Modern Applied Science; Vol. 9, No. 2; 2015
ISSN 1913-1844 E-ISSN 1913-1852
Published by Canadian Center of Science and Education

Effect of Mn^{2+} Addition on Delignification of Water Hyacinth Using *Phanerochaete Chrysosporium*

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Received: March 21, 2014 Accepted: April 8, 2014 Online Published: January 15, 2015

doi:10.5539/mas.v9n2p228 URL: http://dx.doi.org/10.5539/mas.v9n2p228

Abstract

In order to prepare water hyacinth as a bioethanol feedstock, it is necessary to perform a pretreatment process. Biological process is considered to be one of the potential pretreatment that may be developed in the future. This study aims to increase lignin degradation rate and shorten the pretreatment time and increase fermentable sugar yield after enzymatic hydrolysis. Experiment was conducted in batch mode. Cut water hyacinth prepared was inoculated with the fungus and incubated at room temperature for 28 days during which its content of lignin, cellulose and hemicellulose was analyzed regularly. Effect of *co-factor* was studied by adding Mn^{2+} with varied concentrations (between 0.05 – 1%) to water hyacinth

The results showed increase the rate of degradation of lignin and increase fermentable sugar yield results with the addition of *co-factor* Mn^{2+}. In Pretreatment of Water Hyacinth without addition of Mn^{2+} occured lignin degradation reached 24.39% during 16 days incubated with the fungus and fermentable sugar yield reached 36.37%. An addition of *co-factor* Mn^{2+} at a concentration of 0.5% showed that the lignin degradation reached 42.44% during 16 days of incubation, and the fermentable sugar yield in hydrolysis increased to reach 67.66%.

Keywords: water hyacinth, pretreatment, fungus, lignin, fermentable sugar yield

1. Introduction

Water hyacinth is one of potential lignocellulosic feedstock for bioethanol production as it contains almost 62.8% of cellulose and hemicelluloses. Additionally, it is considered as a very fast growing plant [Gunnarson et al., 2007]. Conversion of lignocellulosic materials into bioethanol consists of 3 main stages, namely pretreatment, hydrolysis and fermentation [Talebnia et al. 2010]. The pretreatment aims to eliminate the lignin and reduce the crystallinity of the cellulose, so the cellulose can be hydrolyzed more easily to produce glucose in enzymatic hydrolysis [Taherzadeh and Karimi, 2008]. Biological pretreatment is a process considered to be more environmentally friendly than others, as it requires low energy, does not produce waste and other side products and being relatively cheap compared to chemical pretreatment processes [Kumar et al., 2009]. *Phanerochaete Chrysosporium* is a species of white rod fungus that selectively degrade lignin. This means it has the ability to degrade lignin in wood more than cellulose and hemicelluloses [Hattaka and Hammel, 2010]. A number of researchers [Shi et al. 2008; Wan et al.2011] show that lignin degradation varies for each of the lignocellulosic materials and the fungi used in the pretreatment. The most challenge is to provide the best conditions for fungus to achieve optimum growth in a given system (Akhtar et.al, 1989).

Recent research on the pretreatment of lignocellulosic materials has been focus on increasing the selectivity of fungus in degradation of lignin out of cellulose and hemicelluloses [Shi et al. 2008; Wan et al.2011]. It is generally known that during the degradation of lignin , the fungus produce ligninolytic enzymes [Rothschild et al.. 1999], which break the phenolic and non- phenolic chains in the chemical structure of lignin to produce simple carbon compounds which are water soluble and can be used as a carbon source for the fungal growth [Boyle et al . 1992]. Ligninolytic enzymes are composed of *lignin peroxidase (LiP), Mangannese peroxidase (MnP)* and *laccase (Lac)* [Akthar et al . 1997]. Not all types of fungus produce all of the enzymes. *Phanerochaete Crysosporium* produces only LIP and MnP enzymes [Rothschild et al. 1999] . An increase in lignin degradation can be achieved by increasing the production and activity of ligninolytic enzymes. This can be

done by providing appropriate environmental conditions of the pretreatment process, such as water content and the presence of *co - factor of* Mn^{2+} and Cu^{2+} [Isroi et al . 2011].

This paper deals with the efforts in enhancing degradation of lignin of water hyacinth for preparing to be bioethanol feedstock using *Phanerochaete chrysosporium*. The study was conducted in SSF by initial water content (IWC) (72.2%) and Mn^{2+} as co factor. It is expected that an optimum concentration of Mn can be proposed to achieve maximum degradation of lignin but minimum degradation of cellulose and hemicelluloses. Additionally, it is also expected that pretreatment period also shortened.

