Endo-xylanase Enzyme Production using Agroindustrial Biomass as Feedstock by *Kitasatospora* sp.

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**Abstract.** Endo-β-1,4-xylanases is an enzyme that depolymerize xylan, a major component of lignocelluloses. Lignoseulose is a great source of cheap carbohydrate and thus has been used over the past decade as a raw material for the production of high value products, such as enzymes. However, lignocelluloses are a highly recalcitrant material that is extremely difficult to depolymerize. By using proper pretreatment, lignocelluloses from agroindustrial biomass can replace conventional carbon sources in media preparation for enzyme production. In this study, we used three kinds of agroindustrial biomass, such as sorghum variety of Buleleng, sorghum variety of JP, rice straw LIPI GO1 and these biomasses were pretreated with acid to remove a portion of lignin for use as a carbon source to endoxylanase production. The production of xylanase *Kitasatospora* sp. under submerged fermentation was investigated with different carbon sources using agroindustrial biomass. Optimization steps included studies carbon source concentration and pH medium fermentation. The optimized condition of enzyme production was obtained using the sorghum variety Buleleng biomass at 2% concentration and pH medium is 9.0 with activity 3.04 U/mL.

Keywords: *Kitasatospora*, Endo-xylanase, agroindustrial biomass, sorghum, rice straw, submerged fermentation

1. Introduction

Endo-xylanase is glycosyl hydrolases that catalyze a random hydrolysis of the β-1,4-glycosidic bonds in a xylan via a double displacement mechanism [1]. Endo-xylanase has markedly increased every year due to its widespread potential application in various industry, such as pulping and bleaching processes, textile, waste treatment, the enzymatic saccharification of lignocellulosic materials, animal feed, paper, biofuel industries, enhance digestibility of feed nutrients, improvement of wheat flour quality.

Actinomycetes are recognized as dominant xylanolytic species during several processes of biomass transformation [2], and their enzymes may find new applications in the pulp and paper industry[3,4] (Buchert et al 1992, Leduc et al 1995) and in the recovery of fermentable sugars from hemicelluloses [5]. We already selected and got one strain produce novel xylanases from Actinomycetes in Indonesia [6].

Xylan is the main hemicellulosic polysaccharide found in plant cell walls and is composed of a backbone chain of β-1, 4-linked xylosyl residues and short side chains of arabinosyl, glucoronosyl and acetyl residues [7]. Xylan represents a significant resource of renewable biomass and comprises up to 20-35% dry weight of wood and agricultural wastes [8]. To meet the needs of industry, more attention
has been focused on the enzyme production with lowest cost. Different strategy to enhance xylanase production have been conducted. Xylanase production by xylan commercial is very expensive. Besides that, xylan commercial already discontinuous in some company. Its need alternative carbon source which is high xylan content for xylanase production.

In recent years, there has been an increasing interest towards more efficient utilization of agricultural and industrial residues which are generally considered to be a waste and cause environment pollution, unnecessarily occupy space and require high amount for proper management [9]. Agricultural waste and residues are mostly organic biomass, which through clean microbial technology, can be utilized in various value added applications, such as culture media supplemented for enzyme production [9]. In the present study, xylanase production from *Kitasatospora* sp. using agro residues biomass is reported.

2. Methods

2.1. Microorganisms and growth conditions

*Kitasatospora* sp. was obtained from Biotechnology Culture Collection (BTCC), was used in this study. *Kitasatospora* sp. was maintained in glycerol stock in minus 80 °C. Stock cultures were maintained in ISP2 medium added of 1.7% agar, at 28 °C. The initial submerged cultivation medium was composed of ISP2 medium without glucose with 1% of various biomass. After we get the selected substrate for xylanase production, for the experiments of optimization, the cultivation medium was composed of different concentrations of selected substrate and also varying the medium pH and the time of cultivation.

2.2. Chemicals

All the reagents and chemicals used for the study were standard grades. Standard Xylose, Xyloooligosaccharides were purchased from Megazyme (Ireland). Agroresidue, such as Sorghum biomass were obtained from laboratorium of genomics and crop improvement, rice straw of INPAGO LIPI G0 1 was obtained from laboratory of agronomy for evaluation biotechnology product.

2.3. Biomass preparation and pretreatment

Three biomass (sorghum variety of Buleleng, sorghum variety of JP and rice straw of INPAGO LIPI G0 1) were harvested and then chopped to a size of approximately 10 cm and then continued milled by using a ring flaker until smaller and then the biomass was dried in the sun to be dry. The dried biomass is put into the mill machine to be chopped again until smaller and finally the biomass is sifted with a 40 mesh sieve. These biomass were pretreated by 1% sulphuric acid.

2.4. Culture conditions for enzymes production

The optimization experiments were carried out in 300 mL Erlenmeyer flasks containing 30 mL of cultivation medium. The single colony was inoculated in ISP2 medium for three days at 28 °C, 190 rpm. The shake flasks contained ISP2 medium without glucose plus biomass were inoculated with inocula at 10 % concentration of cells. Shake flasks cultures were maintained at 28 °C, 190 rpm for 144 h and the sampling was conducted at 24 h.

