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Xylanase production from combined *Reutealis trisperma* with potato dextrose broth by *Tricodherma reesei*: the effect of pretreatment

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Abstract. Xylanase is an important enzyme in pulp and paper, food, feed, and textile industries. It is produced by fermentation process with *Tricodherma reesei* as fungi, which grows on several carbon sources such as cellulose and xylan. *Reutealis trisperma* (kemiri sunan) have been used for biodiesel production after being mechanically pressed. The solid waste, which is called *Reutealis trisperma* cake (RTC) contains carbon and nitrogen sources which are valuable to be used for xylanase production by *T. reesei*. Shake flask systems were used to grow *T. reesei* in medium consists of RTC and Potato Dextrose Broth (PDB). The systems were maintained at 28°C, 200 rpm at initial pH of 6.0 for 84 hours. Two forms RTC used in this study were natural and alkaline pretreated. The RTC fermentations were performed for different concentration of PDB (15g/L and 20 g/L) and also for different concentration of RTC (13.33 g/L; 16.67 g/L and 20 g/L). Pretreated systems gave better yield of xylanase production compared to natural systems. Higher concentration of PDB for pretreated system was found to yield lower xylanase production. The highest xylanase activity (198.1 U/mL) was obtained in media containing 15 g/L PDB and 20 g/L pretreated RTC.

1. Introduction

Reutealis trisperma known as "Kemiri Sunan" is a shady tree which is often found in East and West Java. The seeds have been used for biodiesel production after being mechanically pressed to get non-edible oil [1]. The cake called *Reutealis trisperma* cake (RTC) consists of protein, carbohydrate (sugar), fat, moisture, ash, and others (Table 1). Since its protein and sugar content were quite high, we tried to use RTC as nitrogen and carbon sources in a media for *Trichoderma reesei* to produce xylanase. Xylan is a hemicellulose which is normally found in rice plant, wheat, and many other plants. Xylanase is an enzyme produced by fermentation process with liquid culture and has been used as pre-bleaching of kraft pulp to minimize use of harsh chemicals in the subsequent treatment stages of kraft pulp, to improve the ability for feed meal to be easily digested, modified agent for cereal based food, to covert lignocellulose and agricultural waste to fermented products [2][3][4]. Recently, the conversion of lignocellulosic agricultural waste to useful products has been very attractive. Agricultural wastes such as banana peels, rice straw, sugarcane bagasse, wheat bran and soybean hulls have been used as the substrate for xylanase enzyme production [5]. There is no study of using *Reutealis trisperma* pressed cake in xylanase production, hence our study gives new insight of using the cake in xylanase production. Since there is no xylanase yet produced in the country, xylanase is imported for domestic demand. 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]. *Trichoderma reesei* is a mesophilic filamentous fungus which is used at industrial scale for the production of enzymes hydrolyzing biomass such as cellulase and xylanase since it easily grows and well adapted at several substrates in fermentor cultivation condition [7]. *Trichoderma reesei* has two specific gens (*xyn1* and *xyn2*) that specific for xylan degradation and produce endo-β-1,4-xylanases from simple carbon source (xylan) [8][9]. Other fungi, *Aspergillus niger* is also known to produce some enzymes, such as carbohydrases and protease using biomass [10]. Fungal culture for enzyme production is affected by the differences in the nutrient composition. Fungal cultivation on pre-treated corn stover has been reported to perform better in enzyme production [11]. Thus, we used RTC in natural and pretreated forms. Both natural and pretreated RTC were used as additional carbon and nitrogen sources for Potato Dextrose Broth (PDB) in a growth media for *T. reesei* to produce xylanase. This research aims to study xylanase production by *T. reesei* using RTC and PDB and to compare xylanase activity in media contains several concentrations of PDB and pretreated RTC.

2. Materials and methods

2.1. Cake preparation

Reutealis trisperma cake (RTC) used in this research was obtained from Energi Baru Santosa Company, Gresik. Table 1 shows reported compositions of this material [12].

Table 1. Characteristic of RTC

	Content (%w/w)
Protein	34.03
Moisture	6.32
Carbohydrate	18.56
Fat	29.5
Ash	7.76
Others	3.83

Ground RTC (40 mesh) was heated in oven at 50°C. The dried ground RTC is a natural RTC (NRTC), the pretreated RTC was subjected to alkaline solution pretreatment (Alk-RTC). For alkaline pretreatment, RTC was mixed with 0.6% (w/v) aqueous sodium hydroxide (NaOH) with 1: 30 (w/v) solid-liquids ratio. The mixture was heated at 50°C and agitated at 160 rpm for 25 minutes. Finally, The Alk-RTC was washed and dried at 50°C overnight until it reached constant weight. All chemicals used were analytical grade and purchased from Merck.

