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Reutealis trisperma Press Cake Induced Production of Xylanase by *Trichoderma reesei*: Effect of C/N Ratio and Initial pH

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Abstract. *Reutealis trisperma* plant is widely used for nature conservation and the oil seeds have been studied for biodiesel production. The press cake is a solid waste which contains 34.03% protein, 18.56% total sugar, 15.58% lipid, 6.32% moisture and 25.51% others. Since its high content of protein, the cake is used as additional nitrogen source for xylanase production using *Trichoderma reesei*. Xylanase has a wide range of applications such as in pre-bleaching of pulp, improving the digestibility of animal feed stocks, bioconversion of lignocellulosic material to fermentable products and clarification of fruit juices. In pulp and paper industries, pre-bleaching of pulp by xylanase can save 35-40% of chlorine used which would reduce the potential hazard to environment. *T. reesei* is a mesophyllic, filamentous fungus which has been well studied for its ability to produce extracellular enzymes capable of degrading cell wall polysaccharides. The aims of the study are: 1) to formulate media at various C/N ratio in defined medium and various initial pH, and to formulate media containing ground press cake at both optimum C/N ratio and initial pH from defined medium. 2) to calculate xylanase activity in both media. In this study, *T.reesei* was cultivated in shake flask at 28^{o}C in defined medium at various C/N ratio and initial pH and in the culture medium contained ground press cake at optimum C/N ratio and initial pH. The defined medium composition contained cellulose as carbon source at various concentration; organic and inorganic nitrogen sources and mineral salts. Ground cake was used to replace some inorganic nitrogen used in defined medium for media containing ground cake. Sample was withdrawn periodically to measure the pH and xyalanse activity. The best C/N ratio and initial pH in defined medium were 7.8/1 and 6.0 respectively which resulted in 66.93 U/mL of xylanase activity. The xylanase activity was 30.75 U/mL in media containing ground press cake.

INTRODUCTION

The production of enzyme depends on inducers and the effective inducers influence the production of xylanase. Inexpensive substrate and nitrogen sources from plant materials could induce effective xylanase production. *Reutealis trisperma* known as "kemiri sunan" is a shady tree generally grown in east and west Java. Biodiesel production has been studied from the seed oil. After oil extraction by mechanical press, remaining cake contained (% w/w) 34.03 % protein, 6.32 % moisture, 18.56 % Carbohydrate, 29.5 % fat, 7.76 % ash and 3.83% others [1]. The cake was provided by CV. Energy Baru Sentosa located in Gresik, East Java. Since the cake contains nitrogen and carbohydrate, we tried to use the cake for additional nitrogen and carbon sources to produce xylanase. Xylanase production using *Reutealis trisperma* cake as nitrogen and carbon sources have not yet been studied. Most studies used *Trichoderma reesei* Rut C-30, a mutant to produce xylanase [2] by solid state fermentation, SSF. Some studies used soybean hull as substrate in both SSF and submerged fermentation by *Aspergilus niger* to produce xylanase and other enzymes [3,4,5]. Another study used lactose as carbon source for *Trichoderma reesei* Rut C-30 in submerged

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fermentation to produce xylanase [2]. There is no study used *Reutealis trisperma* as carbon and nitrogen sources to produce xylanase by *Trichoderma reesei*.

Xylanase has been widely used in commercial applications, such as in the bleaching of pulp, food, feed meal and clarifying juice. The use of xylanase in pulp bleaching process reduces up to 30 % of chlorine used in the pulp bleaching, leading to 15-20% reduction of chlorine release in the effluent [6]. Xylanase also has been used in bread-processing to delay crumb formation, letting the dough to grow and increase bread volume [7,8]. In animal feed, xylanase may improve the digestion of nutrients in the digestive tract. Xylanase is used with cellulase and pectinase to clarify juices and for liquefying fruits and vegetable [9]. Currently, Indonesia is importing xylanase from overseas for domestic demand since no xylanase has been produced in the country. *Trichoderma reesei* is a mesophyllic, filamentous fungus which has been studied for its ability to produce an extracellular enzyme capable in degrading cell wall polysaccharides. The fungus is able to grow on many carbon sources to produce enzyme [10]. The composition of lignocellulose materials differs from each other based on the source and demography.Thus, fungal culture for enzyme production is affected from differences in nutrient compostion. Fungal cultivation on pre- treated corn stover has been reported to perform better in enzyme production [11]. Thus, we used *Reutealis trisperma* cake (RTC) in natural and pretreated forms. In this study, *T. reesei* was cultivated in both media containing cellulose as carbon source and ground press cake of *Reutealis trisprema* as carbon and nitrogen sources. The aims of the study are 1) to formulate media with various C/N ratio and at various initial pH in defined medium, and 2) to formulate media containing ground press cake at both optimum C/N ratio and initial pH from defined medium experiment. The xylanase activity in both media was also evaluated.

