



## Effect of Lime Pretreatment on Microstructure of Cassava Stalk Fibers and Growth of *Aspergillus niger*

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### Abstract

Cassava stalk can be converted into sugar-based product by using microorganism. Unfortunately, lignin act as a barrier of optimal bioconversion. Cassava stalk needs pretreatment process for removing this barrier. The effect of lime pretreatment on microstructure of cassava stalk fibers and the growth of *Aspergillus niger* FNCC 6114 were observed in this research. The cassava stalks were reduced into 0.147- 0.297 mm size and pretreated with 1 % Ca(OH)<sub>2</sub>. Lime pretreated and unpretreated cassava stalk was used as solid medium for *Aspergillus niger* FNCC 6114. The effect of pretreatment method on fibers microstructure of cassava stalk was evaluated through SEM micrograph. The growth and metabolism activities of *Aspergillus niger* FNCC 6114 were monitored through SEM micrograph of media after fermentation. The other parameters examined were changes in glucosamine, reducing sugar levels, and spores' quantity. Lime pretreatment altered microstructure of cassava stalk fibers. However, cassava stalk without lime pretreatment gave better growth of *Aspergillus niger* FNCC 6144 based on metabolism activities parameters. Cassava stalks is suitable as media for *Aspergillus niger* FNCC 6144 through solid state fermentation. For better growth of *Aspergillus niger* FNCC 6144 fine-sized cassava stalk should not be lime pretreated. The results of this study provide information about the pretreatment of cassava stems which was effective in supporting the growth of *Aspergillus niger*. Enhancements the utilization of cassava stems by using fungi, for example *Aspergillus niger* can overcome the accumulation of organic waste that can interfere with environmental sustainability.

### How to Cite

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## INTRODUCTION

Cassava stalks are the waste from agroindustry. They contain cellulose, hemicelluloses, and small amount of lignin. Cassava stalks are classified into lignocellulosic material. Cassava stalks' components can be converted into simple sugars and further into sugar-based products by some appropriate microorganisms. Optimal utilization of cassava stalk requires pretreatment process for enhancing the degree of depolymerization of complex compounds. In the bioconversion process of lignocellulosic biomass, pretreatment is an important step. Easier access of microorganism in the cellulose, hemicelluloses and/or lignin degradation process is the positive impact of pretreatment in improving the efficiency of bioconversion (Jönsson & Martín, 2016).

Pretreatment with alkali is the best chemical pretreatment than any other. More environmental friendly and non-corrosive chemical usually used in this pretreatment method. This method is effective for removing lignin because the chemicals used react mainly with the lignin (Kim *et al.*, 2016). Alkaline pretreatment with calcium base is cheaper than with other bases (such as sodium, potassium, or ammonium hydroxide). Furthermore, alkaline pretreatment at low temperature and low base concentrations (mild alkaline method) will reduce the formation of inhibitory substances (Jönsson & Martín, 2016). Kim *et al.* also stated that to choose the proper pretreatment method, consideration should be given to the emergence of unwanted side reactions, especially carbohydrate and lignin degradation resulting in inhibitory compounds (Kim *et al.*, 2016).

Widyahapsari *et al.* (2016) has examined the effect of alkaline pretreatment by using lime solution on rice straw bioconversion into lactic acid by *Rhizopus oryzae* AT3. Previously, pretreated rice straw was hydrolyzed into sugar enzymatically by using enzyme extracted from *Trichoderma reesei* PK1J2. The results showed that lime pretreated media gave higher levels of sugar than those without the pretreatment. Unfortunately, this higher sugar level did not followed by the higher lactic acid production that may due to the other compounds synthesis (Widyahapsari *et al.*, 2016).

The possibility of lignin release through the alkaline pretreatment of lignocellulosic biomass will encourage the growth of *Aspergillus niger* better. It is proved by Sridevi *et al.* that cellulase production increased when *Aspergillus niger* grown on pretreated sawdust by using NaOH and H<sub>2</sub>O<sub>2</sub> (Sridevi *et al.*, 2015). In addition, Salihu

*et al.* found that the production of cellulolytic enzymes by *Aspergillus niger* from soybean hull was the highest when using the pretreatment process with NaOH (Salihu *et al.*, 2015).

