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Immobilization of Endo-B-1,4-D-Xylanase Using Alginate/Nanocellulose for Xylooligosaccharide Production

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Abstract: Free endo-β-1,4-D-xylanase cannot be used more than once, so it needs to be modified by immobilizing the enzyme. Endo-β-1,4-D-xylanase was obtained from termite abdomen sources by isolation, ammonium sulfate purification, and dialysis methods. Endo-β-1,4-D-xylanase was immobilized with an alginate/nanocellulose matrix. This study aims to determine the activity, protein content, and repeated use of immobilized Endo-β-1,4-D-xylanase. This study used variations of Alginate/nanocellulose (0; 2.5; 5; 7.5; 10) %. Protein levels of Endo-β-1,4-D-xylanase were tested using the Bradford method and activity using the Miller method. The total protein bound to the immobilized Endo-β-1,4-D-xylanase was stated with the immobilized yield data. The immobilized yield with the composition of Alginate Nanocellulose (ANC) (0%) was 45.33% greater than the other compositions. Immobilized Endo-β-1,4-D-xylanase activity is efficient. ANC 5% produces an efficiency of 62.384% at the 12th hour, which is greater than the other ANC compositions.

Keywords: Endoxylanase, Nanoparticle, Xylooligosaccharide.

INTRODUCTION

Endo-β-1,4-D-xylanase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.8) is one of the xylanolytic enzymes that acts by cleaving the β-1,4 linkages in the main chain of the xylan polysaccharide complex found in plant cell walls [1]. The utilization of Endo-β-1,4-D-xylanase is advantageous for producing xylooligosaccharides, however, it has a drawback in that it is difficult to recover the enzyme after the reaction ends [2]. One approach to overcome this limitation is through enzyme immobilization techniques. Enzyme immobilization is a method of combining enzymes with water-insoluble matrices through chemical or physical interactions so that the enzyme can be reused [3]

One of the commonly used matrices is alginate [4]. Alginate is used as a matrix due to its biocompatibility and non-toxic nature [5]. However, alginate has a drawback of low mechanical stability, making it brittle and fragile [6]. Several efforts have been made to overcome this limitation. [7] conducted xylanase enzyme immobilization using Ca-alginate-chitosan with an efficiency of 51.28% and it could be reused five times. Immobilization using Ca-alginate restricts the active site of xylanase, limiting its mobility and resulting in low substrate hydrolysis. Subsequently, [8] utilized nanocellulose as a reinforcing agent in alginate. Nanocellulose particles exhibit properties such as increased crystallinity, surface area, aspect ratio, and enhanced dispersion ability as well as biodegradability. These characteristics make nanocellulose suitable for use as matrix reinforcement fillers, thickeners in dispersions, and as carriers in drug delivery systems and implants [9].

This study aims to immobilize the Endo-β-1,4-D-xylanase enzyme produced from a Bacillus sp. isolate derived from termite gut microorganisms. The research investigates various compositions of the alginate/nanocellulose matrix to evaluate the immobilization yield and the activity of the immobilized Endo-β-1,4-D-xylanase enzyme (immobilization efficiency). The xylooligosaccharides produced from the hydrolysis by the immobilized enzyme are analyzed using Thin Layer Chromatography (TLC) and by determining the reducing sugar content.

EXPERIMENTAL

The materials used in this study are categorized into main materials, chemicals, and supporting materials. The main material used is oat xylan. The enzyme used is purified Endo-β-1,4-D-xylanase derived from the termite abdomen. The nanocellulose used was obtained from the hydrolysis of corn cobs.

The equipment used in the research includes an autoclave (Tomy ES-315), centrifuge (HITACHI CF15RXII), Eppendorf centrifuge, laminar air flow (LAF), shaker incubator, hot plate (Stuart UC-152), fume hood (JAVVA FH180), water bath (Stuart SBS40), magnetic stirrer, vortex mixer (Vortex Genie 2), refrigerator (Sharp), oven (Froilabo), pH meter (Navih F-15), inoculating loop, micropipette and tips, ball pipette, spray bottle, Bunsen burner, hairdryer, autoclave, incubator, shaker, water bath, magnetic stirrer, stir rod, heating bath, Hitachi U-2900 spectrophotometer with cuvettes, and digital camera.