MATERIAL AND METHODS

Materials

Strain and Pre-cultures

T.reesei was obtained from the University Gadjah Mada culture collection.The culture was stored at 4^{o}C on potato dextrose agar slant (PDA, 40 g/L) and sub cultured regularly. Pre-cultures were prepared by adding three loops of cells from slant to a flask containing 15 mL of the PDB (Potato Dextrose Broth). Inoculum was ready for inoculation after three days incubation at 28^{o}C. Both PDA and PDB were obtained from Sigma Aldrich.

Reutealis trisperma Press Cake (RTC)

Ground RTC (40 mesh) was used as carbon and nitrogen sources. The natural RTC and pretreated RTC were used in the experiment. The pretreatment was carried out by mixing ground RTC with 0.6% (w/v) aqueous sodium hydroxide (NaOH) at 1:30 ratio [1]. The mixture was heated at 50^{o}C and agitated at 160 rpm for 25 minutes. Finally, RTC was washed and dried at 50^{o}C overnight until it reached constant weight. All chemicals used were analytical grade and purchased from Merck, unless stated otherwise.

Media

Three different media were used in the experiments: defined medium, natural RTC (NRTC) medium and Alkaline pretreated RTC (Alk-RTC) medium. The defined medium consists of various concentration of cellulose to get the certain ratio of C/N , $(NH_4)_2SO_4$ 1.4g/L, urea 0.3g/L protease peptone 1g/L, KH_2PO_4 2g/L, $CaCl_2{\cdot}2H_2O$ 0.4g/L, $MgSO_4{\cdot}7H_2O$ 0.3g/L, tween 80 0.2 mL/L, $FeSO_4{\cdot}7H_2O$ 5mg/L, $MnSO_4{\cdot}H_2O$ 0.1212 mg/L, $ZnSO_4{\cdot}7H_2O$ 1.4mg/L, and $CoCl_2{\cdot}7H_2O$ 2mg/L. RTC contains both carbohydrate and protein. We have used the RTC carbohydrate as additional carbon source and protein as the only nitrogen source. The natural and pretreated RTC media (C/N 7.8) consist of natural RTC or Alk RTC 16.5 g/L, Cellulose 7 g/L, KH_2PO_4 2g/L, $CaCl_2{\cdot}2H_2O$ 0.4g/L, $MgSO_4{\cdot}7H_2O$ 0.3g/L, tween 80 0.2 mL/L, $FeSO_4{\cdot}7H_2O$ 5mg/L, $MnSO_4{\cdot}H_2O$ 0.1212 mg/L, $ZnSO_4{\cdot}7H_2O$ 1.4mg/L, and $CoCl_2{\cdot}7H_2O$ 2mg/L. All chemicals are analytical grade and were purchased from Merck.

Methods

Fermentation

In shake flask cultivations, 15 mL inoculum was inoculated to 135 mL culture medium in a 250 mL flask. Cultures were incubated for 5 days at 28°C in an orbital shaker at 200 RPM. The initial pH was adjusted to certain values as parameter for the experiment. Samples were taken aseptically every day to measure pH and enzyme activity.

Protein and Carbohydrate Analysis

Protein in press cake was analyzed using Kjeldahl method and the carbohydrate was analyzed using phenol sulfuric acid method [12]

