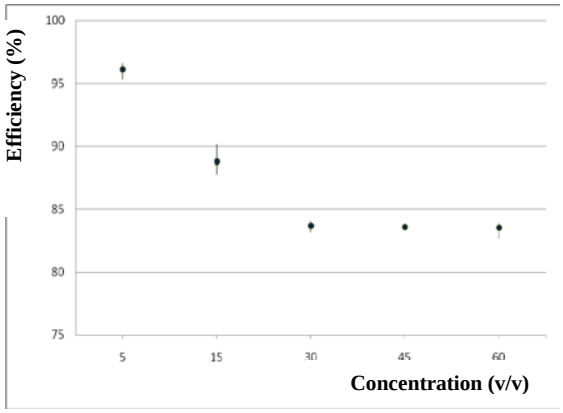


Figure 8. Immobilized enzyme efficiency at different submersed periods

We select the submersed period 30 minutes for further experiments.

3.4 Effect of pH to immobilized enzyme activity

In this experiment, immobilized enzyme hydrolises with CMC solution in pH range 3-7, within 30 minutes, 55^{o}C. We also perform the control test using free enzyme. Results of immobilized enzyme activity on CMC at different pH values are shown as follow:

Table 4. Immobilized enzyme and free enzyme activity on CMC at different pH values

pH	Immobilized enzyme activity (NCU/g protein enzyme)	Correlation activity (%)	Free enzyme activity (NCU/g protein enzyme)	Correlation activity (%)
3	91.97[a]	69.75	533.46 [a]	62.48
3.5	106.68[b]	81.72	627.63[b]	75.08
4	118.21[c]	89.21	786.19[c]	93.90
4.5	132.27[d]	100	835.66[d]	100
5	128.14[e]	96.73	754.10[e]	92.86
5.5	124.25[f]	94.64	736.62[f]	87.57
6	115.50[g]	87.42	600.81[g]	73.97
6.5	103.15[h]	78.55	436.38[h]	45.11
7	91.63[a]	70.38	126.17[i]	15.06

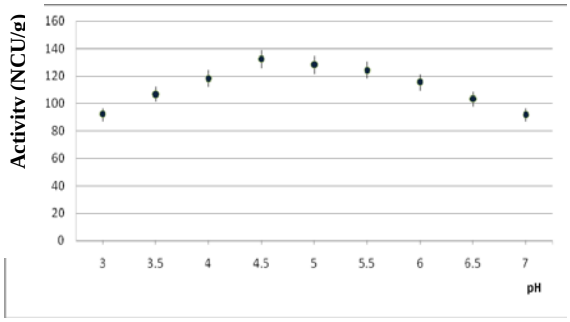


Figure 9. Immobilized enzyme activity on CMC at different pH values

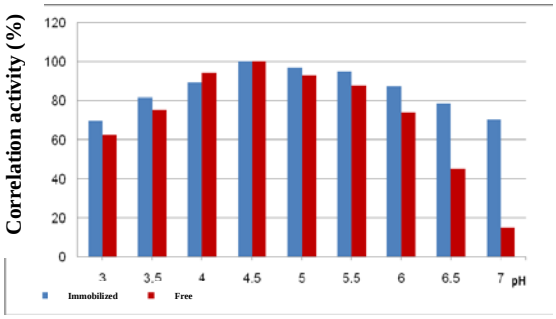


Figure 10. Correlation activity of immobilized enzyme and free enyme on CMC at different pH values.

We decide to choose pH 4.5 for further experiments.

3.5 Effect of temperature to immobilized enzyme activity

In this experiment, immobilized enzyme hydrolises with CMC in the temperature range 30^{o}C to 90^{o}C, in 30 minutes, at pH 4.5. We also conduct the control tests with free enzyme. Results of immobilized enzyme activity on CMC at different temperature values are shown as follow:

Table 5. Immobilized and free enzyme activity on CMC at different temperature values