Production of Endo-β-1,4-D-xylanase

Bacterial isolates producing Endo-β-1,4-D-xylanase were rejuvenated on Luria-Bertani (LB) Borth. The LB Broth was incubated at 37°C for 16 hours. A single colony from the LB Broth was inoculated into a liquid inoculum medium. A total of 1 mL of this liquid inoculum was added to 50 mL of production medium. The enzyme obtained is referred to as crude Endo-β-1,4-D-xylanase extract. The supernatant obtained was then referred to as crude enzyme extract. The crude enzyme extract was purified by ammonium sulfate fractionation, which involved mixing 1 mL of crude extract with 50% (w/v) ammonium sulfate. Enzyme purification was carried out by dialysis at 4°C using dialysis tubing immersed in a beaker filled with citrate-phosphate buffer.

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Isolation of Cellulose from Corn Cobs

Corn cobs were ground using a grinder and sieved through a 60 mesh sieve. A total of 20 grams of corn cobs were added to 500 mL of 3% (w/v) NaOH solution at 100°C with stirring for 3 hours. The material was then bleached using 500 mL of 1.4% NaClO solution at 80°C for 2 hours [10]. The resulting solid was washed with distilled water until a neutral pH was reached. The bleached material was then dried for 24 hours at 40°C using an oven [11].

Preparation of Nanocellulose by Acid Hydrolysis

A total of 10 grams of cellulose were placed in a three-neck flask containing 200 mL of 49% H_2SO_4 solution. Hydrolysis was carried out by heating the solution at 45°C for 60 minutes with stirring [10]. After hydrolysis, 5% (w/v) Na_2CO_3 solution was added with stirring to neutralize the pH. The resulting solid was mixed with 200 mL of distilled water and sonicated for 30 minutes using a sonicator.

Immobilization of Endo-β-1,4-D-xylanase Using Beads (ANC)

The nanocellulose concentrations used were 0%, 2.5%, 5%, 7.5%, and 10% with a constant alginate concentration of 3%. First, nanocellulose suspension was mixed with distilled water, then alginate was added until a final concentration of 3% was achieved. The homogenized mixture of Endo-β-1,4-D-xylanase and ANC was placed into a 10 mL syringe and dropped into 5% $CaCl_2$ solution to form beads [12]. The resulting beads were filtered and rinsed with distilled water. The formed beads were dried at room temperature for 1 hour before use [13].

Qualitative Analysis of XOS

A sample of 5 µL was spotted onto a chromatoplate and placed in a chamber containing the eluent 1-butanol, acetic acid, and distilled water in a 2:1:1 ratio [14]. After drying, the chromatoplate was sprayed with a staining reagent containing α-naphthol, sulfuric acid, and ethanol in a ratio of 1:10:200 and heated in an oven at 100°C for 5 minutes. Based on the migration data, the retention factor (Rf) was calculated using the following formula:

$$Rf = \frac{r_s}{r_{sp}}$$

Where, r_s: migration distance of the sample (cm); and r_{sp}: migration distance of the solvent front (cm).

Analysis of Reducing Sugar

The total reducing sugar content was measured by taking 1 mL of each xylooligosaccharide sample. The samples were centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant obtained was then used for reducing sugar determination using the Miller method [15].

Determination of Immobilization Efficiency and Yield

According to Singh et al. (2010), immobilization efficiency is calculated by:

$$\text{Immobilization Efficiency} = \frac{\alpha_i}{\alpha_f} \times 100\%$$

Where, α_i: total activity of immobilized enzyme; and α_f: total activity of free (non-immobilized) enzyme.

Immobilization yield is calculated using the formula:

$$\text{Immobilization Yield} = \frac{p_i - (p_w + p_s)}{p_i} \times 100\%$$

Where, p_i: total protein content in Endo-β-1,4-D-xylanase; p_w: total protein content in wash solution; and p_s: total protein content in supernatant after immobilization.

RESULT AND DISCUSSION

Endo-β-1,4-D-xylanase is the enzyme immobilized in this study. The enzyme was isolated from a *Bacillus* sp. isolate originating from termite abdomens. The endo-β-1,4-D-xylanase enzyme was obtained through a series of steps including isolation, 50% ammonium sulfate fractionation, and dialysis. The hydrolysis products of the crude enzyme extract, the 50% fraction, and the dialysate with oat xylan as the substrate—producing XOS—were reacted with Miller's reagent containing 3,5-dinitrosalicylic acid (DNS). The reducing sugars formed reacted with DNS to produce 3-amino-5-nitrosalicylic acid (ANS) through a heating process. The reaction was heated at 100 °C, resulting in a yellowish-brown solution. The absorbance of this solution was then measured at a wavelength of 550 nm. Protein concentration was also measured to obtain the enzyme's specific activity data. The protein content of the enzyme was determined by adding Bradford reagent to the enzyme sample, and the absorbance was measured at a wavelength of 594 nm.