2.2. Media

Two different media were used in the experiment, Natural RTC (NRTC) with PDB and alkaline pretreated RTC (Alk-RTC) with PDB. Generally, a 24 g/L PBD was used to cultivate *T. reesei*. The sugar content in RTC (Table1) was used as basic calculation to calculate the combined use of RTC and PDB to be equivalent with 24 g/L PDB. A 20 g/L RTC which consists of 3.7 g/L sugar and 20 g/L PDB will give the sugar content equivalent to 24 g/L PDB. We started with 20 g/L RTC and 15 g/L PDB used in the initial experiment, the composition of PDB and RTC both Alk-treated and natural (untreated) were varied by scaling down 17-20% the amount of pressed cake to optimize media used for the systems. Both NRTC and Alk-RTC without PDB were used as control since we would like to investigate the effect of pressed cake for enzyme induction potential in shake flask systems. PDB was purchased from Sigma Aldrich. All media were adjusted to pH 6.0 prior to autoclave (TOMY,ES-315, Japan) sterilization at 121°C for 15 minutes.

2.3. Culture conditions

Tricoderma reesei was obtained from Gadjah Mada University and stored on Potato Dextrose Agar (PDA) at 4°C and subcultured monthly. *T. reesei* were inoculated into 20 mL culture bottle that

contain Potato Dextrose Broth (PDB) for 3 days at 28°C as pre-culture. After 3 days, pre-culture was added into shake flask systems which consisted of media that contained RTC and PDB at various compositions. Culture was incubated for 84 hours in an orbital shaker operating at 200 rpm for 28°C with initial pH of 6.0. Daily samples were removed aseptically in a laminar flow hood for measurement of pH and enzyme activity. Sample was separated between the solid state and supernatant using centrifuge at 10,000 rpm for 10 minutes and clear supernatant was collected and used for enzyme assay. The analyses were done in duplicate and the mean value presented.

2.4. Enzyme assay

Xylanase activity was determined by a modified method from Bailey [13]. The method was best applied to properly diluted samples with xylanase activities in the range of 0.5-2 U/mL. A 1 wt% beechwood xylan (Sigma Aldrich, St. Louis, MO) was prepared in 200 mL 0.05 M sodium citrate buffer (pH 5.3). A 100 ⌈L enzyme broth was added to 25 mL test tube and then followed by 900 ⌈L of suspended xylan in buffer. For blanks, only suspended xylan was added to the test tube. The samples and blanks were incubated at 50°C for 5 minutes in a water bath. After 5 minutes, a 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 and added with distilled water up to 25 mL. The absorbance values were measured using UV-Vis Spectrophotometer (Hewlett Packard 8453) at 560 nm. Xylanase activity was measured based on xylose release, which can be represented by following equation:

$$Xylanase\left(\frac{U}{mL}\right) = \frac{xylose\ released(mg)}{(5\ mins)(0.1\ mL\ enzyme\ sample)} \times \frac{1\ mmol}{150.13\ mg} \times \frac{1000\ \mu mol}{1\ mmol}$$

$$= 13.32 \times xylose\ released\ (mg) \quad (1)$$

3. Results and discussion

In this research, we used RTC as additional substrate to grow *Trichoderma reesei* for xylanase production. The variables used in this research both for NRTC and Alk-RTC were 13.33 g/L, 16.67 g/L and 20 g/L. The PDB concentrations were 15 g/L and 20 g/L.

3.1. Effect of natural and pretreated RTC on xylanase production

There were two systems used in the experiment, both NRTC with PDB and Alk-RTC with PDB. The PDB used was 15 g/L. The initial pH used for this study was 6.0. The xylanase activity profiles for all systems can be seen in Figure 1. The Alk-RTC gave higher production of xylanase compare to NRTC. The better performance of Alk-RTC in xylanase production may be due to better access to protein in the cake since the alkaline treatment opens the cellulose structure for cell metabolism [14]. Higher concentration of Alk- RTC gave better xylanase activity during the fermentation period since the protein and carbon sources were available enough for cell to induce xylanase production. The NRTC system shows that higher concentration of NRTC resulted in lower xylanase activity which implied that NRTC prevented access to the protein for cell metabolism. With the exception of 20 g/L of pretreated RTC, most of the optimum production of xylanse occurred on the second day. Though, it was a delay of optimum xylanase production on 20 g/L pretreated RTC, the xylanase produced in the system was higher than other pretreated RTC concentration which is due to more nitrogen and carbon sources in the pressed cake. After achieving the optimum production, the xylanase activity decreased which may be due to its interaction with the other component in media which is known as fermentation product [15]. The decrease of xylanase activity can be indicated by the significant decrease of pH as shown in Figure 2.