2. Experiment

2.1 Material

The water hyacinth (*Eichhornia Crassipes*) was obtained from Yogyakarta, Indonesia. The water hyacinth stems and leaves were used and in this study had a composition of 7.98% lignin, 27.27% cellulose, 34.27% hemicellulose, and 13.24% ash and other substances. Preparation of the water hyacinth for the pretreatment process was carried out in accordance with the method set out by-Eka Sari et al., (2011; 2013).

Phanerochaete Chrysosporium used in this reseacrh was obtained from the collection of The Department of Biology, Indonesia Institute of Science and Research (LIPI). The enzyme for the hydrolysis process was obtained from *Suntaq cellulast International Limited Sqzyme CS P (Cellulase acid*), activity of 10,000 U / g, Batch No. 1120604 CSP. For various additions of *co-factor* Mn^{2+} a pure substance of $MnSO_4$ from Merck was used.

2.2 Pretreatment Process

2.2.1 Preparation of Water Hyacinth

Dried water hyacinth was placed in a 500 ml bioreactor made of glass. Water was then added to obtain initial water contents of 72.2% (dry basis). For various additions of *co-factor* Mn^{2+} we used concentrations of Mn^{2+} of 0.05%, 0.1%, 0.5% and 1%. After the bioreactor had been filled with water hyacinth, water and a co-factor Mn^{2+} in accordance with the variation studied, the bioreactor was then wrapped with heat resistant plastic, heated and sterilized in an autoclave for 20 minutes at 121^{o}C and then cooled to room temperature prior to inocculation.

2.2.2 Preparation of Fungus

The culture of *Phanerochaete Chrysosporium* was grown on Poetato Dextrose Agar (PDA) medium or slant and liquid media. The preparation of the was carried following the method applied by Eka Sari et.al (2011; 2013).

2.2.3 Solid State Fermentation

The addition of variations of the concentration *co-factors* Mn^{2} to bioreactor. The fermentation process was carried out in a batch bioreactor for 28 days. Analyses of the chemical composition of water hyacinth i.e. lignin, cellulose, and hemicellulose were made using *Chesson* methods [Datta, 1981]. Analysis of the dry weight of fungus used approach to the analysis of proteins using Kjeldal Total Nitrogen. Converting protein concentration of TKN be fungal dry weight followed the analysis conducted by Shi et al. (2008) with the value of factor conversion of protein to the dry weight of fungus (k_T) is 10.87 (g DW of Biomass / g DW of TKN).

2.3 Enzymatic Hydrolysis

Following the *pretreatment* of water hyacinth, sample was subjected to enzymatic hydrolysis. It was carried out for 5 g pretreated water hyacinth with *cellulase* enzyme. pH was adjusted to pH 5.5 addition sodium acetat of 150 mL as buffer solution. Hydrolysis process was carried out for 72 hours and sampling were performed every 24 hours. *Somogyi-Nelson Method* was used to analysis the concentration of fermented sugar yield.

3. Results and Discussion

3.1 Fungus Growth and Substrate Degradation

Figure 1 presents the effect of various concentration of Mn^{2+} on the fungal growth and lignocellulose degradation.