The purpose of this study was to investigate the effect of lime pretreatment process on microstructure of cassava stalk fibers and *Aspergillus niger* FNCC 6114 growth through solid state fermentation. If pretreatment using lime solution has a positive effect on *Aspergillus niger* growth through the change of cassava stem fiber structure, then pretreatment with lime solution can be chosen to prepare cassava stems as growth medium of *Aspergillus niger* in the process of bioconversion into sugar-based products. Bioconversion of cassava stalks will increase the economic value of waste. Optimal bioconversion process of cassava stalks also contribute to organic waste processing efforts for environmental quality maintenance.

## METHODS

### Microorganism and Starter Powder Preparation

*Aspergillus niger* FNCC 6114 was obtained from Laboratory of Biotechnology, Faculty of Agricultural Technology, Universitas Gadjah Mada as a pure culture on agar slant. Cassava stalks were obtained from Sleman, Yogyakarta. Firstly, the culture was grown on PDA for 5-days, and then the resulting spores were harvested using 0.05 % Tween 80 and used as starter of powder preparation.

Starter culture medium contained rice bran, rice grain and cassava stalk powder in a ratio of 1:1:2. Rice grain (50 g) was previously cooked in distilled water (50 ml) which was added with 0.25 ml lactic acid for avoiding bacterial growth. The cooked rice was mixed with 50 g of rice bran and 100 g of cassava stalk powder and sterilized at 121 °C for 30 min. This medium was then inoculated with spore suspension and incubated at 30 °C for 6 days. After that, the culture media were dried, ground into rough powder and ready for use as inoculums powder.

Inoculums powder contained 3.5 x 10<sup>9</sup> spores/g and 0.003 g of inoculum was inoculated into 30 g sterilized cassava stalk (0.01%, w/w) to reach 10<sup>5</sup> spores density. The remaining inoculums were packed with resealed plastic bag and stored in refrigerator for further use.

### Substrate Preparation and Pretreatment Process

Cassava stalks were chopped and dried, followed by grinded into fine particle size (50-100

mesh). 6.67 g grounded cassava stalk was soaked in 1 % Ca (OH)<sub>2</sub> 100 mL for 16 hours at 85-90 °C and stirred for 30 min. After that, it was washed with tap water, neutralized by 20 % H<sub>2</sub>SO<sub>4</sub>, drained with cheese cloth, and dried in cabinet dryer at 60 °C for 2 days. These pretreated cassava stalks were ready to use for solid substrate.

### Solid State Fermentation

Solid state fermentation was done in 300 mL plastic boxes (6 x 8.5 x 12 cm<sup>3</sup>). The bottoms of boxes were perforated in 1 mm diameter for each 5-10 cm in distance. Every box was filled with 30 g sterilized pretreated media with a thickness of 1.8 cm. The moisture content was maintained to be 53 % by the addition of 30 mL distilled water before the sterilization. These media were inoculated with 10 % (w/w) diluted inoculums powder. Every box was covered with sterilized paper.

Fermentations were carried out for 7 days in covered plastic boxes incubator. Sampling was done every 24 h by taken 1 box of pretreated media. The humidity inside incubators were maintained by putting a beaker glass of water inside the incubator.

### Effect of pretreatment on microstructure of cassava stalk fibers

The effect of alkaline pretreatment on microstructure of cassava stalk fibers were observed by Scanning Electron Microscope (SEM) micrograph. A double-sided adhesive carbon tape used for mounting the powdered sample of media before fermentation on brass stubs. By using sputtering tools, the samples were coated with gold-palladium. Then, the samples were examined with SEM (FEI, Type Inspect S50) at 15kV, high vacuum and 10 mm distance according to the Standard Operating Procedure of SEM analysis, Central Laboratory, State University of Malang, Indonesia. The SEM result was used for explaining the differences of *Aspergillus niger* growth on pretreated cassava stalk media.

