DOI: 10.21776/ub.jpacr.2018.007.02.408 *J. Pure App. Chem. Res.*, **2018**, 7(2), 180-189
16 May 2018

Xylanase Production from *Trichoderma viride* and its Immobilization on the Sea Sands Matrix

Sutrisno[1*], Anna Roosdiana[I], Suratmo[1]

[1]Chemistry Department
Faculty of Mathematic and Natural Science
Brawijaya University

[*]corresponding author : tris_mc@ub.ac.id

Received 1 February 2018; Revised 23 March 2018; Accepted 26 March 2018

ABSTRACT

The study was aimed to investigate the production and characterization of xylanase from *Trichoderma viride* by determining the optimum condition of xylanase immobilization using sea sand matrix. The parameters were shaking time variations of 1, 2, 3, 4, and 5 hours and xylanase concentration of 0.5, 1.5, 2.5, 3.5, and 4.5 mg/mL. Xylanase was purified by ammonium sulfate using the optimum fraction of 40-80%. The optimum shaking time for immobilization of xylanase was reached in 4 h, and the optimum xylanase concentration was obtained in 4.5 mg/mL, with immobilized xylanase activity of 118.55 $\mu g.g^{-1}.min^{-1}$ and adsorbed xylanase amount in sea sand matrix of 20.51 mg. Immobilized xylanase can be used up to 4 times, with the percentage of 62.47%. The surface characterization of immobilized xylanase was conducted using SEM. The FT-IR result obtained peaks at wavenumber 3431.13 and 875.62 cm^{-1} which indicated the existence of N-H bond from enzyme and the sea sand bind with enzyme, respectively.

Keyword: immobilized xylanase, sea sand matrix, shaking time, xylanase concentration, reusability

INTRODUCTION

Enzyme is a biopolymer molecule which comprises a series of amino acid in a chain structure which plays a vital role in various reactions in the cell. Enzyme is produced and applied by human cell for reaction catalysis, such as cell-defense metabolism and energy conversion where enzyme acts as biocatalyst [1]. Enzyme has the specific and beneficial characteristic, such as selective, efficient, also environmental friendly [2].

Xylanase is an extracellular enzyme that hydrolyzes xylan to xylose and xylo-olygosaccharide. It can be produced from various microbes such as bacteria and fungi [3]. Xylanase can be isolated from mold types species such as *Trichoderma harzianum, Trichoderma koningii, Trichoderma lignorum, Trichoderma viride, Trichoderma longibrachium* dan *Trichoderma pseudokoningii*. It has high commercial value in the industry, such as paper pulp and textile, which widely used for diminishing chlorine amount in bleaching process [4]. Xylanase is also applied for feedstock to enhance digestibility. In food industry, it is also utilized for juice color brightener, low calories sweetener production, coffee extraction, and plant oil [5,6].

The journal homepage www.jpacr.ub.ac.id
p-ISSN : 2302 – 4690 | e-ISSN : 2541 – 0733

Cellulase free xylanase has disadvantages characteristics such as not stable in pH, temperature, and metal ion and hard to use repeatedly or continuously. To overcome this problem, xylanase requires being immobilized to enhance its stability and efficiently utilized regularly [7]. Various methods can be applied for enzyme immobilization, such as physical adsorption, covalent bonding, and trapping [8,9,10].

In this research, Immobilization of xylanase was performed by physical adsorption method using sea sand matrix. Sea sand composes of silica up to 50%, which can be applied to natural adsorbent due to its big size pores. The utilization of sea sand matrix as adsorption media is easy because that will not change the conformation or damage in the active site and stable activity of xylanase [11].

Some previous reports regarding xylanase immobilization have been conducted. The optimum concentration of Eudragit^{TM} S100 solution in the xylanase immobilization from *Streptomyces sp.* isolated at concentration 1% (w/v) in the crude extract of xylanase which afforded the effectivity immobilization percentage of 5.51%, while the increase of enzyme volume (6:1) can decrease immobilization. The highest activity occurred at pH 6 and temperature of 40^{o}C [12].

Enzyme immobilization can be influenced by some factors, such as enzyme concentration and shaking time. In the optimum condition, the enzyme amount and activity showed maximally [13]. This work discusses the optimization of xylanase immobilization from *Trichoderma viride* in the sea sand matrix by varying the parameter such as shaking time and xylanase concentration.