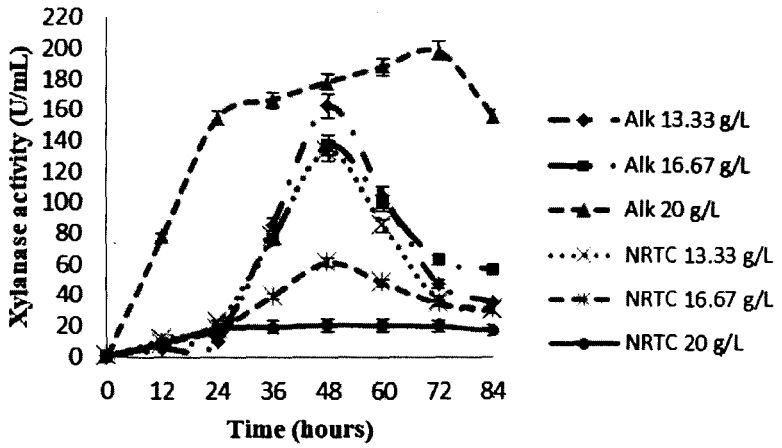

Figure 1. Profiles on xylanase activity for RTC in different concentration of RTC and 15 g/L PDB.

Table 2. Maximum xylanase activity at different concentration of RTC with 15 g/L PDB

RTC (g/L)	Xylanase activity (U/mL)	
	NRTC	Alk-RTC
13.33	133.378	162.28
16.67	61.262	137.12
20	20.534	198.13

Table 2 shows the maximum xylanase activity at different concentration of natural and pretreated RTC. It is clear that pretreated RTC gave a better xylanse activity compare to natural RTC. Natural RTC consists of lignin and cellulose which may hinder *T. reesei* to use the carbon and nitrogen sources in RTC. Having said that, the lower concentration of NRTC used in the production of xylanase may less inhibit xylanase induction, hence higher xylanase activity was found in the lower concentration of NRTC. However, in Alk-RTC (16.67 g/L and 20 g/L) the production of xylanase is 2 fold and approximately 10 fold compared to the same composition used of NRTC, which suggests adequate accessibility of the RTC's proteins and carbon sources to fungus. Other studies reported the effect of natural and pretreated soybean hull in a fermentation process to produce xylanase enzyme showed that the enzyme production was largely unaffected by the presence of pretreated biomass or natural biomass [16].

3.2. Effect of alkaline-pretreated RTC and PDB concentrations on the xylanase activity

Based on data in Table 2, we used 2 concentrations of Alk-RTC (13.33 g/L and 20 g/L) to compare the xylanse activity at different concentration of PDB (15 g/L and 20 g/L).

Table 3. Summary of evaluated fermentation system at different concentration alkaline pretreated (Alk) RTC and PDB

system	Carbon and nitrogen sources	Initial pH	Maximum xylanase activity (U/mL)
1	Alk 13.33 g/L , PDB 15 g/L	6.0	162.28 ± 0.97
2	Alk 20g/L , PDB 15 g/L	6.0	198.13 ± 0.03
3	Alk 13.33 g/L , PDB 20 g/L	6.0	151.19 ± 0.72
4	Alk 20g/L , PDB 20 g/L	6.0	157.93 ± 0.17
5	Alk 20 g/L as control	6.0	13.5 ± 5.1
6	NRTC 20 g/L as control	6.0	8.7 ± 0.64

Table 3 shows the best xylanase activity was achieved in a system with 20g/L Alk-RTC and 15 g/L PDB. Natural RTC and alkaline pretreated RTC were used as control. As has been mentioned in the above explanation, higher concentration pretreated RTC in systems with 15 g/L PDB will give higher xylanase activity. It seems that the lignin content in the cake has been degraded by alkali treatment, hence it give access for the cell to induce xylan. On the other hand, higher PDB concentration for both 13.33 g/L and 20 g/L of Alk- RTC gave similar maximum xylanase activities which were noted in system 3 and 4. The excess of PDB may hinder the production of xylanase which may be due to substrate inhibition. The data shows that natural RTC and pretreated RTC were able to induce xylanase production though the activity was low. The pretreated RTC gave better xylanase activity than natural RTC. It shows that alkaline pretreatment for RTC can give access for cell metabolism since the structure of lignin has been broken by the pretreatment process. There was more than 10 fold higher of xylanase activity in media using both Alk and PDB compare to Alk as control. It shows that carbon source in PDB enhanced the production of xylanase compare to Alk itself. Previous studies on xylanase production using *Trichoderma reesei* were conduct at various substrates. Xiong et al, have studied xylanase production by *T. reesei* RUT-30 which were grown at different pH medium with lactose as the main carbon source. The result showed highest xylanase activity (approximately 94.7± 4.8IU/mL) was achieved at pH 6 after 5 days of fermentation. Our result showed that Alk- RTC (20 g/L) combine with PDB (15 g/L) produced higher xylanase activity (approximately 198.13± 0.03 U/mL) compared to Xiong et.al [17].