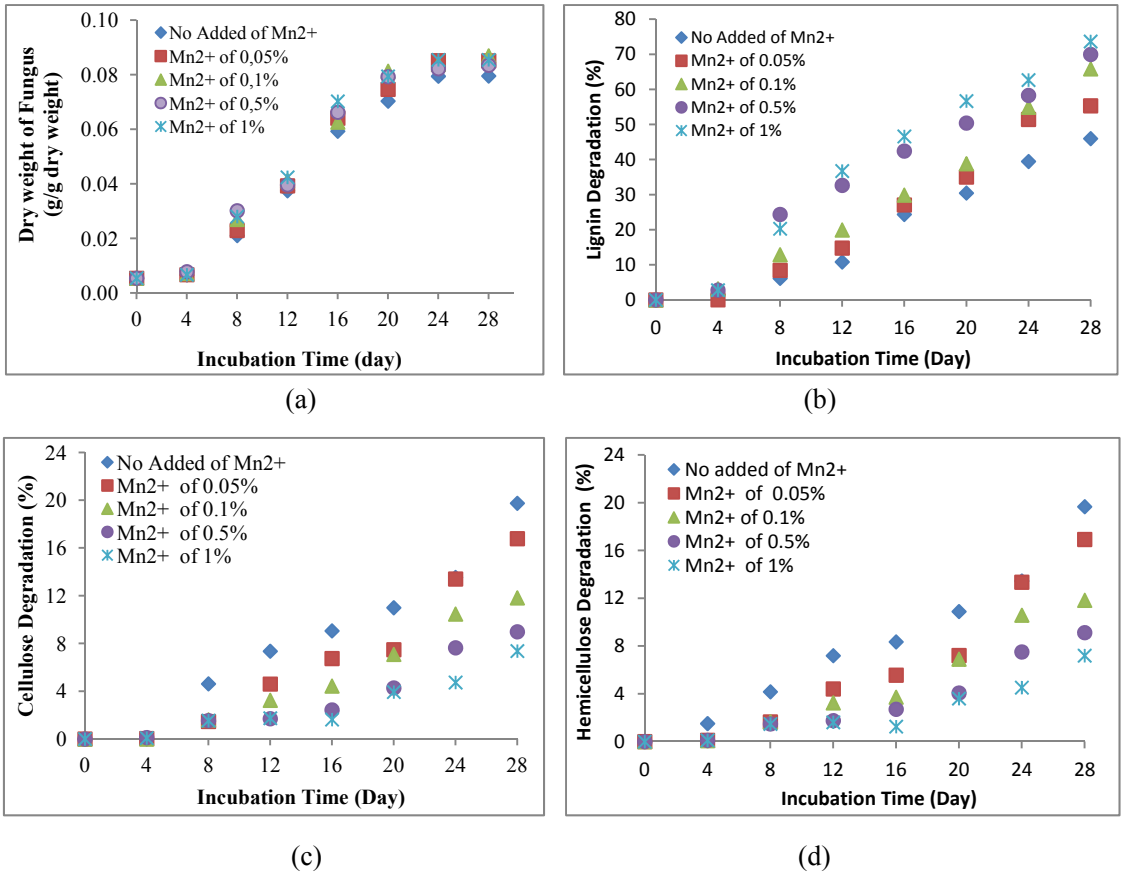


Figure 1. Effect of addition of various concentrations of *Co-factor* Mn^{2+} on a) Dry weight of Fungus; b) Lignin Degradation, c) Cellulose Degradation and d) Hemicellulose Degradation

Figure 1 shows the addition of *co-factor* Mn^{2+} has almost no effect on the growth of the fungi. The addition of *co-factor* Mn^{2+} is very influential in lignin degradation. The results of this study of lignin degradation show that the higher the concentration of co-factors added, the more lignin is degraded. Sequentially, lignin degradation occurred at 45.99% or 0.46 g/g initial dry weight of lignin for the pretreatment without *co-factor* Mn^{2+} during 28 days incubation. While the lignin degradation with the addition of *co-factor* Mn^{2+} at concentrations of 0.05%, 0.1%, 0.5% 1% reached 55.35%, 65.86%, 69.98% and 73.68%, respectively during 28 days incubation.

No more research has been done to study the effects of the addition of various concentrations *co-factor* Mn^{2+} on the degradation of lignin in the pretreatment process. Typically, studies on the addition of a co-factor Mn^{2+} have been evaluated against ligninolytic enzyme production. *Co-factor* Mn^{2+} can increase the production of enzymes LiP and MnP, while the addition of *co-factor* Mn^{2+} can also increase the enzyme laccase (Bonarme et al. 1990). A pretreatment study using PC on various lignocellulosic materials showed that the addition of *co-factor* Mn^{2+} can increase the production of enzymes LiP and MnP. The results of the study by Georgeva et al (2009) showed that the addition of 3.5 mg / L Mn^{2+} can increase the production of enzymes LiP and MnP, the results of Bonarme et al (1990) showed the addition of 11.15 ppm Mn^{2+} significantly increases the production of LiP and MnP enzymes, and the results of research by Urek et al (2007) showed that the addition of 174 µM Mn^{2+} can increase the production of MnP enzymes.

For the degradation of cellulose and hemicellulose it was shown that the higher the added concentration of *co-factor* $Mn^{2+,}$ the greater the decrease in the degradation of cellulose and hemicellulose. In the sequential degradation of cellulose for pretreatment without *co-factor* Mn^{2+} reached 19.74 % during 28 days incubation and with additions of *co-factor* Mn^{2+} at concentrations of 0.05%, 0.1 %, 0.5 % and 1 %, the degradation of cellulose were 16.79 %, 11.83 %, 8.98 % and 7.37 %, respectively during 28 days incubation. While the degradation of hemicellulose for pretreatment without *co-factor* Mn^{2+} reached 19.67 %, and with additions of *co-factor* Mn^{2+} at concentrations of 0.05%, 0.1 %, 0.5 % and 1 % the the degradation of hemicellulose were 16.93 %, 11.83 %, 9.12 % and 7.19 %, respectively. The lowest degradation of cellulose and hemicellulose was achieved by the addition of *co-factor* Mn^{2+} at a concentration of 1%. The results indicated that the addition of *co-factor* Mn^{2+}

stimulated production and MnP enzyme activity, thus leading to increased lignin degradation. It can also inhibit the production of hydrolytic enzymes to degrade cellulose and hemicellulose.