EXPERIMENT

Chemicals and instrumentation

All chemicals used for this research has pro analysis purity grade such as dextrose, Copper(II) sulfate pentahydrate ($CuSO_4.5H_2O$), sodium-kalium-tartrate ($NaKC_4O_6H_4$), potassium dihydrogen phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), ammonium sulphate ($(NH_4)_2SO_4$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), glucose anhydrous, hydrochloric acid (HCl), barium chloride ($BaCl_2$), dinitrosalicylic acid (DNS), sodium hydroxide (NaOH), phenol crystalline, potassium permanganate ($KMnO_4$), and sodium sulfite ($NaSO_3$).

The material used in this research were *Trichoderma viride* in pure culture obtained from Biochemistry Laboratory, Brawijaya University, corn husks collected from Blimbing Market, Malang City, East Java, and sea sands as the immobilized enzyme matrix picked up from Tanjung Aan Beach, Lombok Island. The material used for microbiology such as potato, peptone, oleic acid, casein (Merck), agar starch, xylan, corn husk flour, and distilled water.

Instrumentation applied for this research such as, incubator (Heraus B type 50 Memmert), autoclave (LS-C35L type), ose needles, grinder, furnace (Nabertherm), cold centrifuge (Denley), shaker (Edmund Buhler SM 25 24B), magnetic stirrer, pH meter (Inolab WTW), oven (Memmert), freezer, Spectronic Genesys 20, cellophane bag, Whatman paper no. 40, and water bath (Memmert W 200), sieve 120 and 150 mesh.

Procedure reaction

The fabrication of corn husk flour

A 50 gram of corn husks were washed and dried in the oven. The dried corn husk then was sliced in small size about 0.5 cm and blended. The smooth dried husk was then sieved by sieve 150 mesh and separated into different particle sizes by passing the sample through

sieves of different mesh sizes (120 and 150 mesh). The corn husk passed from sieves was called as the inducer.

The production of solid media

The solid media used in this research was Potatoes Dextrose Agar (PDA). 20 gram of potatoes were sliced and the distilled water was added to 100 mL. The mixture was boiled for 60 min while distilled water was added to keep the constant volume in 100 mL, the boiled mixture was filtered to obtain potato extract. 2 gram of dextrose was added. The mixture was maintained by adding 1 mL of acetate buffer solution pH 5 then was boiled. 1.5 gram of agar flour was added and stirred. PDA solution was sterilized by autoclave at 121^{o}C and 15 psi for 15 min. The sterilized PDA was cooled at room temperature or keep in the refrigerator at a tilted position.

The pure culture rejuvenation of *Trichoderma viride*

The culture of *Trichoderma viride* had been rejuvenated in the laminar air flow. Ose needle was prepared, then pure culture tube of *Trichoderma viride* and media was heated in the Bunsen in order to be sterilized. One single loop of *Trichoderma viride* spore was taken out and streaked in the sterile solid media aseptically. Afterward, the tube was closed by sterile cotton, then kept and incubated for 6 days (144 h) in the incubator at 30^{o}C.

The fabrication of inoculum

The inoculums were fabricated by taking spore from pure culture of *Trichoderma viride* in the age of 6 days from one of tilted agar and was suspended in 10 mL of distilled water by using graduated pipette. 2 mL of suspense was grown in Erlenmeyer flask which contained 13 mL of sterile liquid media. Afterward, the media was incubated in an orbital shaker until mid-logarithmic phase (36-hour).

Production and isolation of xylanase crude extract

150 mL of liquid media in Erlenmeyer flask was sterilized in autoclave at 121^{o}C in pressure of 15 psi for 15 min. After that, the sterilized media was cooled at room temperature, and then 15 mL of inoculum was added aseptically by using a graduated pipette. And then, media was incubated in the shaker at room temperature in 150 rpm until reached initial stationary phase (60-hour). At the end of incubation period, xylanase enzyme was isolated using centrifugation method. 15 mL of acetate buffer solution pH 5.0 was added into incubated liquid media and then was centrifugated for 30 mins at 4^{o}C and 3000 rpm. The obtained supernatant is the crude extract of xylanase which separated from its precipitate and used for purification. The crude extract of xylanase was concentrated by freeze dryer until reached ¼ of initial volume prior to purification.