Reaction temperature (^{o}C)	Immobilized enzyme activity (NCU/g protein enzyme)	Correlation activity (%)	Free enzyme activity (NCU/g protein enzyme)	Correlation activity (%)
30	115.77^{a}	88.24	611.63^{a}	60.42
40	124.96^{b}	95.24	658.55^{b}	65.06
50	128.64^{c}	98.04	766.91^{c}	75.76
55	131.21^{d}	100	1012.30^{d}	100
60	138.99^{e}	105.93	931.20^{e}	91.99
70	122.63^{f}	93.46	707.85^{f}	69.93
75	111.83^{g}	85.23	538.74^{g}	53.22
80	70.54^{h}	53.76	249.93^{h}	24.69
90	66.27^{i}	50.51	166.23^{i}	16.42

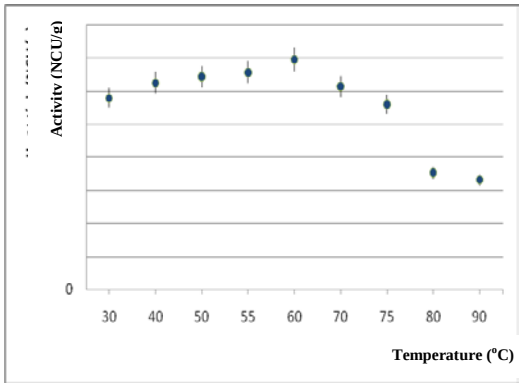


Figure 11. Immobilized enzyme activity on CMC at different temperature values.

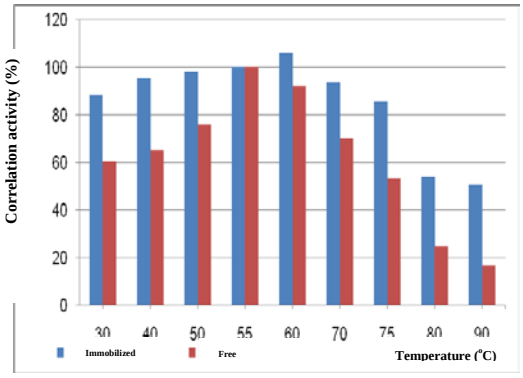


Figure 12. Correlation activity of immobilized enzyme and free enyme on CMC at different temperature values.

At pH4.5 and temperature 60°C, immobilized enzyme activity is 14.93% of free enzyme.

3.6 Immobilized enzyme recycles

One of benefit of using immobilized enzyme is the recycle use so we can save cost at industrial production. Recyling process can be applied by filtration or centrifugation after each usage. In this experiment, immobilized enzyme hydrolises with CMC at 55°C in 30 minutes, pH 4.5. Replicate until activity remained 20%. Immobilized enzyme activity on CMC after each recycle is shown in the table below.

Table 6. Immobilized enzyme activity on CMC after each recycle

Recylce	Enzyme activity (NCU/ g protein enzyme)	Correlation activity (%)
1	134.438[a]	100
2	120.599[b]	89.7
3	112.146[c]	83.4
4	104.136[d]	77.5
5	93.058[e]	69.2
6	64.956[f]	48.3
7	52.485[g]	39.0
8	27.336[h]	20.3

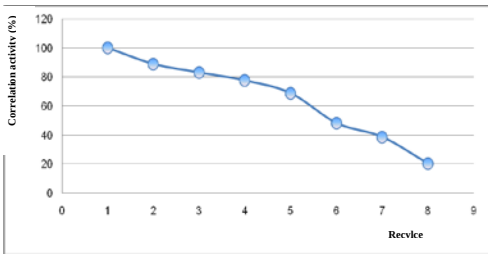


Figure 13. Correlation immobilized enzyme activity CMC on each recyle.

Table 7. Enzyme lost to substrate solution

Submersing time (minutes)	Enzyme lost (%)	Enzyme activity (NCU/ g protein enzyme)	Correlation enzyme activity (%)
0	0.141	129.805[a]	100
5	6.838	121.099[b]	95.66
10	8.601	118.808[c]	92.29
15	9.094	118.167[c]	91.81
20	12.266	114.043[d]	87.86
25	13.394	112.577[e]	86.73
30	15.227	110.195[f]	84.89