Enzyme Activity Analysis

Xylanase activity was determined by a modified method from Bailey et.al [13]. A 1 wt% beechwood xylan (Sigma Aldrich, St. Louis, MO) was prepared by mixing 2 g beechwood xylan in 200 mL 0.05 M sodium citrate buffer (pH 5.3). The substrate was kept at -20°C prior to use. A 100 µL enzyme broth and 900 µL of subtrate was added to 25 mL test tube consecutively. The samples and blanks were incubated at 50°C for 5 minutes in a water bath. After 5 minutes, 3 mL DNS reagent was added to the test tubes to terminate the enzyme reaction. A 100 µL enzyme-containing sample was added to the corresponding blank. The samples and blanks were boiled for 10 minutes to develop color. Distilled water was added into the test tubes to make total volume 25 mL. The absorbance values were measured using UV-Vis Spectrophotometer (Hewlett Packard 8453) at 540 nm. Xylanase activity was measured based on xylose release, which can be represented by following Equation (1).

$$Xylanase\left(\frac{U}{mL}\right)=\frac{xylose\ release(mg)}{(5\ \text{min})(0.1\ mL\ enzyme\ sample)}\ x\ \frac{1\ mmol}{150.13\ mg}\ x\ \frac{1000\ \mu mol}{1\ mmol}=13.32\ x\ xylose\ release\ (mg) \qquad (1)$$

.

RESULTS AND DISCUSSION

Growth on Defined Media

Effect of C/N Ratio

The carbon to nitrogen ratios were calculated from all carbon and nitrogen sources present in the media. We used cellulose concentration of 6, 8, 10, 12 and 14 g/L to get the C/N ratio of 4.7/1; 6.3/1; 7.8/1; 9.4/1; 11/1. We started the fermentation with initial pH 6.0. Fig.1 shows the profile of xylanase activity at initial pH of 6.0 for various C/N ratio. As shown in Fig. 1, the xylanase activity increases with the increased of carbon sources up to the ratio of C/N 7.8 and decreases when the C/N ratio increased (C/N 9.4/1 and C/N 11/1). The N sources (ammonium) may have been depleted on high C/N which resulted in lower yield of xylanase, which is also proved by the decrease of pH during the fermentation process (Fig. 2). Low C/N ratio means limited carbon sources in the system which may lead to lower yield of xylanase. The lowest enzyme activity is 40,14 U/mL at C/N ratio 14/1. Low pH value of 3.6 suggest that the pH did not support optimum xylanase production [2]. The lack of carbon source may reduce the effectiveness of xylanase induction and resulted in lower xylanase yield. The optimum yield of xylanase (66.93 U/ml) occurred at C/N ratio 7,8/1 after 4 days of fermentation. Bailey et al. [13] conducted a research used cellulose (Solka Floc cellulose) as carbon source and got the xylanase activity of 52.7894 U/mL. The measurement of pH during fermentation process was obtained to indicate the growth of *Trichoderma reesei* which relates to the induction of xylanase. Figure 2 shows the decrease of pH during fermentation process. The decrease of pH indicates that *T. reesei* took the TCA cycle to produce organic acid such as citric, oxalic and acetic acid during the induction of xylanase [14].

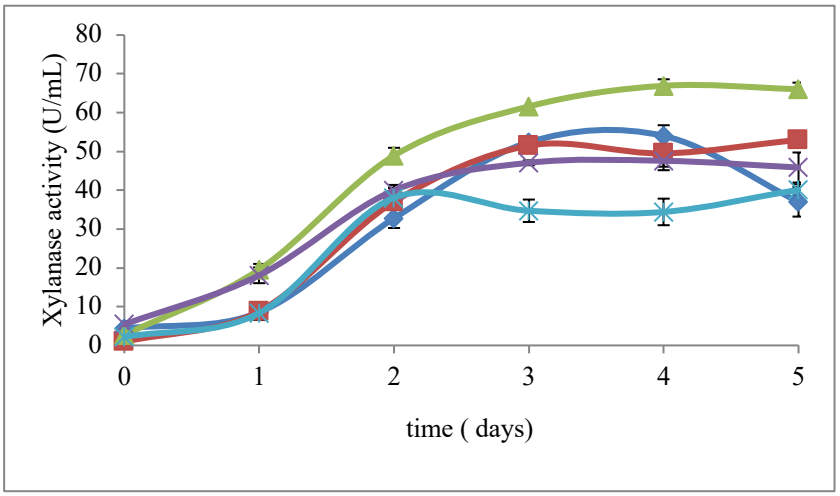

FIGURE 1. Profile of xylanase activity at different C/N ratio and initial pH 6.0 (♦ C/N 4.7;■ C/N 6.3; ▲C/N 7.8; x C/N 9.4;* C/N 11)