2.5. Effect of carbon source on enzymes production

Liquid cultures were prepared in ISP2 medium without glucose supplemented with the carbon sources. Initially, the medium was supplemented with each carbon sources at 1% (w/v) concentration. Erlenmeyer flasks containing 30 mL of medium were inoculated with 3 mL of the preculture and incubated at 28 °C, 150 rpm for 144h.

2.6. Effect of carbon sources concentrations
After we get the selected substrate for xylanase production, the cultivation medium was composed of different concentrations of selected substrate (1.0, 1.5, 2.0 and 2.5%). Erlenmeyer flask (300 L) containing 30 mL of medium were inoculated with 3 mL of the preculture and incubated at 28 °C, 150 rpm for 144 h.

2.7. Effect of pH medium on enzymes production
To verify the influence of the initial pH medium, cultures supplemented with 2% sorghum variety of JP in pH 5, 7, 9 adjusted with 0.1 M HCl or NaOH and Erlenmeyer flask (300 L) containing 30 mL of medium were inoculated with 3 mL of the preculture and incubated for 144 h. The experiments were carried out at 28 °C, 150 rpm for 144 h.

2.8. Preparation of crude enzymes
The crude enzyme solution was initially subjected to filtration to separate the actinomycetes cell and residual biomass. Afterwards, a clear enzyme solution was extracted by centrifuging the culture fermentation at 12,000 rpm for 20 min at 4 °C. The crude xylanase was used for enzyme activity analysis.

2.9. Enzymatic activities
Xylanase activity was measured according to [10] with slide modification. Xylanase activity was assayed in a mixture reaction with xylan beechwood in 0.05 M sodium acetate buffer pH 5.0 and approximately diluted enzyme solution. Reducing sugar was quantified with the DNS acid reagent and the absorbance was measured at 540 nm. One unit of activity was defined as the amount of enzymes required to release 1 umol of product equivalent per min in the assay conditions at 60 °C.

3. Result and Discussion
In the previously study shown that strain Kitasatospora sp. was selected as a good producer for xylanase enzyme from Actinomycetes [6]. Xylan is the most common carbon source utilized in xylanase enzymes studies [11]. For larger scale production processes, however, this rather expensive substrate is not suitable [12]. Besides that, some commercial xylan products are no longer produced by private companies. Different lignocellulosic material, residue from forestry, agricultural practices, pulp and paper industry and many agroindustries have been used as a low price alternative for xylanolytic enzyme production. Therefore, in this study, we investigated for the alternative carbon source for xylanase production from agricultural residues. Three kind of biomass, such as sorghum variety of Buleleng, sorghum variety of JP and rice straw of INPAGO LIPI GO 1 were used in this study shown in Figure 1. Kitasatospora sp. was cultivated in liquid cultures with various carbon sources from three pre-treated biomasses at 28 °C. Xylanase activity were observed in all of carbon sources during seven days the production of xylanase. Fig. 2 shown the results qualitative (2A) and quantitative (2B) analysis of crude enzyme xylanase from Kitasatospora sp. from three different of biomasses. The results obtained from qualitative analysis in Fig. 2A shown that three different biomasses positively produce xylanase enzymes after staining by congo red which are marked by the formation of clear zone. We can see from day zero to day seven, clear zone appears around the third and fourth days and continue to expand into the seventh days. Based on this results shown that three biomasses are capable of
producing xylanase enzymes, but sorghum variety of Buleleng shown a larger clear zone than sorghum variety of JP and rice straw of INPAGO LIPI GO 1. Whereas by quantitative analysis shown in Fig. 2B that among the three biomasses evaluated, the highest level of xylanase was sorghum variety of Buleleng. The xylanase activity on sorghum variety of Buleleng and rice straw of INPAGO LIPI GO 1 biomass decreased from 0 to 96 hours. So, we can see that from zero to 96 hours there was no xylanase enzyme activity resulting from the fermentation process. However, at the 120th hour until the 168th hour, the xylanase enzyme activity was produced at the 120th hour with activity 2.49 U/mL (sorghum variety of Buleleng) and 2.01 (rice straw of INPAGO LIPI GO 1) and increased until the end of the hydrolysis process with activity 2.71 U/mL and 2.28 U/mL, respectively. Whereas in sorghum variety of JP biomass, xylanase enzymes was produced at 120 hours with activity 2.30 U/mL, but their activity decreases at the 144th (2.24 U/mL) and 168th hours (2.19 U/mL).