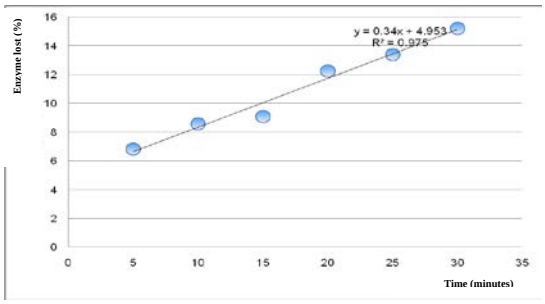


Figure 14. Enzyme lost by hydrolising time

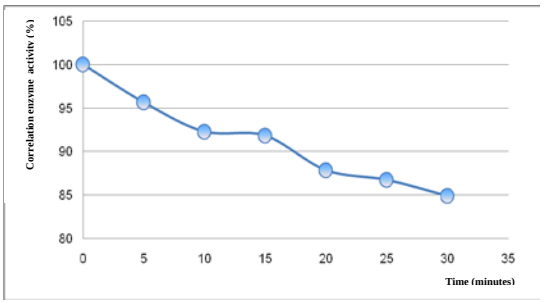


Figure 15. Enzyme activity lost by submersing time.

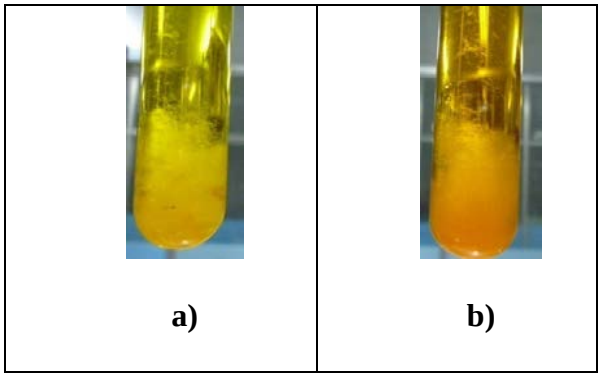
We observe enzyme lost continuosly at 30 minutes and the immobilized enzyme activity decrease dramatically to 84.89%.

3.7 Hydrolizing activity of immobilized enzyme on insoluble substrate

The most important concern is the immobilized enzyme activity on cellulose substrate. In this experiment, the immobilized enzyme hyhrolises with 20 mg Whatman filter at 55^{o}C, in 30 minutes, pH 4.5. Then we observe the hydrolyzed filter; determine the immobilized enzyme and free enzyme activity. The results are as follow:

Table.8. The immobilized enzyme and free enzyme activity on Whatman filter

	Enzyme activity (FPU/ g protein enzyme)	Correlation activity (%)
Immobilized enzyme	**11.47**	**7.2**
Free enzyme	**159.366**	**100**



a) **b)**

Figure 16. Hydrolized filter a) by immobilized enzyme, b) free enzyme

4. CONCLUSION

From our research, we draw out some conclusions:

- ✓ **Cellulase immobilization:**
  - Concentration of sodium alginate for gel formation: 2%
  - Diameter of Calcium alginate bead for immobilization: 3mm
  - Concentration of immobilized enzyme 5- 15 % ($V_{enzyme}/V_{enzyme\ -\ alginate}$)
  - Submersing time for cellulase immobilisation into $CaCl_2$ 0.1M: 30 minutes
  - Immobilization efficiency: 83.645%.
  - Activity of immobilized cellulase 14.93% compared to free enzyme.

✓ **Chacracteristics of immobilized enzyme:**

- Optimum pH for enzyme immobilization is 4.5, the same pH for free enzyme. At pH =7 activity of immobilized enzyme equals to 70.38% compared to pH 4.5; activity of free enzyme equals to 15. 06% compared to pH 4.5.

- Immobilized enzyme is quite stable with high temperature. Optimum temperature for immobilisation is 60^{o}C. At 90^{o}C, activity of immobilized enayme equals to 47.67% compared to temperature 60^{o}C; free enzyme equals to 16.40% compared to temperature 60^{o}C.

✓ Immobilized enzyme has high recycles. Activity of immobilized enzyme remains 69.2% after 5 recycles and 20.3% left at the 8th recyle.

✓ Insoluble hydrolizing activity of immobilized enzyme is low 11.47 (FPU/g protein enzyme), equals to 7.2% compared to free enzyme.

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