

## **Effect of immobile isolated enzymes from rumen liquid by using alginate matrices on the bay leaf extraction**

Vita Paramita, Mohammad Endy Yulianto, Eflita Yohana, Fahmi Arifan, Hanifah, and Muhammad Taqiyuddin Amjad

Citation: [AIP Conference Proceedings](#) **1699**, 060009 (2015); doi: 10.1063/1.4938363

View online: <http://dx.doi.org/10.1063/1.4938363>

View Table of Contents: <http://aip.scitation.org/toc/apc/1699/1>

Published by the [American Institute of Physics](#)

---

---

# Effect of Immobile Isolated Enzymes from Rumen Liquid by using Alginate Matrices on the Bay Leaf Extraction

Vita Paramita<sup>1</sup>, Mohammad Endy Yulianto<sup>1</sup>, Eflita Yohana<sup>2</sup>, Fahmi Arifan<sup>1</sup>, Hanifah<sup>1</sup>, Muhammad Taqiyuddin Amjad<sup>1</sup>

<sup>1</sup>Chemical Engineering of Diploma Programme, Faculty of Engineering, Diponegoro University  
Jl. Prof. Sudharto, Tembalang, Semarang, Central Java, 50275, Indonesia  
E-mail: vita.paramita@gmail.com

<sup>2</sup>Department of Mechanical Engineering, Faculty of Engineering, Diponegoro University  
Jl. Prof. Sudharto, Tembalang, Semarang, Central Java, 50275, Indonesia

Correspondence: Vita Paramita<sup>1</sup> Chemical Engineering of Diploma Programs, Faculty of Engineering, Diponegoro University, Semarang, 50275, Indonesia; E-mail: vita.paramita@gmail.com

## BSTRACT

**Abstract.** This research aims to develop the enzymatically of bay leaves phytochemical extraction process. The novelty and the main innovations of this research is the development of extraction process by using enzymatic extractor and isolate the enzymes from rumen liquid to shift the equilibrium phase, increase the extraction rate and increase the extraction yield. The activity of rumen liquid enzyme was represented by the activity of cellulase and protease. The analyze of total flavonoid content was performed by using UV-Vis Spectrofometry. The activity of immobilized enzyme of cellulase ( $0.08 \pm 0.00$  U/ml) was lower than the un-immobilized one ( $0.23 \pm 0.00$  U/ml). However, there was no difference activity of the immobilized ( $0.75 \pm 0.00$  U/ml) and un-immobilized ( $0.76 \pm 0.01$  U/ml) of protease. The model of mass transfer of un-immobilized enzyme can be fitted on the experimental data, however the model of mass transfer of immobilized enzyme did not match with the experimental data. The mass transfer coefficient of enzymatic extraction flavonoids bay leaf without immobilization was  $0.17167 \text{ s}^{-1}$  which greater than the reported value of obtained KLa from extraction by using electric heating.

**Keywords:** bay leaf; rumen liquid; enzymatic hydrolysis-extraction; enzyme immobilization; alginate matrices

## INTRODUCTION

Recently, antimicrobial properties from herbal and spice ingredients gained scientific community's attention. Simultaneously, consumers are also demanding food with a long shelf life and are free from the risk that can cause food borne illness. Therefore, future food industry will progressively replace chemical preservatives and adopt natural preservatives regarding to safety from the microbes. One of the possible strategies is a bioactive phytochemical compounds based on localization spices. Antimicrobial activity of herbal spices are commonly used like bay leaves, became the basis of a variety of applications in preservation of raw food materials and food ingredients, pharmaceuticals, alternative medicine and natural therapies.