Table 1. Activity and Protein Concentration of Endo-β-1,4-D-xylanase

	Enzyme Activity (U/mL)	Protein Concentration (mg/mL)	Specific Enzyme Activity (U/mg)
Crude Extract	0.061	0.059	1.034
50% Fraction	0.078	0.051	1.529
Dialysate	0.128	0.045	2.844

Based on Table 1, the crude enzyme extract exhibits lower activity compared to the 50% fraction and the dialysate. The crude extract is not yet pure, as it still contains proteins other than the endo-β-1,4-D-xylanase enzyme. The presence of other proteins in the crude extract is evidenced by its higher protein content compared to the 50% fraction and the dialysate. The purification steps resulted in a decreasing protein concentration, indicating the removal of non-target proteins. As the enzyme becomes purer, its specific activity increases. The enzyme with the highest purity was selected for immobilization into the alginate/nanocellulose matrix.

Alginate/nanocellulose beads were prepared using the ionic gelation method with Ca^{2+} ions from a $CaCl_2$ solution. The $CaCl_2$ solution acts as a cross-linking agent. The formation of alginate/nanocellulose gel is modeled on the "egg-box" structure. Calcium ions bind between alginate monomers and entrap the nanocellulose. Gel formation occurs when the mixture is dropped into the $CaCl_2$ solution. The interaction between Ca^{2+} ions and guluronate monomers in the alginate causes cross-linking, which results in a denser and more solid gel structure.

Table 2. Immobilization Yield Data

NCC Concentration (%)	Pre-immobilization Protein Level (mg/mL)	Released Protein Concentration		Total Released Protein (mg/mL)	Total Bound Protein (mg/mL)	Immobilization Yield (%)
		During Washing (mg/mL)	After Hydrolysis (mg/mL)			
0	1.125	0.263	0.352	0.615	0.510	45.33
2.5		0.232	0.467	0.699	0.426	37.87
5		0.292	0.345	0.637	0.488	43.38
7.5		0.206	0.533	0.739	0.386	34.31
10		0.256	0.504	0.760	0.365	32.44

The immobilization yield represents the ratio of immobilized enzyme protein to the initial protein at the mixing stage. Based on **Table 2**, 5% concentration of nanocellulose (NCC) resulted in an immobilization yield of 43.38%. This value is lower than the immobilization yield obtained without NCC, which was 45.33%. Alginate can bind enzymes more effectively compared to the presence of nanocellulose. However, alginate has a drawback in its poor mechanical stability, making alginate beads prone to breakage [6].

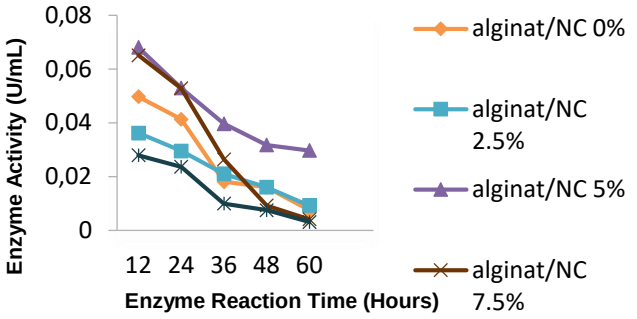

Figure 1. Enzyme Activity of Immobilized Endo-β-1,4-D-xylanase in Alginate/Nanoselulose with Various Compositions

The enzyme activity of immobilized enzyme in all compositions of alginate/NCC beads (Figure 1) shows the highest enzyme activity compared to the subsequent hydrolysis times. Repeated enzyme use leads to a decrease in enzyme activity across all bead variations, as the enzyme is released after the previous hydrolysis process. This results in a reduction in the product produced, as indicated by the decrease in enzyme activity.