Purification of xylanase crude extract

The crude extract of xylanase was fractionated using ammonium sulfate. 22.6 gram of ammonium sulfate was added into 100 mL of xylanase crude extract slowly while stirred until dissolved. The solution was cooled in freezer for 30 min, then centrifuged at 4^{o}C in 8000 rpm for 30 min. The supernatant was obtained and separated from its precipitate. 26.3 gram of ammonium sulfate was added into 100 mL of supernatant and was treated as the previous fraction to obtain the fraction of 40-80%. Afterward, the dialysis process was undertaken in the fractionation of ammonium sulfate.

A 10 mL of 0.2 M acetate buffer solution pH 5 was added to precipitates from fraction 40-80%. Enzyme solution was put into cellophane membrane which was bind to keep enzyme isolated. Cellophane bag which contain enzyme was immersed in 250 mL of 0.1 M acetate buffer solution pH 5 and stirred wisely at 4°C. Dialysis was continued until ammonium sulfate was removed from the purified enzyme, substituted with 0.1 M acetate buffer solution pH 5 every 2 h.

Gel filtration

A 1 gram of Sephadex G-100 put into beaker glass and 10 mL of acetate buffer solution pH 5 was added , then that was observed until the gel formed. 1 gram of glass wool and gel was put into a column. 2 mL of xylanase enzyme from dialysis process then put into column and by 0.2 M acetate buffer solution pH and collected 10 mL for each fraction. Gel filtration was stopped if there was the decrease in enzyme activity significantly.

Determination of free xylanase activity

The free enzyme has 1 unit activity which equivalent with 1 µg of xylose that afforded from xylan per min by each mL of enzyme. Measurement of xylanase activity was conducted by plotting the absorbance value which resulted from standard curve equation, to obtain reducing sugar concentration of xylan hydrolysis by xylanase. The equation for determining 1 unit of enzyme activity is below:

$$EA = \frac{X \times V \times df}{p.q} x \frac{MW\ of\ xylose}{MW\ of\ glucose}$$

where : EA = enzyme activity ($\mu g.mL^{-1}.min^{-1}$)
X = reducing sugar concentration ($\mu g.mL^{-1}$)
V = total volume of sample (mL)
df= dilution factor
p = xylanase extract volume (mL)
q = reaction time (min)
molecular weight of xylose = 150.13 g/mol
molecular weight of glucose = 180 g/mol

Determination of the initial yield protein

2 mL of casein solution in 5000 µg/mL was added by 8 mL of Biuret reagent and 2 mL of 0.2 M acetate buffer solution pH 5. The mixture was shaken until homogenous and incubated at 50°C for 30 min. Afterward, the casein mixture was measured its absorbance at 400-600 nm to determine the maximum wavelength. And then, it was applied to estimate solution absorbance in concentration casein solution of 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, and 9000 µg/mL by Spectronic Genesys 20. The results were plotted standard curve with the correlation between casein concentration (µg/mL) on the x-axis and absorbance on the y-axis. The initial yield protein was investigated by plotted the absorbance value on the standard curve equation of casein.

Immobilization of xylanase enzyme on the activated sea sand matrix

Preparation of sea sand matrix

The sea sand was grinded until smooth and sieved by sieve 120 mesh and continued by sieve 150 mesh. The sand detained on the sieve 150 mesh was utilized as immobilized

matrix. And then the sand was homogenized, washed using distilled water, filtered and dried in the oven at 105 ^{o}C until reach the constant weight.

Activation of sea sand matrix

10 gram of sea sand matrix was immersed in 200 mL of HCl 0.4 M. After that was shaken at room temperature for 4 h at 100 rpm. The immersed sea sand was filtered using Whatman paper no. 40 and washed by distilled water until the washed water being neutral. The sea sand was dried in the oven at 105^{o}C and was calcined at 500 ^{o}C for 4 h.

Determination of optimum shaking time

2 mL of the purified enzyme was added by 0.2 M acetate buffer solution pH 5 until 5 mL in volume and xylanase concentration of 1.087 mg/mL. A purified enzyme solution was mixed with 0.1 gram of activated sea sand. The mixture was incubated in the shaker in 100 rpm at room temperature for (1, 2, 3, 4, 5) h in order to investigate the optimum shaking time of enzyme adsorbed in sea sand. After that, the immobilized result was filtered using Whatman no.40 to separate filtrate and precipitate. Immobilized xylanase and determined its activity and identified using SEM analysis and FT-IR spectrophotometry.

