# EFFECT OF pH AND MANGANESE KATION ON THE ACTIVITY OF COW RUMEN FLUID CELLULASE ENTRAPPED IN CALCIUM ALGINATE BEADS

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*Enzyme entrapment in calcium alginate beads is considered as one of the important methods of immobilization. In order to utilize cow rumen fluids as source of cellulase and in order to increase the cellulase activity, we were isolated cellulase of cow rumen fluid and investigated the influence of pH (5-9) and concentration of $Mn^{2+}$ (25-100 mM) towards the activity of the cellulase that is entrapped in calcium alginate beads. The research showed that the immobilization of cow rumen fluid cellulase in alginate beads is lead to a higher optimum pH. Free cellulase exhibit the highest activity at pH 4 while the entrapped cellulase exhibit the highest activity of 145 U/ml at pH 6. $Mn^{2+}$ is significantly stimulate the activity of entrapped cow rumen fluid cellulase.*

**Keywords:** ***alginate beads, cow rumen fluids cellulase, immobilization, pH, Mn***

## Introduction

Enzyme technology is increasingly important as a tool for chemical synthesis. The application of enzyme is driven by consumer demand for new products and by industrial attempts for increasing profits and cost reduction. Highly specific, mild operation condition, high reaction rate, small amount of enzyme is needed, the reactions are controlled easily, and reduce the impact of manufacturing on the environment are advantages of the utilization of enzyme (Sheldon, 2007). Beside all of the advantages described above, the use of enzyme has been limited by some factors, mainly are their stability, high cost of production, availability in small amounts, non-reusability, high sensitivity to several denature agents, and expensiveness of the recovery process (Sarrouh et al, 2012). Many of the undesirable constraints may be removed by the application of enzyme immobilization.

Immobilization of enzyme means that it has been confined or localized so that it can be reused continuously (Brena and Viera, 2006). Commonly, enzyme immobilization is carried out by three principle means, matrix assisted entrapment of enzyme, adsorption on a solid support, and ionic or covalent binding. Entrapment method prevents excessive loss of enzyme activity after immobilization, increases enzyme stability in microenvironment of matrix, and protects enzyme from microbial contamination. Hence it is considered as the most preferable method (Meena and Raja, 2000; Riaz et al, 2009; Anwar et al., 2009; Gulay, 2009). Among several matrix used as medium for entrapment, enzyme entrapment in calcium alginate beads is also considered as one of the important methods of immobilization. Sodium alginate beads are prepared by dripping aqueous sodium alginate solution into a solution of calcium ions. Entrapment within insoluble calcium alginate gel is recognized as a rapid, nontoxic, inexpensive and versatile method for immobilization of enzyme (Meena and Raja, 2000; Riaz et al, 2009; Anwar et al., 2009; Gulay, 2009).

Moreover, in order to overcome the high cost of enzyme, finding new source for enzyme isolation is an important area in enzyme production. Commonly, commercial cellulases are produced by Trichoderma species and Aspergillus species. One of potential source of cellulase is cow rumen fluids. It is reported as a rich source of cellulolytic bacteria. Cow rumen fluid can be collected from slaughterhouse. It is now considered as waste since it is recently just being composed and used as fertilizer. Annually, 1.75 millions of local cows are slaughtered in Indonesia. While it is reported that each of cow produces rumen fluid up to 31 L. Hence, the potency of the cow rumen fluid is up to 54.25 millions of Litre per year (Berutu, 2007).

In order to utilize cow rumen fluids as source of cellulase and in order to increase the cellulase activity, we were isolate cellulase of cow rumen fluid and investigate the influence of pH and concentration of $Mn^{2+}$ towards the activity of the cellulase that is entrapped in calcium alginate beads.

## Materials dan Methods

### Sample collection

The main material in this study was rumen fluid of local Indonesian cow from the Penggaron Slaughterhouse, Semarang. Rumen fluid sample was obtained from gut right after cow's death by filtering it into a prewarm (39°C) thermos flask (Wahyudi et al, 2010). Oxygen was removed from gas by filling $CO_2$ into flask and covering it with a sterile butyl rubber stopper.

### Isolation of cow rumen fluid cellulase

Cow rumen fluids were collected and centrifuged at 10.000 g for 10 minutes at 4 $^oC$ in order to separate the supernatan from the cell and cell debrix. The supernatan then taken as crude enzyme. The supernatan was reacted with 60% ammonium sulphate and agitated in a magnetic stirrer for an hour and stored for a night at 4 $^0C$. The supernatan was centrifuged at 10.000 g for 15 minutes at 4 $^oC$. Phosphat buffer pH 7.0 was added into the sedimen of enzyme in 1:10 ratio.