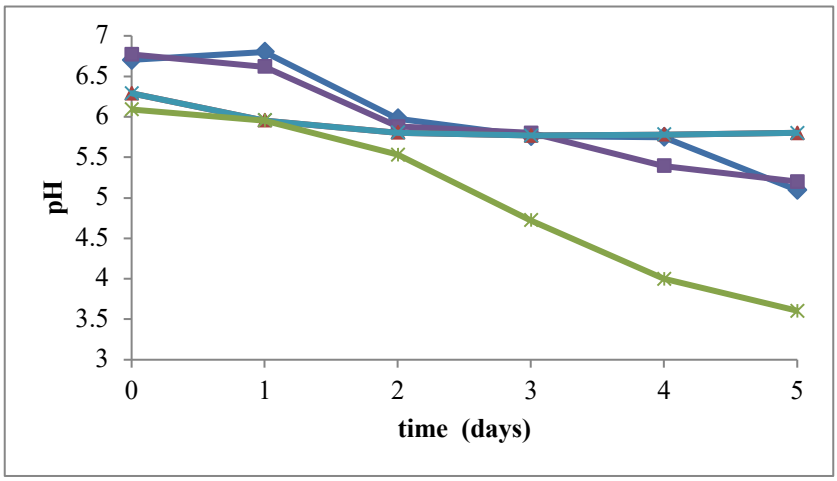

FIGURE 2. pH profiles during fermentation (♦ C/N 4.7; ■ C/N 6.3; ▲C/N 7.8; x C/N 9.4; * C/N 11)

Both cellulose and xylan have been known to induce effectively cellulase and xylanase production. However, xylan is very expensive and the availability is not sustainable either. Therefore, the use of cellulose as carbon source for xylanase production in defined media is more economically practical than xylan.

Effect of Initial pH for Fermentation at Optimum C/N Ratio

pH is an important parameter in the production of enzymes by *T. reesei*. Previous study shows that the production of xylanase by *T. reesei* on cellulose was at pH 7.0 [13]. We used various initial pH in the experiments to look at the influence of initial pH on xylanase production.

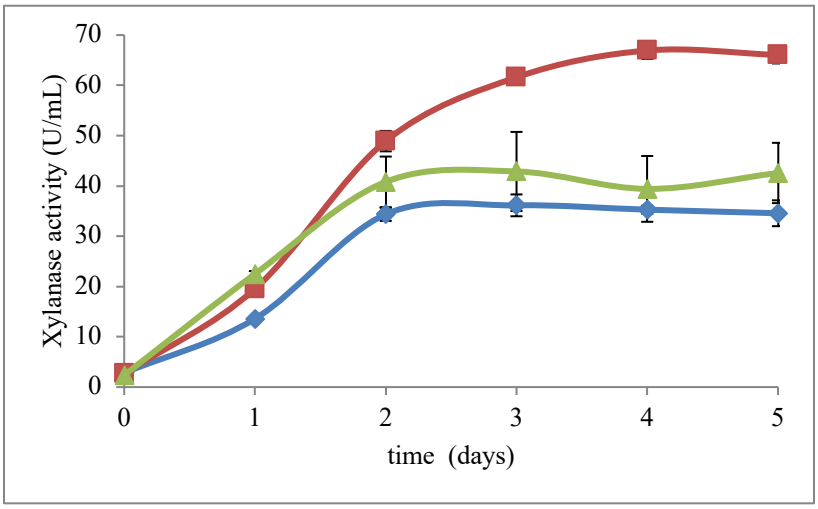

FIGURE 3. Xylanase activity at different initial pH, C/N 7.8 (♦ pH = 5; ■ pH = 6; ▲pH =7)

The most favorable initial pH for xylanase production was at pH 6.0 as can be seen in Fig. 3. The lowest xylanase production was at initial pH of 5.0. The optimum xylanase activity is at pH 6.0-6.5 [2]. The results are in line with the findings of previous studies. Other studies also showed that the best pH for xylanase production was at pH 6-7 [13,15]. Higher pH (pH > 7.0) and lower pH (pH < 4.0) decrease xylanase activity and also the soluble protein concentrations [2]. Lower initial pH will lead to rapidly decrease of pH causing early autolysis, thus it is important to start with pH at higher level [16].