### Effect of alkaline pretreatment on the growth of *Aspergillus niger* FNCC 6114

Effect of alkaline pretreatment on the growth of *Aspergillus niger* FNCC 6114 was evaluated by using SEM micrograph after fermentation. And another parameters of fungal growth evaluated were glucosamine and reducing sugar levels, and also spores quantity produced per gram media.

### SEM micrograph after the fermentation pro-

### cess

Cultured media after 6 days fermentation were dried and prepared for SEM analysis to observe the mycellium growth after being inoculated by *Aspergillus niger* FNCC 6114. A double-sided adhesive carbon tape used for mounting the powdered sample of culture media on brass stubs. A gold-palladium coating was also done through same method mentioned above. SEM analysis was done at 20 kV, high vacuum and observed at 9.8 - 10 mm distance.

### Glucosamine

The biomass of *Aspergillus niger* FNCC 6114 in media during the fermentation was estimated by determining glucosamine content. The glucosamine content was measured by the method of Souza *et al.* (2011) based on colorimetry at  $\lambda = 530$  nm. The samples were dried previously in oven at 50 °C, milled with waring blender, and weighed to 0.2 g for each.

### Reducing sugar

Metabolic activity of *Aspergillus niger* FNCC 6114 in utilizing cellulose and hemicelluloses was estimated by determining reducing sugar content. The reducing sugar content was measured spectrophotometrically by the method of Miller using DNS reagent (Miller, 1959). Samples (1 g) in sterile plastic bag was added to 50 mL distilled water, homogenized in stomacher for 60 sec and filtered. The filtrate was used as a sample in spectrophotometric assay.

### Spores quantity

Cultured media (1 g) was added to 9 mL of 0.1 % Tween 80 solution and mixed thoroughly. The calculation of spores content was then conducted by using Neubauer haemocytometer under light microscope (Olympus BX 41) at magnification of 520 x.

### Statistical Analysis

All data were collected through duplicate measurements and analyzed using the Excel Program (Microsoft). Average results were expressed as the mean (+) standard deviation.

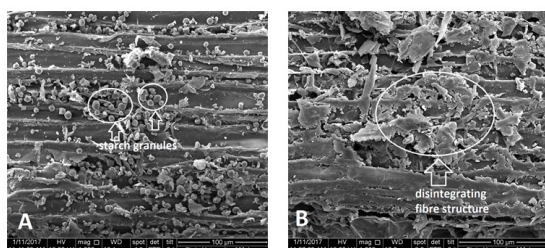
## RESULT AND DISCUSSION

### Effect of lime pretreatment on microstructure of cassava stalk fibers

Figure 1 shows the differences of cassava stalk microstructures after lime pretreatments observed with SEM analysis at magnification of 1000 x. Lime pretreatment resulted in disinteg-

rating fibre structures compared to that structure without the lime pretreatment. This result was in line with the finding of Guilherme *et al.* (2015). They found that various pretreatment of sugarcane bagasse resulted in disruption of sugarcane bagasse fiber. This phenomenon does not occur in untreated ones.

Lime pretreatment also altered the presence of starch granules. This result is consistent with research result by Sudha *et al.* (2016). Sudha and friends conducted pretreatment on cassava stems using an alkaline solution combined with microwave heating. They found that there's no starch granules on alkaline pretreated cassava stalk.



**Figure 1.** SEM analysis (at magnification of 1000 x) of cassava stalk after alkaline pretreatment (A. Fine media without lime pretreatment; B. Fine media with lime pretreatment)