Qualitative phytochemical analysis of bay leaf spice indicated many components of alkaloids, cumarin, flavonoids, saponins, terpenes, and tannins. These components are relatively high and may apply as antimicrobials. Extraction of antimicrobial components from several sources has been studied by several researchers. This extraction process is generally carried out using methanol, ethanol or petroleum ether. However, the efficiency is relatively low because the process reached a natural limit (equilibrium phase) since the extraction rate cannot be increased anymore. In addition, most bioactive phytochemical compounds have run into thermal degradation due to the extraction process. Enzymatic extraction technique is a promising and prospective technology because it can shift the equilibrium phase to degrade the structure of plant cell walls. The degradation of cell walls will be followed by the solute extraction [1], [2].

Enzymatic extraction from several sources has been studied by several researchers. Pectin was extracted from mandarin orange peel by using enzymes which derived from *Aspergillus aculeatus* [3]. The results showed that the extracted pectin content can achieve 21.3 % (w/w). Meanwhile, extracted pectin from pumpkin by using a complex enzyme which derived from *Aspergillus owamori* obtained the yield of pectin at 14 % with dextrose equivalent (DE)

of 53 %. These results were higher than the use of enzymes from *Basillus macerans* and *Trichordema viride*. Complex enzymes contained on *Aspergillus owamori* are cellulose, xylanase,  $\beta$ -glucosidase, endopolygalacturonase and pectinesterase [4]. Extracted dioscorin from yam by using cellulase isolated from bovine rumen liquid indicated highly prospective of free dioscorin of yam composite flour for noodle making [5]. More over, rumen liquid was also applied on green vanilla pods curing in order to degrade the tissue of vanilla green pods. This application can avoid the curing process on vanillin formation and extraction. Vanillin content of vanilla green pods was found higher by treating the vanilla green pods at 30°C (376 ppm) than treating at 40 °C (178 ppm) [6].

Enzymatic extraction technique is believed to have many advantages over conventional extraction, including high yield of product with high selectivity as well as environmentally friendly [1]–[5]. Although there are several schemes patents enzymatic extraction process [7], [8], but the efficiency of the process is less than 80%. This condition is because of the un-effective cost of production, about 15-20% of the total cost of production is used to supply enzymes. Fundamental weakness of the enzyme extraction process is the expensive price of a commercial enzyme with disposable enzyme application.

Therefore to reduce the cost of production, cellulase and protease enzymes needed are sourced from cow rumen liquid [2], [9], [10]. Enzyme isolated from bovine rumen has advantages compared to commercial enzymes, among others, is more stable at higher temperatures, higher specific activity, optimum pH is higher, and also lower production costs [10], [11]. Rumen liquid provide a great ability to hydrolyze lignocellulosic components by many microbial communities remaining in the rumen. However, these large microbial communities can not be culture yet [12]. Many microbes lives in the reticulorumen which is responsible for the disruption of plant material tissue, such as celluloses and hemicelluloses [13].

The significant effect of this work was modifying the enzyme from rumen liquid by immobile using alginate matrices. The rumen liquid obtained more attention due to their advanced polysaccharide-degrading enzymes, including, xylanase, amylase, glucanase and arabinase [14]. This enzyme activity of ruminant anaerobic fungi was found higher about 3–6 times than that of aerobic fungi (e.g. *Tricoderma reesei*, *T. Viridae*, *Aspergillus oryzae*, etc.) used widely in industrial process. Therefore, it have a potential for industrial in biotechnology.

This research aims to develop the enzymatically of bay leaves phytochemical extraction process. The novelty and the main innovations of this research is the development of extraction process by using enzymatic extractor and isolate the enzyme bovine rumen to shift the equilibrium phase, increase the extraction rate and increase the extraction yield.

## **MATERIALS AND METHODS**

### **Rumen Liquid Isolation**

Rumen fluid extracted from bovine rumen contents by filtration under cold conditions. The filtrate was centrifuged at 10,000 g for 10 min at 4°C to separate the supernatant from the cell and microbial cell contents. Supernatant was then taken as the source of crude enzyme. Supernatant was then treated with 60% ammonium sulphate and stirred using a magnetic stirrer for 1 hour, and allowed to stand for 24 hours at 4°C. Supernatant was centrifuged again at 10,000 g for 15 min at 4°C. Precipitation (enzyme) that is obtained is taken then dissolved in phosphate buffer pH 7.0 with a ratio of 10:1 (precipitated from 100 ml of rumen fluid supernatant was dissolved in 10 ml of phosphate buffer pH 7.0).