### Immobilization of Cellulase

The enzyme solution was mixed with sodium alginate solution (1%) in 1:2 of ratio. The cellulase-alginate mixture was added dropwise into calcium chloride (0.2 M) solution with continuous shaking at 4 $^0C$. As soon as the drop of cellulase-alginate solution mixed with $CaCl_2$ solution, $Na^+$ ions of sodium alginate were replaced by the $Ca^{2+}$ ions of $CaCl_2$ solution, which finally formed Ca-alginate beads. The beads thus formed were washed 3-4 times with deionized water and finally with 50 m Tris HCL buffer of pH 7.5. These beads were dried for further studies.

### Enzyme Assay

The activity of Cellulase was assayed using DNS method (Khan et al., 2011). The total reaction mixture of 2 ml contained 1 ml of 1 % (w/v) CMC solution in phosphat buffer (50 mM, pH 7) and 1 ml of the free enzyme or immobilized enzyme made from 1 ml of free enzyme. The reaction mixture was incubated at 50 °C for 30 min. After incubation, the enzyme activity was stopped by adding 3 ml DNS reagent; tubes were then placed in a water bath at 90 °C for 15 minutes, 1 ml of sodium potassium tartrate was added to each tube before cooling. The adsorbant of samples was immediately measured at 575 nm using spectrophotometer.

The enzyme activity was then calculated based on formula:

$$\text{enzyme activity} = \frac{\text{glucose concentration x dilution factor}}{\text{mass molecule of glucose x incubation time}}$$

One unit of cellulase activity is the amount of enzyme that release µmol of glucose in 1 minute of the assay. The dilution factor was 1, the mass molecule of the glucose was 180 and the incubation time was 30 minutes.

### Effect of $Mn^{+2}$ concentration

For investigating the effect of $Mn^{+2}$ concentration, the enzyme was immobilized at four different $Mn^{+2}$ concentration ( 25, 50, 75 and 100 mM).

### Effect of pH

For studying the effect of pH, the enzyme assay was carried out at four different pH (5,6 and 8 and 9).

## Results and Discussion

Alginate is a biopolymer with many applications. Alginate is water soluble linear polysaccharide extracted from brown sea weed. It is composed of alternating blocks of 1-4 linked α-L-guluronic and β-D-mannuronic acid fragments (Nita et al, 2007). This polymer comes along with sodium ions (sodium alginate). Alginates are able to produce gels with cations. Gelation occurs by cross-linking of the uronic acids with divalent cations. The most suitable divalent cation is calcium due to its low toxicity (Braccini and Perez, 2001; Liu et al., 2002; Gilhort et al., 2010).

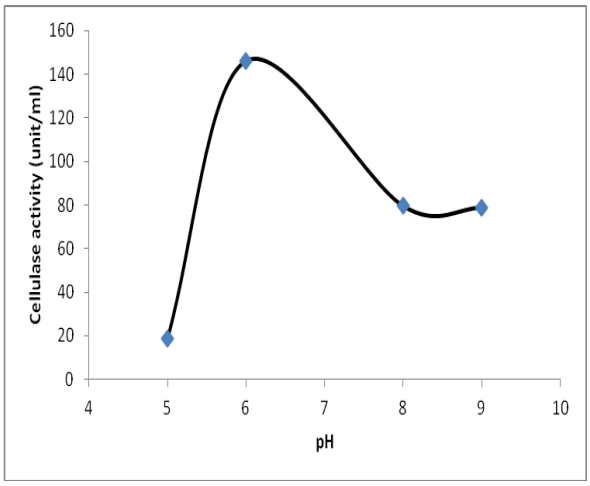


**Figure 1. Effect of pH on Activity of Immobilized Cellulase**

The gelification process of the alginate beads initiates in the outer shell at the interface with the $CaCl_2$ solution. The inbound process is continously proceeed until the entire droplets forms a solid

micro bead. It is also stated that the hardening of the outer shell occurs quite rapidly upon contact with the curing agent, the $Ca^{2+}$ ions have to diffuse through an increasingly thick layer of hardened alginate at the later stages. The curing process of the beads described above completes within several seconds (Haerbelle et al., 2007). The primary mechanism of this gelation involves extended chain sequences which adapt a regular two fold conformation and dimerize with specific chelation of $Ca^{2+}$, the so-called 'egg-box' structure. Each $Ca^{2+}$ ion takes part in nine coordination links with an oxygen atom, resulting in three-dimensional network of calcium alginate (Braccini and Perez, 2001; Liu et al., 2002; Gilhort et al., 2010).