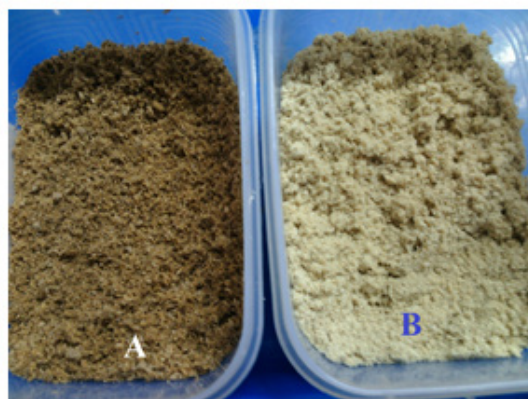
Lime pretreatment was a better choice for making the fibre structure destroyed. However, lime pretreatment could also remove the amyllum granules from the cassava stalk, so that this seemingly made the growth of *Aspergillus niger* FNCC 6114 hindered. Poorer growth of *Aspergillus niger* FNCC 6114 on lime pretreated media will be discussed in further section of this paper.

#### Effect of pretreatment on the growth of *Aspergillus niger* FNCC 6114

Figure 2 shows that lime pretreatment could change the color of cassava stalk to be darker/brownish. This could be resulted from the browning reaction which encountered during lime treatment. According to the visual observation, the growth of *Aspergillus niger* FNCC 6114 was better on media without lime pretreatment (data not shown). This visual observation was similar with the result of glucosamine content (Figure 3).

Browning reaction is a reaction of the formation of brown compounds that can occur enzymatically or non-enzymatically. Enzymatic browning reactions occur when the phenol compounds are oxidized into quinone by the polyphenol oxidase enzyme that usually takes place in fruit, vegetable and seafood products. The non-

enzymatic browning reaction consists of two types of reactions, namely caramelization reactions and Maillard reactions. The caramelization reaction occurs when the sugar gets a very high heat treatment, and by the presence of water produces a brown caramel compound with a distinctive aroma. While Maillard reactions occur between carbonyl groups of sugars with free amino groups of amino acids, peptides or proteins, produces a brown compound whose type depends on the reacting sugar compound.



**Figure 2.** Difference performance of cassava stalk with (A) and without (B) lime pretreatment.

The type of browning reaction that occurs during lime pretreatment of cassava stem is probably Maillard's reaction. Maillard reaction can occur at low temperatures, for example at room temperature and reaction rate will increase under alkaline conditions. The occurrence of Maillard's reaction can lead to the loss of essential amino acids which are important for the growth of fungi, eg. *Aspergillus niger*.

In alkaline conditions, various reactions may occur, including: carbohydrates degradation to produce carboxylic acids; polysaccharide degradation produces saccharinic acid, lactic acid, formic acid, and dicarboxylic acids; formation of phenolic compounds; and saponification of acetyl groups released by hemicellulose. These compounds may inhibit enzymatic reactions or growth of microorganisms (Jönsson & Martín, 2016).

Beside of the changes in the presence of starch granules, and discolouration to brownish suspected due to Maillard reaction during the process of lime pretreatment, the lime pretreatment process also leads to changes in chemical composition as can be followed through Table 1. The chemical composition of cassava stems further observed are cellulose, hemicellulose and lignin.

**Table 1.** Weight and composition of cassava stems before and after chemical pretreatment

Cassava stalk	Before	After	Changes (%) <sup>*</sup>
Weight (g)	520.00	433	Lower 16.7
Cellulose level (%)	33.70	35.32	Lower 12.7
Hemicellulose level (%)	31.61	22.74	Lower 40.1
Lignin level (%)	27.04	22.85	Lower 29.6

Note: <sup>\*</sup> Counted according to Pelaez *et al.* (2013) formula

The decrease in cellulose content was 12.7%. The reduction in cellulose occurs because the lignocellulosic complex bonding has decomposed and the cellulose becomes dissolved during washing. Reduced hemicellulose levels reached a very high value, ie 40.1%. Chemical pretreatment carried out using alkali is effective in removing lignin, but most of the hemicellulose is lost during chemical pretreatment. Hemicellulose and cellulose are sugar polymers which are a source of carbon for fungal growth. The reduction of hemicellulose and cellulose leads to poor growth of *Aspergillus niger* FNCC 6114.