Determination of optimum xylanase concentration

Immobilization in the various enzyme concentrations was conducted as well as various shaking times. Purified xylanase applied (0.55; 1; 2.77; 3.87; and 4.98) mL until xylanase concentration reached (0.5; 1.5; 2.5; 3.5; and 4.5) mg/mL. Afterward, the mixture was incubated for 4 hours as optimum shaking time in the shaker at 100 rpm. The immobilization result was filtered using Whatman no 40 to obtain filtrate and precipitate. Filtrate was tested its residual yield protein. The optimum xylanase was investigated by plotting the curve correlation between xylanase concentration versus adsorbed xylanase amount and xylanase concentration versus immobilized xylanase activity.

Determination of the residual protein yield

2 mL of casein solution 5000 ppm and 8 mL of Biuret reagent was added to 2 mL of immobilized enzyme filtrate. The mixture was shook until homogeneous and was incubated at 50^{o}C for 30 mins. After that, the mixture was cooled to room temperature and measured its absorbance at the maximum wavelength of casein. The protein yield was determined by plotting the absorbance value in the standard regression curve of casein.

Activity test of immobilized xylanase enzyme

Determination of immobilized xylanase activity

1 mL of xylan substrate 1% (/v) in tube reaction was heated in water bath for 15 mins at 60^{o}C. And then, the substrate was added 0.1 gram of immobilized xylanase enzyme, 1 mL of buffer acetate 0.2 M pH 5 and 1 mL of free reductant water. The mixture was incubated in the water bath for 55 min at 60^{o}C and was added by 2 mL of DNS reagent and was heated in the water bath for 5 min and cooled by running water. And then, it was put into volumetric flask 25 mL and was added by distilled water until the limit line and homogenized. The solution was measured its absorbance at maximum wavelength using Spectronic Genesys 20.

Efficiency test of immobilized xylanase enzyme

Determination of reusability of immobilized xylanase using activated sea sand matrix was conducted by activity test as the step before. This treatment was repeated 6 times until

obtained absorbance with activity up to 50%. After that, enzyme activity was determined in each repetition. The immobilized xylanase is still efficient in activity up to 50%.

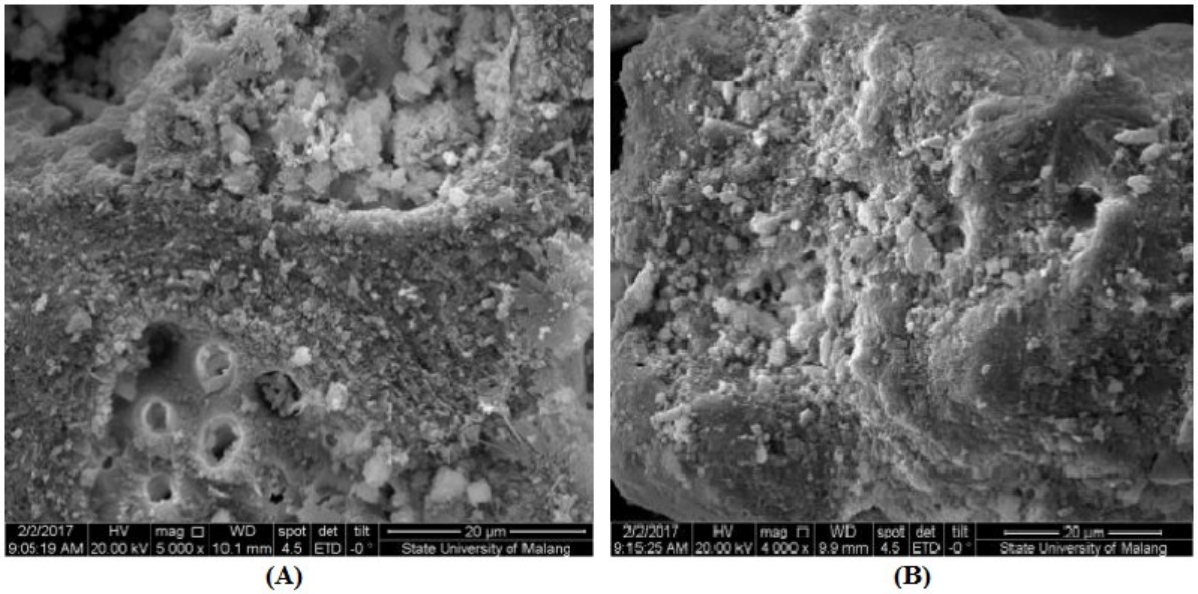
Data Analysis

The optimum condition and the effective usage of immobilized xylanase were analyzed by F test using Randomized Block Design and treatment was repeated 3 times showed in the reference. If F-calculated is larger than F-value in the table (α db), then continued to analysis using the smallest real difference test (5%).

