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Enhanced Fermentable Sugars Production from Sago Waste by *Trichoderma reesei* Pk_1J_2 and *Aspergillus niger* FNCC 6114 Fermentation

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Abstract: ***Lignocellulosic compounds from sago waste are abundant, inexpensive, untapped carbon sources, and even tend to pollute the environment. Trichoderma reesei and Aspergillus niger are two fungi that are widely used to degrade lignocellulose compounds into simple sugars. T. reesei plays the first role to degrade cellulose. Then A niger will play a role in continuing the process of changing the product of cellulose degradation into simple sugars. The right combination of time to inoculate A. niger into media after T. reesei growing in could maximize the degradation process of lignocellulose compounds. In this study, T. reesei Pk_1J_2 inoculation was carried out 48 hours before the A. niger FNCC 6114 inoculation. Fermentation was continued in 48 h (total 96 h). The result is that the production of fermentable sugar increases 3-5 times compared to only using monoculture of T reesei or A.niger. Thus, this combination can be recommended for the preparation of potential carbon sources for fermentation and industrialization.***

Keywords: lignocellulose, sago waste, *Trichoderma reesei* Pk_1J_2, *Aspergillus niger* FNCC 6114, mixed culture

1. Introduction

Sago is widely grown in eastern Indonesia, such as Maluku, Irian Jaya, and Sulawesi and usually used as a staple food instead of rice (such as papeda, kapurung, etc). Distribution of sago plants in Indonesia covers 51.30% of the total world sago, including the Irian Jaya region which has 6 million hectares of sago plants [1]. In the utilization of sago plants, starch pith is processed until sago flour and sago waste are obtained. Konawe Regency, Southeast Sulawesi produces 9536.18 tons/year of sago waste [2].

The sago waste is one of the lignocellulosic materials, which is abundant (75-83%) and has not utilized optimally even allowed to decompose in the sago processing area. Sago waste contains 58% starch, cellulose 23%, hemicellulose 9.2%, pectin 5.8% and lignin 3.9% [3] therefore sago waste has potential as a source of inexpensive carbon fermentation processes, including the production of cellulases [4], biofuel [5], biohydrogen [6], biocellulose [7], and bioethanol [8].

Generally, the utilization of lignocellulosic wastes requires pretreatment, such as physical/mechanical pretreatment [9] and/or pre-chemical [10]. Preliminary research found that sago waste does not require the physical/mechanical process (grinding), but a sifting to separate softwood from hardwood. Softwood sago waste contains lignin levels low enough to be hydrolyzed microbiologically/enzymatically. Microbial hydrolysis is considered to be more environmentally friendly and low cost. Microbes that play a role in degrading lignocellulose into simple sugars, for example, *Phanerochaete chrysosporium* [11] *Trichoderma reesei* RUT-C30, *Aspergillus niger*, *Aspergillus saccharolyticus*, dan *Aspergillus carbonarius* [12],[13]. *Trichoderma reesei* is one of the microbes that are efficient for the lignin degradation process of the lignocellulosic compounds [14]; whereas *Aspergillus niger* has a high β-glucosidase activity and can increase the delignification process of hemicellulose [15]. The combination of the growth of these two fungi will be able to produce high degradation products. However, inoculation of both microbes at the same time will cause the growth of *T. reesei* to be inhibited [13]. Additionally, the right combination of time to inoculate *A. niger* into media after *T. reesei* growing in has not been much explored. Therefore, this study aims to find out the best combination of the time of inoculation of *A. niger* in biodegradation of lignocellulosic sago waste using a mixture of *T reesei* and *A. niger* cultures in providing potential carbon sources. It is also to study the effect of chemical pretreatment on changes in the components and structure of sago waste.

2. Materials and methods

2.1 Material and microorganisms

Trichoderma reesei Pk_1J_2 and *Aspergillus niger* FNCC 6114 obtained from Laboratory of Biotechnology, Faculty of Agricultural Technology, Universitas Gadjah Mada. Sago waste was obtained from the Konawe Regency, Southeast Sulawesi, Indonesia.

2.2 Sago waste preparation

Sago waste preparation was carried out by the method of Fang and co-workers with some modifications [8]. Sago waste was dried by sun drying for 4 d and continued by using a drying cabinet at 60 ° C for 6 h. After that, sago waste was separated using a 10 mesh size sieve to get soft- and hardwood. Softwood sago waste with ± 10% moisture content was stored in a closed plastic container until use.