The xylanase activity at zero hour is zero. The reducing sugar produced by this strain at zero hours shown the glucose or sugar content in the culture medium fermentation. We guest that there are inhibitors that interfere with xylanase enzyme activity, so we continue to check the crude supernatant by Thin Layer Chromatography (TLC) (Fig. 2C). [13] described that the presences of sugar in the production of enzymes can affect the results of the fermentation process. The addition of glucose in the cellulase enzyme fermentation process greatly affects to the amount of enzyme activity formed. The higher the glucose concentration in the hydrolysis medium will affect to the accumulation of the cellobiose, which is acts as a secondary inhibitor. Therefore, the activity of the enzymes formed is very low.

**Figure 1.** Three biomass (A) sorghum variety of JP, (B) sorghum variety of Buleleng and (C) rice straw of INPAGO LIPI GO 1 were used in this study. Fresh biomass (upper side) and pre-treated biomass (under side) were pretreated by 1% of sulphuric acid.
Figure 2. Qualitative (A), quantitative (B) and TLC (C) analysis crude enzyme xylanase produced by Kitasatospora sp. from three different of biomasses used as a carbon sources

The highest enzyme activity was seen on the seventh day from each biomass, which had the highest enzyme activity is sorghum variety of Buleleng with activity 2.69 U/mL then followed by sorghum variety of JP with activity 2.29 U/mL and rice straw of INPAGO LIPI GO 1 with activity 2.27 U/mL. Considering these result, sorghum variety Buleleng was selected for the subsequent optimization experiments.

The optimization process was continuing for substrate concentration by using sorghum variety of Buleleng which is consist of 0.5%, 1%, 1.5%, 2.0% and 2.5%. Xylanolytic enzymes production profiles in the culture supernatant of Kitasatospora sp. in submerged culture with sorghum variety of Buleleng at different concentration for 7 days at 28 °C was shown in Fig. 3A. From these figure shown that substrate concentration of 0.5% has decreased from zero hour to 48 hours, then has an increase from hour 72 to 168 hours. Enzyme activity at substrate concentration 1% has decreased from zero hour to 96th hour, then the enzyme activity rises at the 120th hour to 168th hour. In a concentration of 1.5% decreased enzyme activity from the zero to 96th hour, then increased at the 120th hr to the 168th hour. Enzyme activity at 2% decreased from zero until 144h, and just displaying activity at 168h. Then finally, at 2.5% enzyme activity decreased from zero until 144h, and just displaying activity at 168h. The best substrate concentration was selected at 2% with the activity 2.75 U/mL at 168h. While the lowest enzyme activity was at a substrate concentration of 0.5% at 48h with the amount of enzyme activity 2.18 U/mL. In this experiment also indicated that presence of sugar compound that act as inhibitors that inhibit the activity of the enzymes. Therefore, TLC analysis was carried out to check the sugar content during the fermentation process (Fig. 3B).
Figure 3. Xylanase enzymes production profiles in the culture supernatant of Kitasatospora sp. in submerged culture fermentation with sorghum variety of Buleleng at different concentration for 7 days at 150 rpm, 28 °C.

The final optimization fermentation was conducted using sorghum variety of Buleleng at 2% concentration at different pH medium fermentation. pH media is one of important environmental parameters that determine growth rates of microorganisms and significantly affect the level of enzyme production. Enzyme activity is influenced by the pH of fermentation media, synthesis and expression of certain genes and the activity of microorganism metabolism is influenced by internal pH, which is in response to the external environment [14]. Xylanase enzymes production profiles in the culture supernatant of Kitasatospora sp. in submerged culture with 2% sorghum variety of Buleleng at different pH for 7 days at 28 °C was shown in Fig. 4. Based on the data in Fig. 4 shown that the highest xylanase activity was at pH 9.0 at 168 h 3.04 U/mL. Whereas there is no indicated xylanase activity at pH 5.0 because the xylanase activity is constant. This result was supported by [15] that described Actinomycetes cannot growth optimally at environments pH below 6.5. Xylanase enzyme production from Streptomyces albidoflavus has the higest activity at pH 8.0 with value of activity 0.21 U/mL, whereas xylanase enzyme produced by Streptomyces sp. has higest value at pH 7.2 which was activity 3.86 IU/mL. The xylanase activity produced by different pH shown similar pattern results with xylanase activity at various different biomass and concentration. We guess the presences of sugar compound which inhibit of the xylanase activity during of fermentation process. Therefore, TLC also was performed to determine the presences of these sugar in culture supernatant (Fig. 4B).
Figure 4. Xylanase enzymes production profiles in the culture supernatant of *Kitasatospora* sp. in submerged culture with 2% sorghum variety of Buleleng at different pH for 7 days at 28°C.

4. Conclusions
The resulting enzymes profiles found in the different cultivation using three different of biomasses demonstrated *Kitasatospora* sp. is a good producer of the xylanase enzymes. The optimized condition of enzyme production was obtained using the sorghum variety of Buleleng biomass at 2% concentration and pH medium is 9.0 with activity 3.04 U/mL. The presences of inhibitor contain sugar in the culture supernatant need recheck again of the biomasses pretretment method to obtain low amount of sugar in the pretreated biomass which is suitable for enzyme production.
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6. References
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