RESULT AND DISCUSSION

Identification of immobilized xylanase enzyme

Immobilized xylanase enzyme was characterized using Scanning Electron Microscope (SEM) to investigate the morphology of sea sand and matrix which was bonded with enzyme. SEM images of sea sand matrix and xylanase enzyme are represented in Figure 1.



(A) **(B)**

Figure 1. SEM images of (A) sea sand matrix and (B) immobilized xylanase enzyme

Figure 1 represented that in the sea sand matrix was found the cavities where enzyme and matrix was bonded each other. While in the immobilized enzyme matrix was not found cavities.

Identification of immobilized xylanase was performed by FT-IR spectrophotometry to investigate the functional group which comprised in the compound. The FT-IR result showed that some bonds were possessed by one of sea sand and immobilized enzyme matrix. Sea sand matrix spectrum as shown in Figure 2 indicated the N-H bond at wavenumber 3431.13 cm^{-1} (9) and Si-OH bond at wavenumber 875.62 cm^{-1} (4)N-H bond indicated the binding between the active site of enzyme and sea sand matrix.

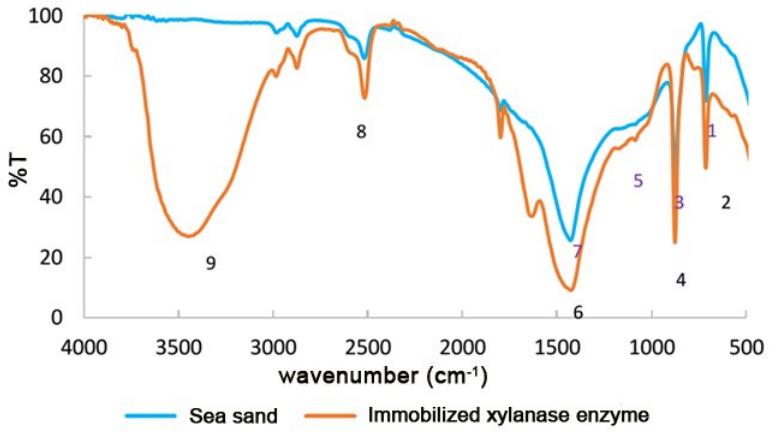


Figure 2. FT-IR spectra of sea sand matrix and immobilized xylanase enzyme

The purified xylanase contained protein of 4.517 mg/m with activity of free xylanase was 15.911 $\mu g.g^{-1}.min^{-1}$.

Determination of optimum shaking time

The optimum condition of immobilized xylanase was determined by various shaking time to investigate time needed by enzyme interacted with the adsorbed matrix in immobilization process. The more shaking time, xylanase amount absorbed into sea sand also increased therefore it will reach saturation level which means adsorption rate was equivalent to desorption rate. The curve which illustrated the correlation between shaking time toward adsorbed xylanase amount was depicted in Figure 3.