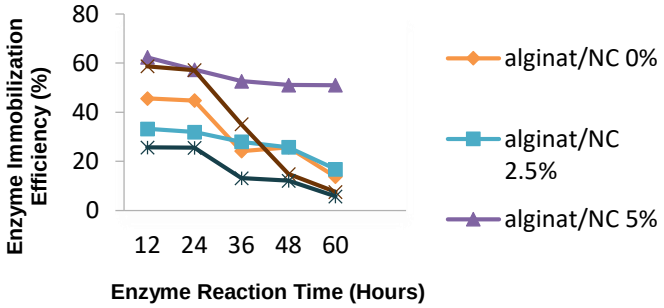

Figure 2. Efficiency Percentage of Immobilized Endo-β-1,4-D-xylanase in Alginat/Nanoselulose at Various Compositions

Figure 2 shows the efficiency of immobilized endo-β-1,4-D-xylanase in Alginat/Nanoselulose (ANC) at various compositions. Repeated enzyme use results in a decreasing efficiency. The calculated enzyme efficiency of immobilized endo-β-1,4-D-xylanase in Alginat/Nanoselulose (ANC) shows that enzyme efficiency decreases in the following order: Alginat/NC 5% > Alginat/NC 7.5% > Alginat/NC 0%, and so on. It is clear that Alginat/NC 5% produces the highest efficiency. This is due to the stronger interaction between the composite and the enzyme compared to the other compositions.

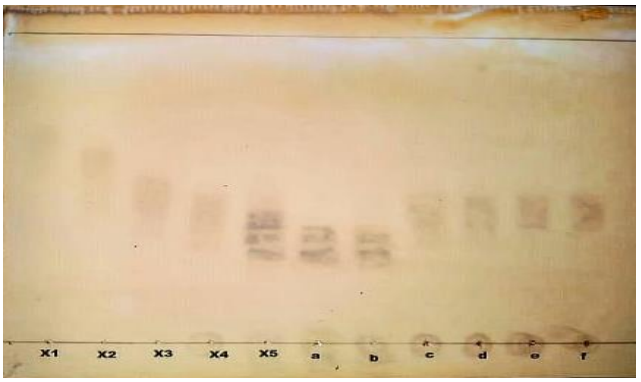

Figure 3. Chromatogram of Standard and Samples (a) Free Enzyme; (b) ANC 0%; (c) ANC 2.5%; (d) ANC 5%; (e) ANC 7.5%; and (f) ANC 10%

The retention factor (Rf) is the ratio of the distance traveled by the sample to the distance traveled by the solvent. Based on Figure 3, the results of oat xylan hydrolysis using free and immobilized endo-β-1,4-D-xylanase positively produced xylooligosaccharide products. The standard Rf values for xylosa are X1, X2, X3, X4, and X5, which are 0.70, 0.60, 0.50, 0.40, 0.34, and 0.30, respectively. The control with inactive enzyme did not produce any spots because no xylooligosaccharide product was formed. The Rf values for the samples obtained in this study from hydrolysis with free enzyme, Alginat Nanoselulose (ANC) 0%, 2.5%, 5%, 7.5%, and 10% are 3.125, 3.125, 1.818, 1.818, 1.818, and 1.818, respectively. The free enzyme sample and the Alginat/Nanoselulosa 0% enzyme sample each formed spots in the X5 and X6 regions. The Alginat/Nanoselulosa 2.5%, 5%, 7.5%, and 10% samples only formed spots near X3 and X4.

Reducing sugar content indicates the amount of xylooligosaccharides (XOS) produced during the hydrolysis process. The sugar content was measured using the Miller method. Table 4.4 shows the total reducing sugar content from the hydrolysis of immobilized enzymes in alginate and alginate/NCC 2.5%, 5%, 7.5%, and 10%, which are 0.581

mg/mL, 0.463 mg/mL, 0.692 mg/mL, 0.612 mg/mL, and 0.495 mg/mL, respectively. All immobilized enzyme variations produced more total reducing sugar compared to the free enzyme, which only produced 0.349 mg/mL. This is because the immobilized enzyme can be used for 5 cycles of hydrolysis, whereas the free enzyme cannot be reused, leading to higher product yields from the immobilized enzyme.

CONCLUSION

The activity of immobilized endo-β-1,4-D-xylanase decreased after 5 cycles of hydrolysis. The yield percentage and immobilization efficiency were influenced by the content of nanocellulose (NC) in the beads. The highest yield percentage of 45.3% was observed with 0% NC, while the highest immobilization efficiency (62.385%) occurred with 5% NC content. The xylooligosaccharides produced in all hydrolysis reactions using the immobilized endo-β-1,4-D-xylanase with an alginate/nanocellulose matrix were X4 and X3. The total reducing sugar content from the hydrolysis of the immobilized enzyme with alginate and alginate/NCC at 2.5%, 5%, 7.5%, and 10% NC were 0.581 mg/mL, 0.463 mg/mL, 0.692 mg/mL, 0.612 mg/mL, and 0.495 mg/mL, respectively. All variations of the immobilized enzyme resulted in higher total reducing sugar levels compared to the free enzyme, which only produced 0.349 mg/mL.

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