### **Enzyme Immobilization in Alginate Matrices**

Sodium alginate solution is prepared by adding deionized water to a concentration of 1.5%. The enzyme was added to the solution of sodium alginate with ratio of 3:2 and stirred. Mixture introduced into macropipette 2 ml and slowly dripped into CaCl<sub>2</sub> solution while stirring with a magnetic stirrer. Gel containing the enzyme was then removed by filtration. Enzyme immobilization with the addition of Mn<sup>2+</sup> performed by adding 50 mM MnSO<sub>4</sub>.

### **Measurement of Protease Activity**

Measurement of protease activity was carried out by using 1% casein as substrate according to the method of Murachi [15]. Five grams of immobilized enzyme was added into 5 ml of substrats. One milliliter of 30 % of trichloroacetic acid (TCA) solution was added into the mixture and then heated at 50 °C for 20 minutes. Coagulated protein was separated and the filtrate was measured the absorbance at a wavelength of 280 nm. Unit of protease activity is expressed as the amount of enzyme which causes an increase in absorbance at a wavelength of 280 nm which is equivalent to 1 ug of tyrosine/ml enzyme/20 minutes.

## Measurement of Cellulase Activity

Cellulase activity was measured by using the method of Miller [16]. One milliliter of extracted enzyme was added into 1 % of carboxymethyl cellulose (CMC) substrate at 0.2 M of phosphate buffer pH 7. This mixture was then incubated at 50 °C for 60 minutes. After 60 minutes, the reaction was stopped by adding 2 ml of dinitrosalicylic acid and boiled for 15 minutes. The mixture was then cooled to room temperature and measured at a wavelength of 540 nm. Sugar produced was determined by standard glucose. One unit of cellulase activity was defined as the amount of enzyme that produces 1 pmol of glucose in one minute.

## Enzymatic Extraction Procedure

Enzymes of extracted rumen liquid were added to the powder of bay leaf with the weight ratio of enzyme and substrate at 0.15. Distilled water was then added and the pH of the solution is set at 4 using phosphate buffer solution. Extraction process was carried out at given temperature (30, 40, 50 °C). Calculation of extraction time ( $t = 0$ ) begins when the stirrer was started on the certain rotation (80 rpm). Every 10 minutes, samples were taken from the extractor. When the sample in the form of a matrix of immobilized enzyme, the mixture was filtered to separate the insoluble biomass. The filtrate was analyzed the density, the viscosity and the composition of the total flavonoid.

## Analysis of Total Flavonoid

One tenth gram of sample was dissolved into 1 ml of demineralized water, then, 0.5 ml of this solution was mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10 % aluminum chloride hexahydrate ( $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ ), 0.1 ml of potassium acetate ( $\text{CH}_3\text{COOK}$ ) and 2.8 ml of demineralized water. This solution was incubated at ambient temperature for 40 minutes and analyzed the absorbance at 415 nm by using Spektrofotometri UV-Vis (UV-1700 PharmaSpec UV-VIS Spectrophotometer, Shimadzu, Japan). Data were done in once [17].

## RESULTS AND DISCUSSION

### Activity of Protease and Cellulase on Immobilized Enzyme and Un-immobilized Enzyme

Figure 1 shows the protease and cellulase activity on rumen liquid. The result indicated that there is no effect of immobilized enzyme on the activity of protease. The protease activity was observed at  $0.75 \pm 0.00$  U/ml for un-immobilized enzyme and  $0.76 \pm 0.01$  U/ml for immobilized enzyme. However, the activity of cellulase shows the significant reduction in activity. Without immobilization, it shows activity at  $0.23 \pm 0.00$  U/ml. When the enzyme was immobilized, its activity decreased to  $0.08 \pm 0.00$  U/ml.