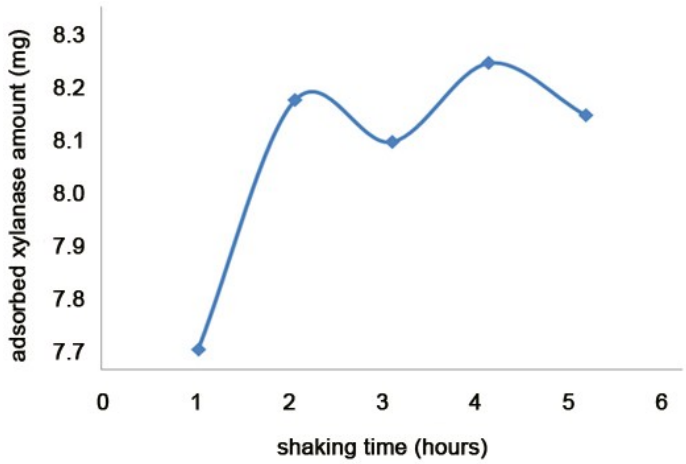


Figure 3. Correlation between shaking time vs. adsorbed xylanase amount

The xylanase amount adsorbed by sea sand matrix was enlarged significantly following the increase of shaking time for 1 until 4 h. This result may due to the equilibrium state which afforded the optimum adsorbed xylanase of 8.206 mg. The shaking time in 5 h occurred the decrease of adsorbed xylanase which caused by weak binding between xylanase with sand. Therefore, xylanase was removed easily. The statistic test result showed the shaking time was influenced toward xylanase mass which adsorbed by sea sand, that represented $F_{calculated\ (34.667)}$

> $F_{table\ (19.250)}$. Based on the smallest real difference test (5%) showed that shaking time 3 to 5 h there is no real difference in the result.

Determination of optimum xylanase concentration

Xylanase concentration can influence the adsorbed xylanase amount, the increase of xylanase concentration may lead to enhance the adsorbed xylanase amount. Determination of optimum xylanase concentration was determined by varying the xylanase concentration and shaking time for 4 h was obtained from optimum shaking time.

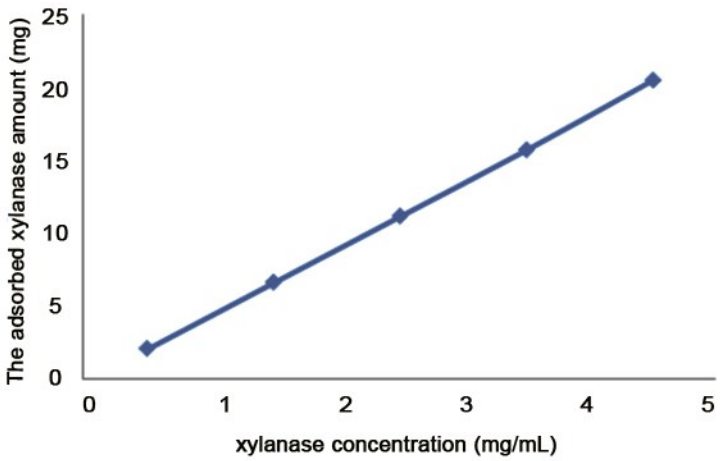


Figure 4. Correlation between xylanase concentrations toward the adsorbed xylanase amount

The adsorption of xylanase in sea sand increased proportionally to the increase of xylanase concentration (Figure 4). It may due to the chemical potential of enzyme solution was more significant than the sand surface. Consequently, the diffusion rate of xylanase into sand surface was enhanced and cause the greater of adsorbed xylanase amount. The optimum xylanase concentration cannot be determined from Figure 4. However, it can be determined by looking at the relationship between xylanase concentration to the activity of adsorbed xylanase (Figure 5). It showed that the activity of adsorbed xylanase had reached maximum at xylanase concentration of 4.5 mg/mL which was adsorbed xylanase amount of 20.5 mg. At xylanase concentration above 4.5 mg/mL increased the number of the adsorbed xylanase, in contrast, the activity of adsorbed xylanase decreased. This may due to a part of xylanase active site was bound to sea sand.

Efficiency of immobilized xylanase utilization

Immobilized xylanase is enzyme which can be utilized repeatedly in some efficiency. The increase use of immobilized xylanase, the activity was decreased. It may due to denaturation process in the incubation and xylanase was released from matrix surface. Immobilized xylanase was conducted for 4 h (optimum shaking time) using xylanase concentration of 4.5 mg/mL and was tested the utilization repetition for 5 times. The result of utilization efficiency was 48.02% for 5 times therefore the xylanase can be reused for up to 4 times repetition due to the activity for 5 times below 50% (Table 1). This result is better than the previous report which is produced immobilized xylanase from *Streptomyces spp.* by using Eudagrit^{TM} S100 that afforded activity of 52.38% after 3 times repetition [14].