2.3 Alkaline Pretreatment

Pretreatment using NaOH solution was done by the method of Zheng and co-workers [16] with some modifications. Add

NaOH solution (1%) to sago waste (softwood) at a ratio of 10:1 v/w and soaked for 3 d at 50 °C. After that, washing was done using tap water until a clear filtrate solution was obtained, then the pH was adjusted to 7-8 using 1 M HCl. Dry it at 50 ° C for 2 d before use.

2.4 Starter preparation

A hundred grams of rice (for each isolate) cooked until half cooked by adding 100 ml of lactic acid (0.1%; pH 4). After that, it was sterilized at 121ºC for 20 min. Add 50 g of sterile sago waste, 100 ml of sterile H_2O and 15 ml of spore suspension (10^7 spores / ml). Then incubated at 25-26ºC for 7 d. After 7 d, it was dried in the sun and then ground to get starter powder [9]. The number of dry starter spores was calculated by growing on Dichloran Rose Bangal Chloramphenicol Agar (DRBC) media.

2.5 Production of fermentable sugars

The isolates were grown in media containing 10 g of pretreated (softwood) sago waste and 30 mL of mineral solution. The mineral solution contained 6 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.3 g/L $CaCl_2$, 0.3 g/L $MgSO_4$, 0.005 g/L $FeSO_4$, 0.0016 g/L $MnSO_4$, 0.0014 g/L $ZnSO_4$, and 0.0037 g/L $CoCl_2$. The initial pH was adjusted to 5 ± 0.2 by adding 0.3 mL of citrate buffer (1 M). After that, the media was autoclaved at 121 ° C for 30 min [12].

The starter powder was inoculated as much as 1% ($1x10^6$ spores/g) for monoculture growth, or as much as 0.5% (each isolate) for mixed culture. In mixed cultures, *T. reesei* was first inoculated and incubated for 48 h (named Mix48) or 72 h (named Mix72) and then *A. niger* was inoculated. Incubation was carried out at temperatures 26-28 C, humidity (RH) 90-95%, moisture content 70%, and incubation time 120 h. Every 24 h the production of fermentable sugar was analyzed.

2.6 Extraction

Extraction was done by adding 60 mL of H_2O into the culture media and stirring slowly. Then filter with a filter cloth and the resulting filtrate was centrifuged at 3000 g for 15 min. The supernatant was analyzed for its reducing sugar levels.

2.7 Analysis

Analysis of hemicellulose, cellulose, and lignin levels was performed using the Datta method [17]. Reducing sugar was analyzed by the DNS method [18]. Surface sago waste microstructure was observed using a Scanning Electron Microscope (SEM). SPSS software (version 22.0) was used for statistical analysis.

3. Results and Discussion

3.1 The effect of NaOH pretreatment

Before pretreatment with NaOH, sago waste was sifted and the result was hardwood (35%) and softwood. (65%). Then softwood was given a pretreatment with NaOH which served to facilitate the subsequent degradation process (Table 1).

Table 1 shows that the highest cellulose content was found in hardwood sago waste (50.64%). While for softwood sago waste, cellulose content was quite low, but after pretreatment, the content increased significantly (± 16%). The same increase also occurred in hemicellulose which increased 13-15% after pretreatment.

In general, treatment with NaOH could increase cellulose levels and reduced lignin levels. NaOH effectively attacked the bonds connecting lignin and hemicellulose in the Lignin-Carbohydrate Complex (LCC) and was useful for breaking ester bonds and carbon bonds (CC) in lignin molecules so that it would reduce lignin levels [19].

Increased levels of lignin obtained after pretreatment were not yet known. It might be caused by phenol compounds from plant tissue that form cross linking, bind esters to the polysaccharide cell walls, and form "lignin objects" that could be measured as lignin [20]. However, the level of lignin in softwood sago waste is still relatively low.

3.2 Changes in the structure of lignocellulose

Observation of lignocellulosic structure aims to show the surface condition of the material before and after the pre-treatment process. The structure of sago waste shows that the NaOH pretreatment can loosen lignin bonds in cellulose and hemicellulose (Figure 1).

Table 1: Changes in the components of sago waste pretreatment with NaOH (% dry basis)

Raw Material	Hemicellulose (%)	Cellulose (%)	Lignin (%)
Sago waste (hardwood)	29.27 ± 1.539^{a}	50.64 ± 5.642^{a}	5.85 ± 0.516^{a}
Sago waste (softwood) : a. without pretreatment	34.22 ± 5.136^{a}	18.42 ± 1.645^{b}	1.30 ± 0.493^{b}
b. NaOH (1%) pretreatment	48.61 ± 5.742^{b}	34.53 ± 2.050^{bc}	3.27 ± 0.871^{bc}

Note: Numbers followed by the same notation in the same column shows no significant differences based on the LSD test (p <0.05).