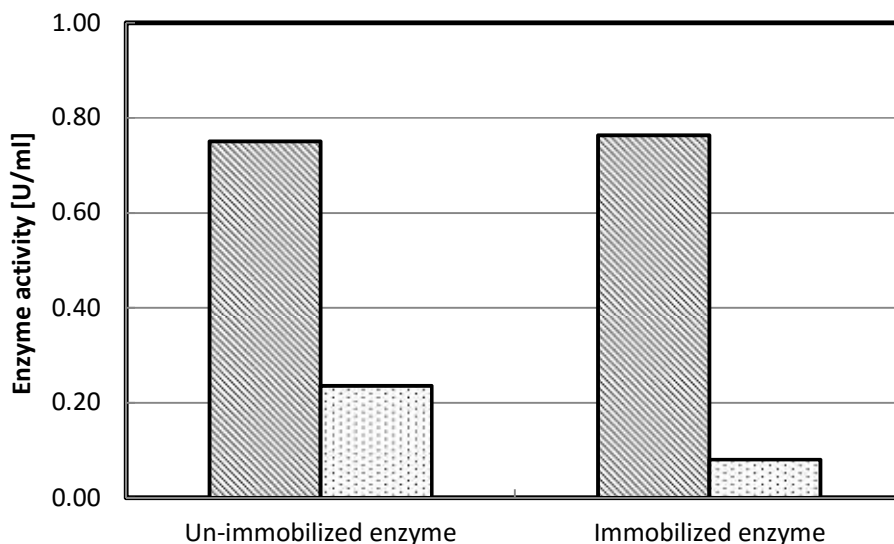




Fig 1. Activity of protease and cellulase regarding on immobilized and un-immobilized enzyme;  protease;  cellulase.

### Physical Properties of Extracted Bay Leaf

Table 1 represent the density and viscosity of extracted bay leaf which were analyzed at the final extraction time. The density of the extracted bay leaf show no significant difference, it vary in the range of 1.029–1.043 gr/mL when it was immobilized. In addition, without immobilized the enzyme, the density of the extracted bay leaf show lower value (1.008 gr/mL) when it was extracted at 40 °C. There was no significant difference on the viscosity both of immobilized and un-immobilized one with the varying of temperature extraction. The viscosity of the extracted bay leaf by using immobilized enzyme slightly decreased with the increasing of temperature, 1.114 mPa.s for 30 °C, 1.050 mPa.s for 40 °C and 1.033 mPa.s for 50 °C. In addition, un-immobilized enzyme show almost similar viscosity at 1.042 mPa.s.

Table 1. Density and viscosity of extracted bay leaf

	Temperature (°C)	Density (gr/mL)	Viscosity (mPa.s)
Un-immobilized enzyme	40	1,008	1,042
Immobilized enzyme	30	1,043	1,114
	40	1,029	1,050
	50	1,037	1,033

### Effect of Enzymatic Extraction Time and Temperature on the Flavonoid Content

Figure 2 shows the total flavonoid content on pH 4 and 0.15 of enzyme-substrate ratio. Un-immobilized enzyme represent an increasing total flavonoid content from 400 ppm, detected at 10th min, to 500 ppm, detected at 40th min. Conversely, un-immobilized enzyme decreased regarding to the time. Total flavonoid content, which were extracted at 40 °C, show about 550 ppm, however, extracting the bay leaf at 30 °C resulted higher total flavonoid content, about 750 ppm.