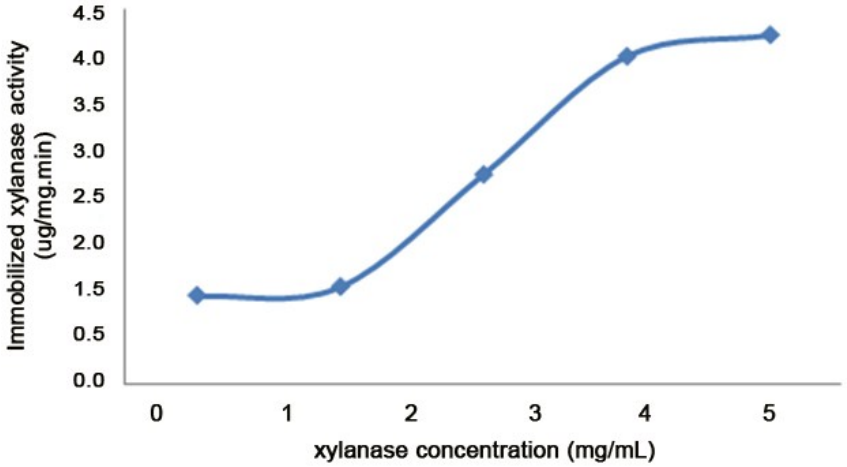


Figure 5. Correlation between xylanase concentrations versus immobilized xylanase activity

Table 1. The influence of activity with utilization repetition of immobilized xylanase

Repetition (times)	Activity (µg g^{-1} min^{-1})	% Efficiency
1	150.299	100
2	124.876	83.08
3	108.907	72.46
4	93.897	62.47
5	72.179	48.02

CONCLUSION

Xylanase enzyme was successfully produced through gradual precipitation enzyme purification method. The optimum condition of immobilized xylanase was investigated after shaking time for 4 h and xylanase concentration of 4.5 mg/mL with the activity of 118.5 µg g^{-1} min^{-1} and xylanase amount adsorbed in sea sand of 20.5 mg. Immobilized xylanase in the sea sand matrix can be utilized for 4 times with activity of 62.47%.

ACKNOWLEDGMENT

The authors wish to thank the DRPM – Ministry of Research Technology and Higher Education –RI for supporting the financial.

REFERENCES

[1] W. A. Khattak, M. Ul-Islam, M. W. Ullah, S. Khan, J. K. Park, *Polysaccharides,* **2015,** 17, 535-579.
[2] F. L. Motta, C. C. P. Andrade and M. H. A. Santana, A Review of Xylanase Production by the Fermentation of Xylan: Classification, Characterization and Applications, *in* Sustainable Degradation of Lignocellulosic Biomass, **2013,** 1st edition, InTechOpen, London, 251-276.
[3] Beg Q. Kapoor K. M., Mahajan L., and Hoondal G. S., *J.Appl Microbiol Biotechnol,* **2001**, 56, 326-338.
[4] A. D. Harris and C. Ramalingam, *JES*, **2010**, 1(7), 1-11.
[5] V. Juturu, J. C. Wu, *Biotechnol Adv.*, **2012**, 30(6), 1219-1227.

[6] Lailah, N., S. Prasetyawan, A. Srihardyastutie, *J. Pure App. Chem. Res.,* **2017**, 6(2), 150-159.
[7] S. Nagar, A. Mittal, D. Kumar, L. Kumar, V. K. Gupta, *Micropor Mesopor Mat.,* **2012,** 161, 1-6.
[8] Esawy, M. A., Mahmoud D. A. R., and Fattah A. F. A., *Braz. J. Chem. Eng.,* **2008**, 25(2), 237-246.
[9] R. A. Sheldon and S. V. Pelt, *Chem. Soc. Rev.,* **2013**, 42, 6223-6235.
[10] Z.-G. Wang, L.-S. Wan, Z.-M. Liu, X.-J. Huang, Z.-K. Xu, **2009**, 56(4), 189-195.
[11] O. S. Bello, I. A. Bello, K. A. Adegoke, *S. Afr. J. Chem.,* **2013,** 66, 117-129.
[12] Z. Ai, Z. Jiang, L. Li, W. Deng, I. Kusakabe, H. Li, *Process Biochem.*, 40(8), 2707-2714.
[13] U. Hanefeld, L. Gardossib, and E. Magnerc, *Chem. Soc. Rev.,* **2009**, 38, 453-468.
[14] Meryandini, A., Sunarti, T.C., Mutia, F., Gusmawati, N.F., and Lestari, Y., *J. Teknol. dan Industri Pangan*, **2009,** 20(1), 9-16.