Calcium alginate beads is used for immobilization of cells in bioreactors, entrapment of plant protoplasts and plant embryos for micropropagation, immobilization of hybridomas for the production of monoclonal antibodies, and the entrapment of enzymes and drugs (Madden, 2007). Invertase of Saccharomyces cereviisiae, esterase of thermophilic bacillus, protease of Bacillus subtilis KIBGE-HAS, lipase of Candida rugosa, and rifamycin oxidase from Chryseobacterium are enzymes entrapped in alginate beads (Tanriseven and Dogan, 2001; Gulay, 2009; Anwar et al., 2009; Sawangpanya et al, 2010; Jobanputra et al, 2011). While cellulase of Aspergillus niger was entrapped in natrium alginate gels and in natrium alginate/silica gel hybrid materials (Dragomirescu et al., 2011).

The activity and stability of enzymes depends largely on the particular operating and storage conditions, and is strongly influenced by factors such as the chemical environment, temperature, pH, and solvent properties. When the immobilization of an enzyme is considered, the above factors need to be taken into consideration (Twyman, 2005). Thus, in this project we only investigated the influence of pH and $Mn^{2+}$ toward the activity of the entrapped cow rumen fluids cellulase.

The effect of pH on the activity of cellulase immobilized on the calsium alginate beads was studied by varying the pH of the reaction medium from 5 to 9 and the pH profile is shown in Fig. 1. It is clearly seen that immobilization led to a higher optimum pH, since Budiansyah et al (2010) reported that free cellulase of cow rumen fluids exhibits maximum activity at a pH of 4.

The pH optimum value of immobilized enzyme shifted to a higher or lower pH, depending on surface charges of the supports. It is reported that when the enzyme is coupled with a polyanionic carrier the optimum pH usually shifts in the alkaline direction whereas if the carrier is polycationic the shift is in the acid direction (Costa et al., 2001, Twyman, 2005). It was also reported that the surface of the beads in which the enzyme is localized has a cationic or anionic nature. This charged surface of beads and entrapped enzyme produces a charged micro environment, which ultimately affects the nature of the active enzyme protein and alters the pH of the entrapped enzyme (Anwar et al., 2009, Jobanputra et al, 2011). The calcium alginate beads are reported present an overall negative surface charge (Celesi, 2003). It might be the reason for the shifting of the optimum pH of the entrapped cow rumen cellulase to the alkaline direction.

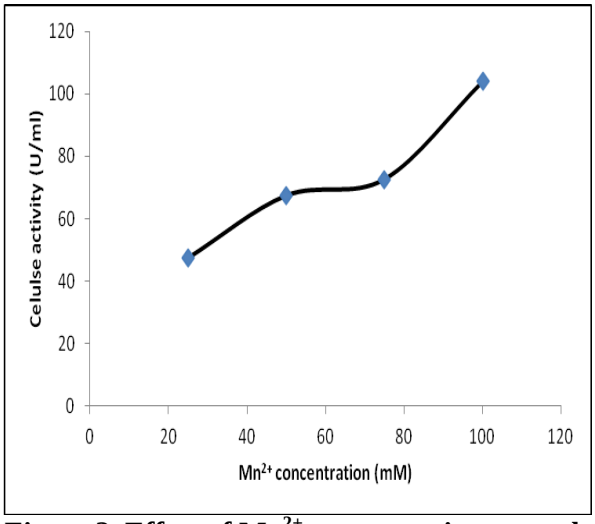


**Figure 2. Effect of $Mn^{2+}$ concentration towards the celulase activity**

Moreover, anzyme activity could be enhanced by the addition of cations or certain compounds. Cations that positively affect the cellulase of cow rumen fluids are Fe, Mg, Mn, Zn, Cu, Co, and Ca (Budiansyah et al. 2010). In this work we investigated the influence of different concentration of $Mn^{2+}$ (25-100 mM). The result showed that $Mn^{2+}$ stimulate the entrapped cellulase of cow rumen fluid (Fig.2).

Similar result of cellulase activity enhancement by cations were reported by several researchers. Ajayi et al. (2007) reported that the activity of the partially purified cellulase from tomato fruits deteriorated by Aspergillus flavus Linn is enhanced by the addition of $Ca^{2+}$, $Na^{+}$, $Mg^{2+}$ and $K^{+}$. While Voget et.al (2006) stated that the activity of metagenome-derived cellulase (endoglucanase), Cel5A, was even enhanced in the presence of low concentrations of manganese.

**Conclusion**

Immobilization of cow rumen fluid cellulase in alginate beads is lead to a higher optimum pH. Since free cellulase exhibit the highest activity at pH 4 while the entrapped cellulase exhibit the highest activity of 145 U/ml at pH 6. $Mn^{2+}$ is significantly stimulated the activity of entrapped cow rumen fluid cellulase.

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