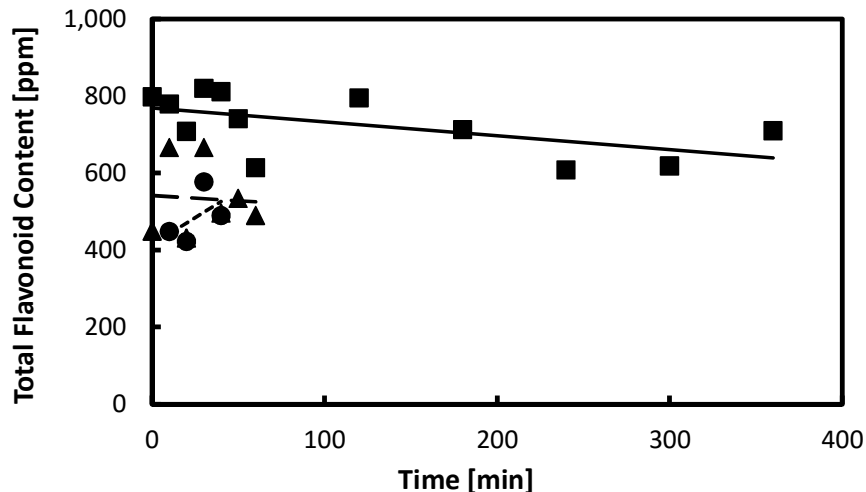


Fig 2. Flavonoid content on the extracted bay leafes as a time function; pH 4, 0.15 of enzyme-substrate ratio; ●, un-immobilized enzyme, extracted at 40 °C; ■, immobilized enzyme, extracted at 30 °C; ▲, immobilized enzyme, extracted at 40 °C.

### Modelling of Enzymatic Extracted Flavonoid of Bay Leaf

The flavonoid extraction of bay leaf is regarded as a series of mass transfer phenomenon which is including the flavonoid diffusion from inside of solid material into surface of solid material, the mass transfer of flavonoid from surface of solid material into liquid solvent on the solid porous and the diffusion of flavonoid into liquid solvent.

Solid-liquid rate extraction depends on the flavonoid diffusion from inside of solid material into surface of solid material and the mass transfer of flavonoid from surface of solid material into liquid solvent. If the differences of diffusion rate and mass transfer were almost the same, the extraction rate is determined by both of two processes. However, if the differences of both rate can be considerable, then the extraction rate is determined by the slowest process rate [18].

Mass transfer rate of the flavonoid diffusion from inside of solid material into surface of solid material is expressed in equation 1 [19]:

$$N_{AS} = -D_e \cdot A \cdot \frac{dc}{dr} \quad (1)$$

where,  $N_{AS}$  = flux of mass ( $\text{kg} \cdot \text{s}^{-1}$ ),  $D_e$  = diffusivity of solute ( $\text{m}^2 \cdot \text{s}^{-1}$ ),  $A$  = surface area of contact diffusion ( $\text{m}^2$ ),  $C$  = solute concentration on the solvent ( $\text{kg} \cdot \text{m}^{-3}$ ),  $r$  = distance of mass transfer (m). Mass transfer rate in the liquid solvent is expressed in equation 2 [19]:

$$N_{AS} = K_{La}V(C_s - C) = \frac{dM}{dt} \quad (2)$$

where,  $M$  = mass of transferred solute (kg),  $V$  = volume of solvent ( $\text{m}^3$ ),  $t$  = time (s),  $C_s$  = solute concentration at the solid surface, which equilibrate with the solute concentration at the saturated solution ( $\text{kg} \cdot \text{m}^{-3}$ ),  $K_{La}$  = volumetric mass transfer coefficient ( $\text{s}^{-1}$ ).