Lignin levels decreased by 29.6%. This value is less than that obtained by (Pelaez *et al.*, 2013), that is 44,24% when doing pretreatment process of cassava stem using 10% (w/v) 2% NaOH solution for 11 hours, at 60 °C in shaker with speed of 120 rpm. The lower percentage value of lignin levels reduction is probably due to the difference in type and concentration of the alkali solution used. Pelaez *et al.* (2013) also used

a shaker, so that the stirring conducted continuously and the chemical reaction becomes more stable.

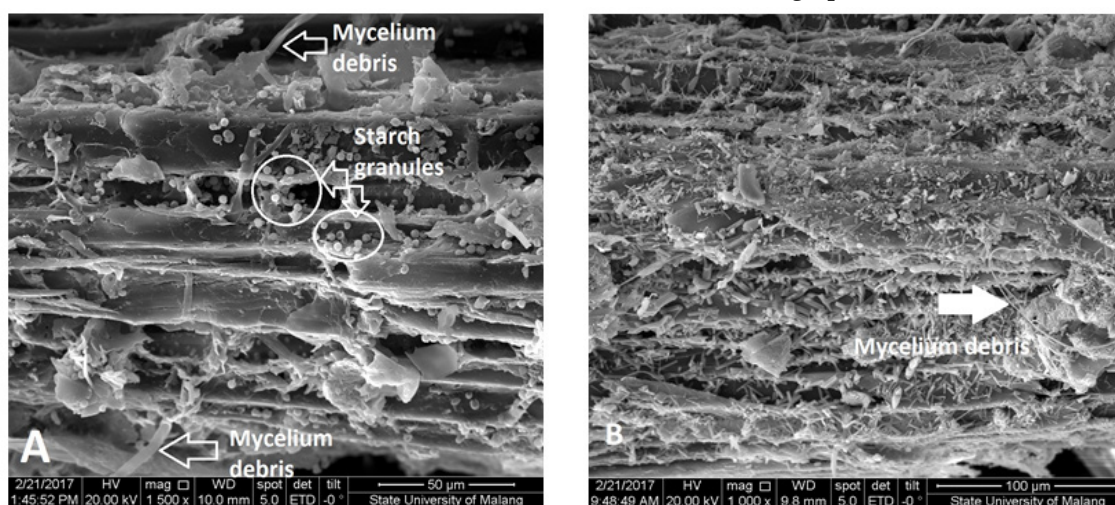
Another factor that may also be the cause of lower fungal growth in chemical pretreatment media is the formation of salt. In the process of pretreatment with alkali will form salts that can not be removed and remain left on the material (Kumar *et al.*, 2009). In this study it was possible to form calcium sulphate salt (CaSO<sub>4</sub>) during the neutralization stage using H<sub>2</sub>SO<sub>4</sub>. Calcium salts, such as CaCl<sub>2</sub> can inhibit fungal growth because they are toxic (Boumaaza *et al.*, 2015).

It was predicted that the reasons why the growth of *Aspergillus niger* on lime pretreated cassava stalk was very poor were 1). nutrients missing in washing step of lime pretreatment; 2). Inhibitory substances which may formed through browning reaction; and 3). Calcium sulphate salt formed in neutralizing step of lime pretreatment (Kumar *et al.*, 2009).

On the other hand, with size reduction pretreatment without immersion in the lime solution, cassava stalks undergo hemicellulose and lignin levels decreased by 63% and 31, 21%. The milling of cassava stalk causes damage to the fiber structure due to the damage of the cell wall of the fiber, thus decreasing cellulose crystallinity. Fiber structure damage and the decrease of cellulose crystallinity leads to the release of hemicellulose and lignin from the lignocellulosic structure. A decrease in lignin levels may support fungal growth. The growth of fungi is not disturbed by the various compounds produced which occurred during the pretreatment process with lime solution.