3.3. pH profiles during fermentation process

The measurement of pH during fermentation process was obtained to indicate the growth of *Trichoderma reesei* related to the induction of xylanase. The decrease of pH means that *T. reesei* takes the TCA cycle to produce organic acid such as citric, oxalic and acetic acid [18]. However, the increase of pH shows that *T. reesei* takes protein to support its growth which may not go to TCA cycle which will end up with a little production of xylanase. The pH profile during fermentation process for alkaline-pretreated (Alk) RTC at various concentrations of RTC and PDB are shown in Figure 2.

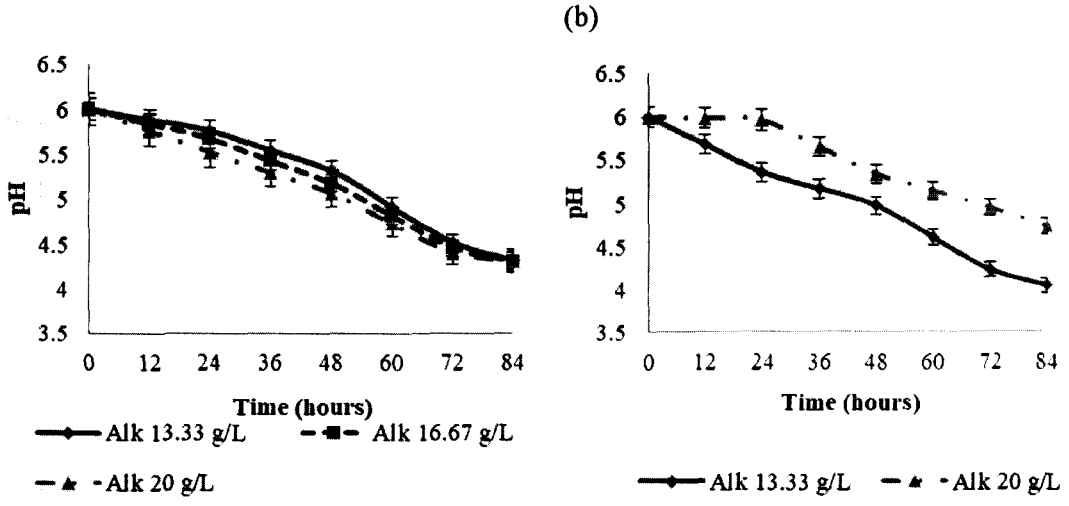

Figure 2. Profiles of pH during fermentation process for alkaline-pretreated (Alk) RTC at various concentration of RTC and PDB concentration (a) 15 g/L (b) 20 g/L

The initial pH used in this research was 6.This pH was taken regarding with previous research from Bailey et al. They found that the highest value of xylanase production was achieved when initial pH for fermentation in the pH range 6-7 [19]. From Figure 2, it can be seen that pH decreased during fermentation process. It means that *T. reesei* grows through carbohydrate metabolism pathway TCA cycle. It produced organic acid, especially citric acid as their final product that slowly decreased pH in the reactor along with the time of fermentation [20]. Most of maximum xylanase activities were

always achieved on 48 hours of fermentation (pH 5- 5.5). The xylanase production decreased significantly at pH below 4.5 after 72 hours of fermentation as can be seen in Figure 1 and Figure 2.

4. Conclusions

Shake flasks systems indicated that *Reutealis trisperma* cake (RTC) can be used as additional carbon and nitrogen sources for xylanase production. Pretreated RTC has been proved to give higher xylanase activity compare to natural RTC both in PDB 15 g/L. The maximum xylanase activity was performed at the system with 20 g/L pretreated RTC and 15 g/L PDB which was 198.13 ± 0.03 U/mL. The fungi were able to use RTC to induce xylan for xylanase production. The pH profile during the fermentation decreased along with the time of fermentation which presumably indicated the production of organic acid during *Trichoderma reesei* growth.