Since the solid grain was prepared in very small size, the diffusion of flavonoid from the solid surface perform rapidly and can be neglected. Therefore, mass transfer of flavonoid from solid into liquid is being decisive [18], [20]. Volumetric mass transfer rate of flavonoid from solid surface into solvent based on equation 2, where  $C_s$ , the concentration of flavonoid in the solid surface, equilibrate with the concentration of solute in the saturated solution,  $C^*$ . Therefore, equation 2 possibly expressed in the form of:

$$N_{AS} = K_{La}V(C^* - C) \quad (3)$$

The relationship between the solute concentration in a saturated solution with initial solute concentration in the solid ( $C^0$ ), solute concentration in the rest of the sample at infinite time ( $C^\infty$ ) and initial mass sample ( $m$ ) can be expressed in the equation:

$$m \cdot (C^0 - C^\infty) = VC^* \quad (4.a)$$

This equation can expressed in the form of:

$$\frac{m \cdot (C^0 - C^\infty)}{V} = C^* \quad (4.b)$$

Mass balance of total flavonoids in solution is expressed in the following equation [21]:

$$N_{AS} = \frac{d(VC)}{dt} \quad (5)$$

Total differential of equation (5) is:

$$N_{AS} = C \frac{d(V)}{dt} + V \frac{d(C)}{dt} \quad (6)$$

Since there is no difference in the volume of the solvent, then equation (6) can be simplified into:

$$N_{AS} = V \frac{d(C)}{dt} \quad (7)$$

By substituting equation (7) and equation (4.b) into equation (3), it can obtained the following equation:

$$V \frac{d(C)}{dt} = K_{La}V \left( \frac{m \cdot (C^0 - C^\infty)}{V} - C \right) \quad (8)$$

Equation (8) can be reordered into:

$$\frac{d(C)}{dt} + K_{La} \cdot C = K_{La} \left( \frac{m \cdot (C^0 - C^\infty)}{V} \right) \quad (9)$$

When  $A = K_{La}$  and  $B = K_{La} \left( \frac{m \cdot (C^0 - C^\infty)}{V} \right)$ , then equation (9) can be converted into:

$$\frac{dC}{dt} + AC = B \quad (10)$$

Equation (10) is a first-order of ordinary differential equation. Analytically solution with the boundary condition of  $t = 0$ ,  $C = 0$  and when  $t = t$ ,  $C = C$  will be obtained:

$$C = \frac{B}{A} - \frac{B}{A} e^{(-At)} \quad (11)$$

The value of  $K_{La}$  can be found by optimizing one variable using MATLAB (Mathworks Inc., United States). This optimization is performed to obtain the sum square of errors (SSE) or the smallest sum of the squared of the difference between the C count ( $C_{count}$ ) with C trial ( $C_{trial}$ ).

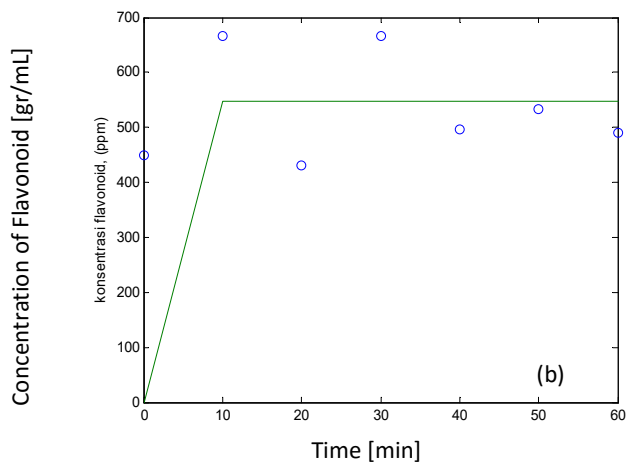
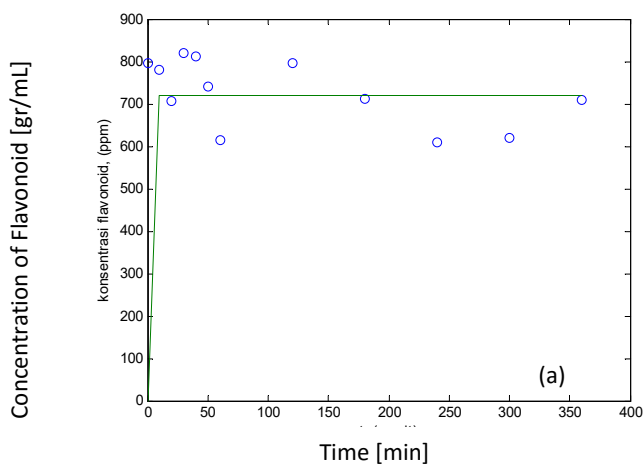
$$SSE = \sum (C_{count} - C_{trial})^2 \quad (12)$$