### SEM micrograph after fermentation

SEM micrograph of media after the fer-



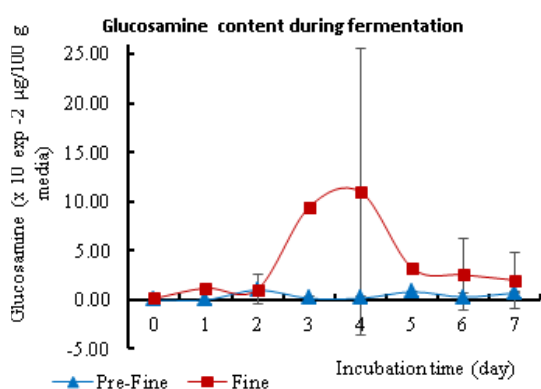
**Figure 3.** SEM micrograph (at magnification of 1500 x) of cassava stalk after fermentation (A. Fine media without lime pretreatment; B. Fine media with lime pretreatment)

mentation were shown at Figure 3. Samples were taken from 6<sup>th</sup> days of incubation. It can be seen that there are more mycelium debris in media without lime pretreatment (3A). There were almost no mycelium debris in lime pretreated media (3B). These SEM micrograph after fermentation was also in line with visual observation. According to visual observation (data not shown), the growth of *Aspergillus niger* FNCC 6114 was better on media without lime pretreatment.

This also indicated that media without lime pretreatment gave better growth of *Aspergillus niger* FNCC 6114. This fact is in line with the result of glucosamine and reducing sugar content in further discussion.

### Glucosamine content

The result of glucosamine content analysis is shown on Figure 4. Glucosamine obtained from media with no lime pretreatment was higher than that one from lime pretreated media. This result is in line with the better growth of *Aspergillus niger* according to visual observation (data not shown). Glucosamine is a component of mycelium or fungal cell wall. The higher the glucosamine, the better growth of fungi.



**Figure 4.** Glucosamine content during fermentation of cassava stalk by *Aspergillus niger* FNCC 6114. Effect of alkaline pretreatment (Fine & Pre-fine size media)

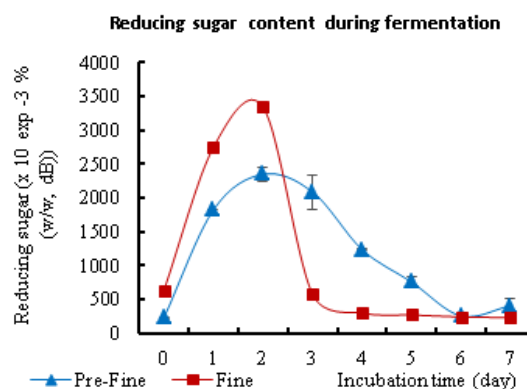
The highest value of glucosamine was reached from unpretreated media at 4 days of incubation, which was  $10.785 \times 10^{-4} \mu\text{g/g}$  media (dry basis). This value was much higher than the one from lime pretreated media, which was  $0.324 \times 10^{-4} \mu\text{g/g}$  media (dry basis). This may be caused by the nutrients missing especially amylose (starch) as seen on Figure 1. According to SEM micrograph, cassava stalk in media without lime pretreatment has more abundant starch granules. Size reduction will give more chance for opening

the fiber structure and let amylose granules rise to the media surface, while lime pretreatment affects cellulose structure becomes more amorphous, but resulted in reduced level of amylose granules.

Particle size of the material affects the success of solid state fermentation process, partly because it determines the porosity and the specific area of the media (Yoon *et al.*, 2014). Size reduction of the cassava stalk into 50 mesh can support better growth of *Aspergillus niger* FNCC 6114, because: 1). There is diffusion of nutrients from and into particles more easily and quickly, and 2). Provides a larger surface area for enzymatic reactions per equal volume of media. Media with a size of 50 mesh is still quite porous. By reducing the size of cassava stalks up to 50 mesh (+ 0.297 mm) also has not been clumped. Media clumps that can inhibit the transfer of mass, heat, and oxygen do not occur due to the fibrous cassava stalk properties.

### Reducing sugar content

Reducing sugar content during fermentation was shown in Figure 5. It was indicated that sugar content was higher in media without alkali pretreatment. Meanwhile, the maximum level from both of media reached at 2 days of incubation, which were 3.14 and 2.55 (% w/w, dB) for media without and with lime pretreatment respectively.