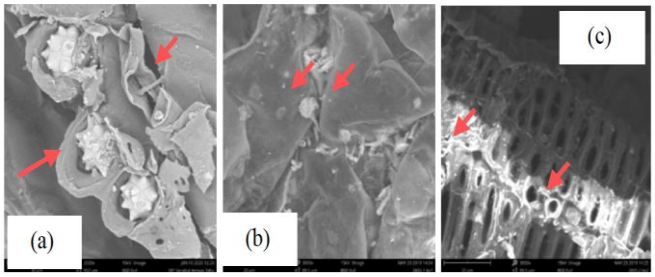

Figure 1: Morphological structure of sago pulp: (a) hardwood sago waste; (b) softwood sago waste; (c) softwood sago waste with pretreatment at a magnification of 2500 - 3000x

Figure 1a shows that hardwood sago waste had a rigid structure to support strong plant tissue. While sago softwood waste had a finer-looking surface and starch granules covered the lignocellulose matrix (Figure 1b). Starch granules were small balls that had a smooth and shallow surface. The pretreatment process using NaOH caused the structure of the softwood sago waste to change shape due to the loosening of the lignin bond to produce a porous structure (Figure 1c). NaOH (1%) could degrade lignin and hemicellulose bonds in the delignification process [21].

3.3 Effect of fermentation time on fermentable sugars production by monoculture and mix culture

Fermentable sugar production is closely related to enzyme production during microbial growth. Microbes will produce various enzymes to hydrolyze the lignocellulose substrate into simple sugars. The results are shown in Figure 2.

During the growth of a single *T. reesei* culture, the production of fermentable sugar increases with time, maximizing incubation at 120 hours ie 107.67 mg/g (Figure 2.a).

This pattern of production and growth of T reesei is different from A niger (Figure 2.b). In A. niger the production pattern increases very rapidly to 48 hours (146.79 mg / g) and then decreases. This indicates that A. niger will use the available simple carbon source and convert it to simple sugar, after the supply of simple carbon sources is depleted because it cannot produce cellulase, the production stops. In contrast to the growth of T. reesei in Figure 2a, because the production of the β-glucosidase is low, the resulting concentrated sugar is also low, but because the cellulase production is quite high, stepwise fermentable sugar levels continue to increase.

Based on the results described in monoculture fermentation (Figures 2a and 2b), mixed culture fermentation was carried out. *T. reesei* was first grown for 48 h (Mix 48) (taking into account the amount of sugar produced as much as 1/2 of the maximum production in 120 h fermentation) and 72 h (Mix72) (taking into account the sugar content that has produced 2/3 from maximum production at 120 hours of fermentation). The results could be seen in Figures 2c and 2d. The increase in sugar production was very fast due to the activity of β-glucosidase produced by *A. niger* and the availability of simple carbon from the degradation of lignocellulose by cellulase produced by *T. reesei*. This synergism increased sugar production 2.75 times higher in Mix48 (after 98 h fermentation) or 3.0 times higher in Mix72 (after 120 h fermentation), compared to when using *T. reesei* monoculture.