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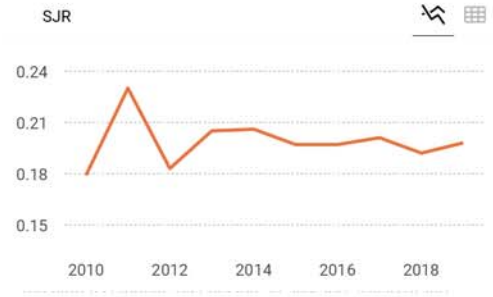

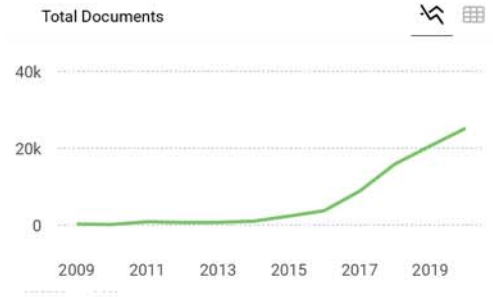

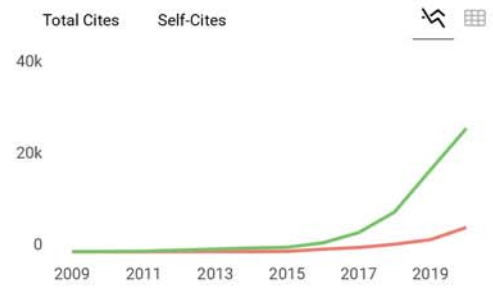

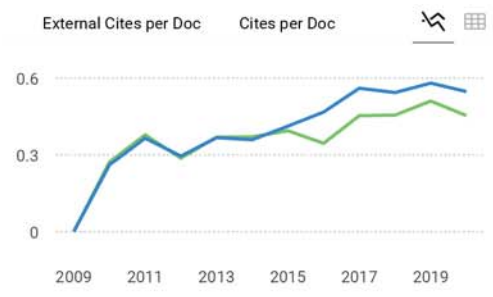

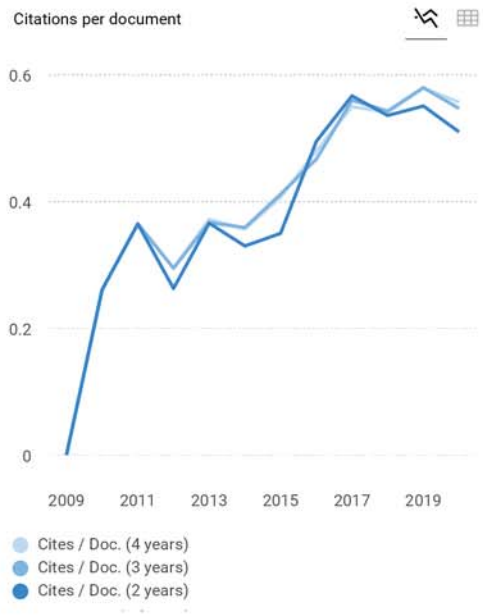

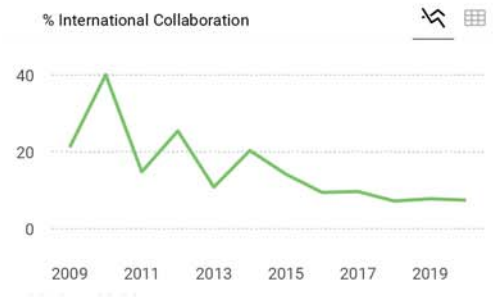

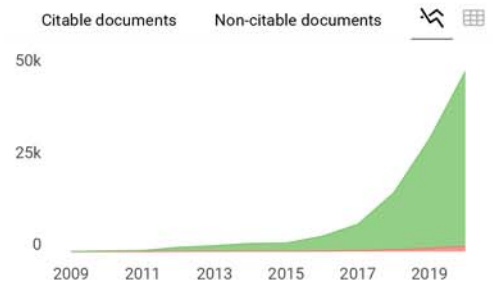

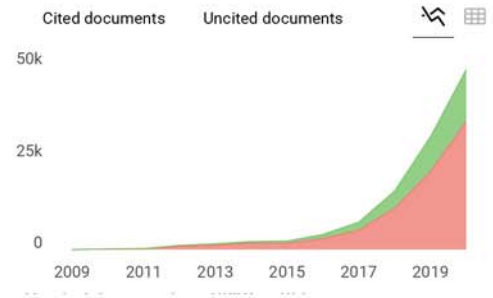

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