Model of mass transfer in enzymatic extraction of flavonoids were verified by experimental data, as shown in Figure 3. Whereas, Table 2 shows the value of SSE of experimental dan counted data. It shows that the extraction rate at the early of extraction process was very fast, however after exceeding 20 minutes, the extract obtained was not increasing further. The proposed model fits the experimental data with the value of constants A and B were found 0.17167 and 91.9717. As shown in Figure 3c, the straight line represents the proposed model and most of the data is attached to the line. Unfortunately, Figure 3a and 3b did not match the proposed model since the data obtained were randomly as a time function.

Table 2. Value of SSE of each condition

Immobilized / Un-immobilized Enzyme	Extraction temperature (°C)	Value of SSE (-)
Immobilized enzyme	30	697991.0068
Immobilized enzyme	40	249242.3433
Un-immobilized enzyme	40	206089.6405

The mass transfer coefficient ( $K_{La}$ ) on mass transfer model of enzymatic extraction flavonoids bay leaf without immobilization (Figure 3c) was  $0.17167 \text{ s}^{-1}$ . The value of  $K_{La}$  were greater than the reported value of  $K_{La}$  ( $0.0055 \text{ s}^{-1}$ ) which obtained from extraction by using electric heating [22].



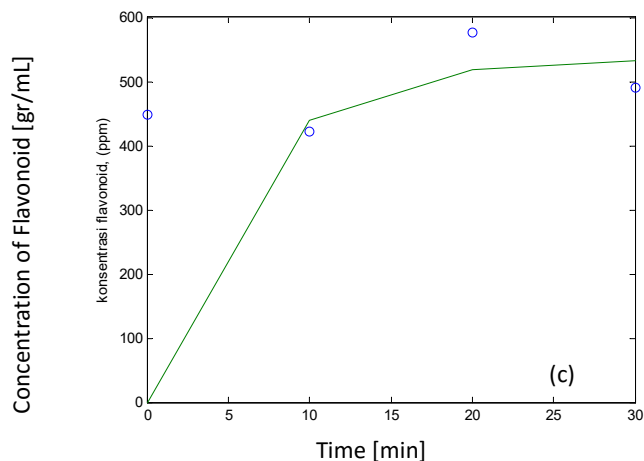


Fig 3. Proposed model and experimental data compatibility, (a) immobilized enzyme, extraction temperature at 30 °C, (b) immobilized enzyme, extraction temperature at 40 °C, (c) un-immobilized enzyme, extraction temperature at 40 °C; ○, experimental data; —, model.

## CONCLUSIONS

The activity of immobilized enzyme of cellulase was lower than the un-immobilized one. However, there was no difference activity on the immobilized and un-immobilized of protease. The model of mass transfer of un-immobilized enzyme can be fitted on the experimental data, however the model of mass transfer of immobilized enzyme did not match with the experimental data.

## ACKNOWLEDGEMENT

This work was supported by the Grant-in-Aid for Scientific Research (No. 302c-2/UN7.5/LT/2013) from the Ministry of Education and Culture of Indonesia.