**Figure 5.** Reducing sugar during fermentation of cassava stalk by *Aspergillus niger* FNCC 6114. Effect of alkaline pretreatment (Unpre & Pre-fine size)

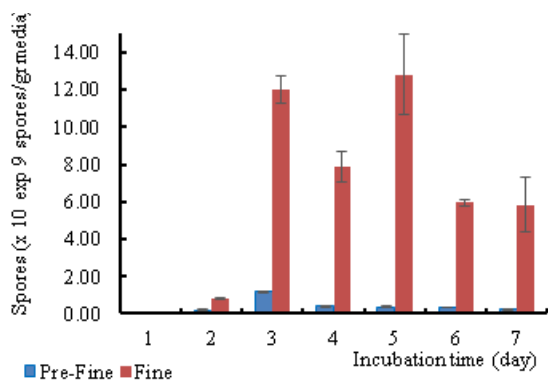
Furthermore, observation on glucosamine content (Figure 4) compared to sugar content (Figure 5) indicates that at 2 days of incubation on unpretreated media, reducing sugar formation reached maximum level although glucosamine level was not at maximum level. This was in accordance with the Desai & Converse (1997, in: Sridevi *et al.*, 2015) statement that polysaccharides

breakdown into reducing sugar will be at maximum rate in early state of incubation since there still an amorf region of polisaccharides on the media (Sridevi *et al.*, 2015).

Figure 5 also shows that at the beginning of fermentation, ie at incubation day-0, the reduction sugar content in the medium without pretreatment with lime solution is higher than the reducing sugar content in the pretreatment medium with lime solution. Monosaccharides, such as glucose, are unstable under alkaline conditions and will degrade to produce lactic acid (Millati *et al.*, 2002).

### Spores quantity

Number of spores from all kind of media during 7 days fermentation can be seen in Figure 5. Pretreatment methods used affects the spores quantity. Mycelium started to rise at 2 days of incubation (data not shown), and followed by sporulation. The level of sporulation is increased with increasing day of incubation. The more mycellium growth the more spores produced.



**Figure 6.** Spores quantity during fermentation of cassava stalk using *Aspergillus niger* FNCC 6114. Effect of alkaline pretreatment (Fine & Pre-fine size media)

Sporulation without lime pretreatment was higher. It can be seen on Figure 5, that the spores produced by *Aspergillus niger* FNCC 6114 on media without lime pretreatment are more abundant. Spores are formed when environmental conditions are unfavorable for mold growth. Unfavorable environmental conditions may be mismatches in humidity, temperature, pH, or nutrient availability. In the medium of cassava stems that are not subjected to pretreatment with lime solution, the growth of mycelia is very high on the second day, so that on the third day there is maximal spore formation because the media has excessive nutrient deficiency.

Fermentation will be more efficient if

sporulation is delayed (Nicolás-santiago *et al.*, 2006). When the fermentation of cassava stalk conducted efficiently, it will support optimal utilization of lignocellulosic biomass. It takes effort to delay the sporulation on cassava stalks without lime pretreatment.

The process of bioconverting cassava stalk to increase its economic value through solid state fermentation is constrained by the presence of lignin. The effective process of cassava stalk pretreatment which can improve its feasibility as a growth medium for microorganisms that play a role in bioconversion through solid state fermentation, has not been studied in depth. The results of this study provide information on the effective process of pretreatment towards cassava stalk to enhance the growth of *Aspergillus niger*. Increasing the utilization of cassava stems using molds, for example *Aspergillus niger* can overcome the accumulation of organic waste that can interfere with environmental sustainability.

### CONCLUSION

Based on the research result, it was known that lime pretreatment alters microstructure of cassava stalk fibers and *Aspergillus niger* FNCC 6114 growth. The better growth of *Aspergillus niger* FNCC 6114 through solid state fermentation was on the fine-sized media without lime pretreatment. For efficiently fermentation of cassava stalk into valuable product by using *Aspergillus niger*, its need an effort for depressing spores formation.

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