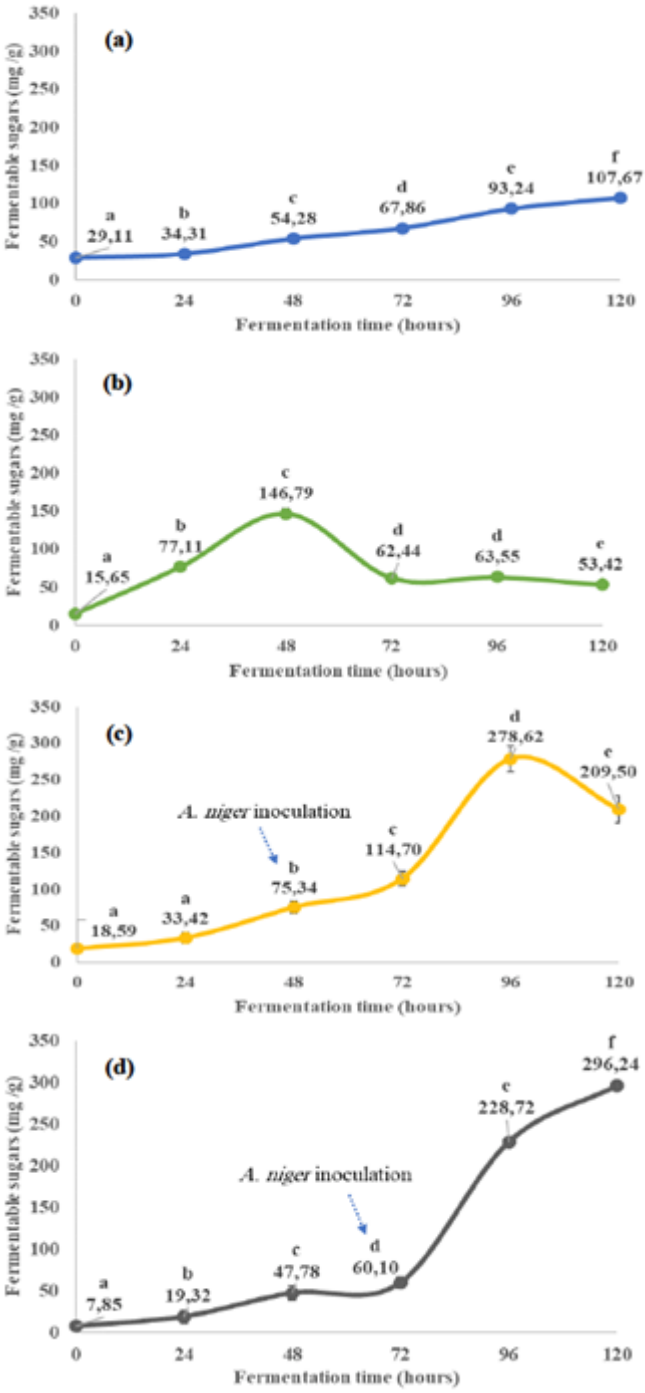

Figure 2: Fermentable sugar production during the growth of *Trichoderma reesei* and/or *Aspergillus niger*. (a) *T. reesei* Pk1J2; (b) *A. niger* FNCC 6114; (c) Mixed culture of Mix48; (d) Mixed culture of Mix72. Figures followed by the same notation do not show significant differences based on Duncan's test ($p < 0.05$).

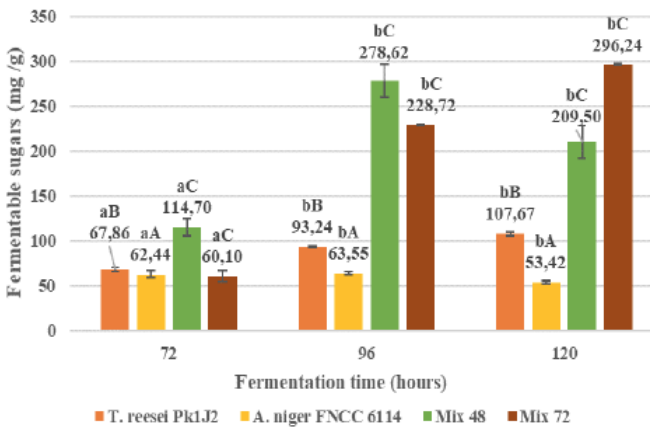

Figure 3: Effect of microbial growth (monoculture and mixed culture) on the production of fermentable sugar at various fermentation times. Figures followed by the same notation do not show significant differences based on Duncan's test ($p < 0.05$). Capital letters represent microbial types, and lowercase letters represent the time of fermentation.

A.niger FNCC 6114 was a local isolate that could produce fermentable sugar faster (48 h) and higher (146.8 mg/g) than previously reported *A. niger* (72 h [22] with the highest was 95 mg/g [23]). However, only using *A. niger* could not achieve high fermentable sugar. Mixed-culture with *T. reesei* could increase fermentable sugar products up to five times at 120 h of incubation (Figure 3). Likewise, if only T. *reesei* was used, the fermentable sugar products reached only one-third compared to using mixed culture.

From these results, it can be concluded that to degrade sago waste, a mixture of *T. reesei* and *A. niger* was needed. *T. reesei* inoculation was carried out 48 hours before the *A. niger* inoculation. Fermentation was continued in 48 h (total 96 h). In this way, a 3-5 times increase in fermentable sugar production can be achieved, calculated if only using a single *T reesei* or *A.niger* culture. Both microbes contributed to the need for enzymes that degrade the lignocellulose component into a simple source of carbon (by *T. reesei*) and the enzyme which subsequently converted into fermentable sugar (by *A.niger*).

4. Conclusion

NaOH pretreatment would damage the structure of softwood sago waste to become more porous to facilitate further degradation by microbes to produce fermentable sugar. Mixed culture could produce fermentable sugar 3-5 times higher than the highest monoculture yield with 48 h fermentation time in mixed culture. The *T. reesei* had to first be grown for 48 h before the growth of mixed culture begins.

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