## REFERENCES

1. M. Panouille, J.-F. Thibault and E. Bonnin, *J. Agric. Food Chem.* **54**, 8926–8935 (2006).
2. M. E. Yulianto and I. Hartati (private communication).
3. C. G. Avilla, M. S. Gallagher, J. C. M. Saenz and R. R. Herrea, *Valnatura*, 21–27 (2005).
4. N. M. Ptichkina, O. A. Markina and G. N. Rumyantseva, *Food Hydrocolloid*. **22**, 192–195 (2008).
5. V. Paramita, S. M. Priyadi, C. P. Sulistyono and N. I. Kartikasari (private communication).
6. V. Paramita and M. E. Yulianto, *J. Food Res.* **2**, 65–69 (2013).
7. G. L. Lehmborg, D. A. Balentine, S. A. Gobbo, and R. S. Hang, *Enzyme Extraction Process for Tea*. US Patents No. 5,952,023A (14 Sept 1999).
8. K. J. Pienta and A. Raz, US Patents No. 5,895,784A (20 Apr 1999).
9. O. Ayuningtyas, “Eksplorasi Enzim Selulase dari Isolat Bakteri Asal Rumen Sapi (Exploration of Cellulase from Cow Rumen Bacterial Isolates)”, Bachelor Essay, Airlangga University, 2008.
10. A. Budiansyah, K. Resmi, K. G. Wiryawan, M. T. Soehartono, Y. Widyastuti, and N. Ramli, *Media Peternakan* **33**, 36–43 (2010).
11. K. J. Rasmussen, D. F. Visser, F. Hennessy, J. Kemp, M. P. Roux-van der Merwe, J. Badenhorst, T. Ronneburg, R. Francis-Pope and D. Brady, *Curr. Microbiol.* **66**, 145–151 (2013).
12. T. A. Cheema, K. Jirajareonrat, T. Sirinarumit, and S. K. Rakshit, *Anim. Biotechnol.* **23**, 261–277 (2012).
13. I. Mizrahi, “Rumen Symbioses,” in *The Prokaryotes*, edited by E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson (Springer, Heidelberg, 2013), pp. 533–544.
14. S. S. Lee, K. J. Shin, W. Y. Kim, J. K. Ha and I. K. Han, *Asian Australas. J. Anim. Sci.* **12**, 988–1001 (1999).
15. T. Murachi, *Biochemistry-US* **3**, 932–934 (1964).
16. G. L. Miller, *Anal. Chem.* **31**, 426–428 (1959).
17. J.-Y. Lin and C.-Y. Tang, *Food Chem.* **101**, 140–147 (2007).



18. W. B. Sediawan and A. Prasetyo, *Pemodelan Matematis dan Penyelesaian Numeris dalam Teknik Kimia (Mathematical Modelling and Numerical Solving in Chemical Engineering)* (Andi Publisher, Yogyakarta, 1997), pp. 22–26.
19. A. Ibarz and G. Canovas, *Unit Operation in Food Engineering* (CRC Press, Boca Raton 2003), pp. 737.
20. J. M. Smith, *Chemical Engineering Kinetics* (Mc. Graw Hill BookCo., Inc., Singapura, 1981). pp. 65.
21. D. Basmadjian, *Mass Transfer: Principles and Application* (CRC Press, Boca Raton, 2004), pp. 43.
22. Samun, *Ekulibrium* 7, 17–21 (2008).



# Certificate

DEPARTMENT OF CHEMICAL ENGINEERING DIPONEGORO UNIVERSITY  
proudly certifies

*Dr. Eng. Vita Paramita, S.T., M.M., M.Eng.*

who has successfully accomplished his/her role  
as

**Presenter**

In the 2<sup>nd</sup> INTERNATIONAL CONFERENCE ON CHEMICAL AND MATERIAL ENGINEERING 2015 (2<sup>nd</sup> ICCME 2015)  
Grand Candi Hotel, Semarang, Indonesia, September 29-30th, 2015

In conjunction with

The Golden Jubilee (50<sup>th</sup>) of Department of Chemical Engineering, Faculty of Engineering, Diponegoro University

On behalf of Dean of Faculty of Engineering,  
Vice Dean for Academic Affairs  
Diponegoro University



Dr. Budiyo



Dr.-Ing. Silviana