Growth on RTC Media

We used natural and pretreated RTC to produce xylanase, the medium was formulated as written in Section 2. The control was also provided. Protein and carbohydrate composition in RTC after pretreatment were 21.53 % w/w and 16.21 w/w respectively. We only measured the protein and carbohydrate contents in Alk-RTC to enable us in formulating the media. Table 1 shows that RTC induced xylanase production, as can be seen in systems 3 and 4 which xylanase was produced in control systems. System 1 consists of natural RTC as additional carbon source and sole nitrogen source and system 2 consists of pretreated RTC as additional carbon source and sole nitrogen source. The time profile of xylanase activity in Fig.4.shows the xylanase activity in both NRTC and Alk-RTC media. A 72 hours production delay was seen in the system for both NRTC and Alk-RTC compare to defined medium which presumably due to the adaptation period of *T. reesei* to induce xylan in media contained RTC.

TABLE 1. Summary of xylanase production in NRTC and Alk-RTC media.

system	Carbon and nitrogen sources	Initial pH	Maximum xylanase activity (U/mL)
1	NRTC as additional Carbon and sole nitrogen sources	6.0	30.75 ± 2.12
2	Alk-RTC as additional Carbon and sole nitrogen sources	6.0	28,013 ± 0.93
3	Pretreated RTC 20 g/L as control	6.0	13.5 ± 5.1
4	NRTC 20 g/L as control	6.0	8.7 ± 0.64

Xylanase activity in defined medium was about two-fold compared to NRTC. It may be due to the fact that protein from RTC as the only nitrogen source was not sufficient to support the growth of *T. reesei* and xylanase production [16]. The nitrogen sources in defined medium are provided by peptone, ammonium sulfate and urea, whereas the nitrogen sources in RTC medium are provided by only RTC. The xylanase activity did not widely differ between both NRTC and Alk-RTC. Hence, whether the RTC needs to be pretreated prior to use should be taken into consideration. The pretreated RTC is not attractive to be used as nitrogen source and additional carbon source, since there is an additional cost for pretreatment.

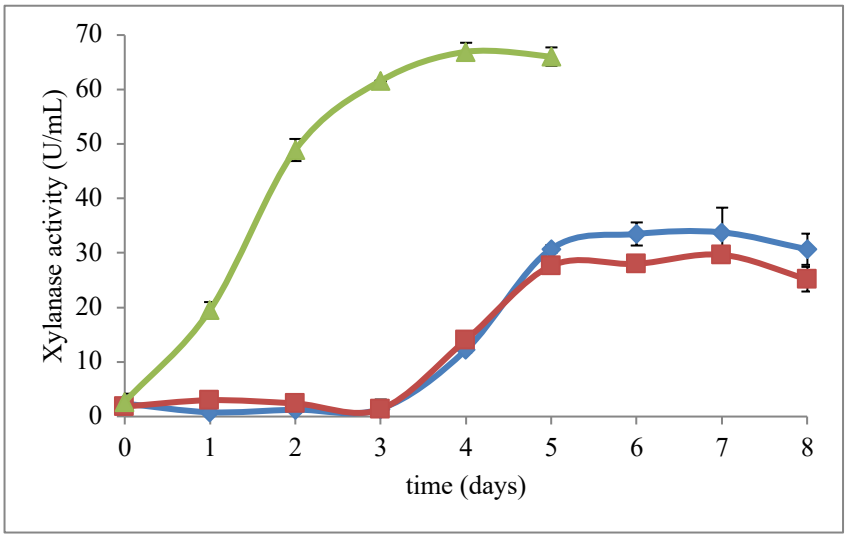

FIGURE 4. Profiles of xylanase activity in defined and RTC media (▲defined media; ♦ NRTC; ■ Alk-RTC)

CONCLUSIONS

T.reesei grew on defined medium was found to produce xylanase during growth period and yield of 66.93 U/mL xylanase activity at C/N ratio 7.8/1 and initial pH of 6.0. The best initial pH for xylanase production was 6.0. *T.reesei* grew in *Reutealis trisperma* cake which was used as additional carbon and nitrogen sources was also found to yield 30.75 U/mLof xylanase activity. The pretreatment of RTC using NaOH presented no benefits to xylanase production from the fermentation study in systems 1 and 2. It has been observed that *Reutealis trisperma* induced xylanase production but did not enhance the production of xylanase when it is used as the sole nitrogen source.

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