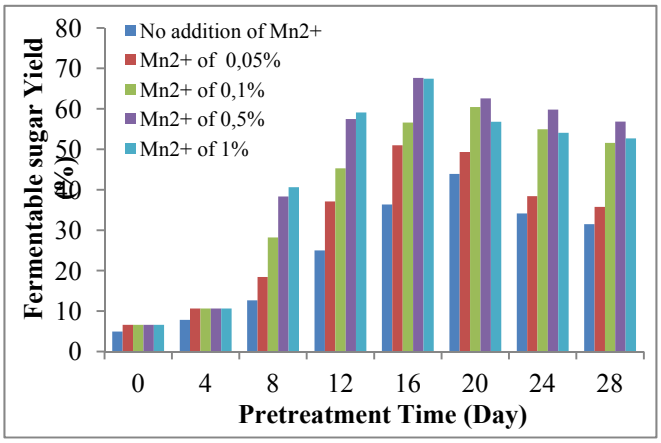


Figure 2. Fermentable Sugar Yield at Various Concentrations of Additions of *Co-factor* Mn^{2+} and Various Pretreatment Time

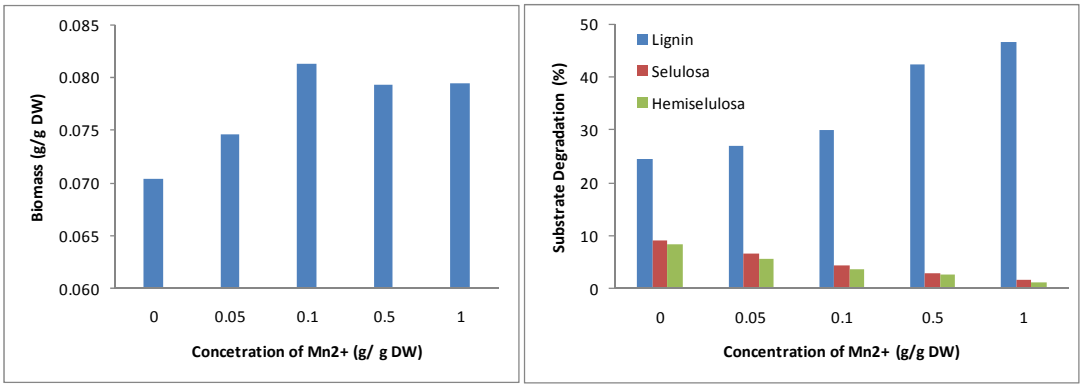
Figure 2 shows the effects of the addition of *co-factor* Mn^{2+} on fermentable sugar yield. Up to the 4th day of pretreatment, there was no significant change in the fermentable sugar yield for all levels of concentrations of *co-factor* Mn^{2+}. After that time, increases in the yield of sugar fermentation corresponded to increases in concentrations of added *co-factor* Mn^{2+}. A significant increase in fermentable sugar yield occurred at 16 days of incubation, and then decreased until the end of the incubation. It happens on all variations of concentration The highest fermentable sugar yield reached 67.66 % at concentration of *co-factor* Mn^{2+} of 0.5 % at 16 days of pretreatment. While, fementable sugar yield for the others concentration only obtained 36.37%, 50.99%, 56.62% and 67.46% for concentration *co-factor* Mn^{2+} of 0%, 0.05%, 0.1% dan 1%, respectively.

Based on the evaluation of the enzymatic hydrolysis results of water hyacinth in the biological pretreatment at various incubation times, if there is a significant increase on the yield of glucose, it is indicated that damage morphological structure of lignin [Eka Sari et al. 2014]. To determine the timing of the morphological structure of lignin breakdown, it is necessary to review the results of the glucose yield in enzymatic hydrolysis for different incubation time and concentration variation of *co-factor* Mn^{2+} that can be seen in Figure 2.

In addition to the selection of the concentration for the addition of *co-factor* Mn^{2+} being necessary for the enhanced recovery of sugar fermentation, the duration of the pretreatment time also affects the fermentable sugar yield. Pretreatment time that gave the maximum fermentable sugar yield occured at 16 days. If the fermentation time was extended to 28 days, the fermentable sugar yield is relatively stable or even declines. There was even a possibility that shortening the time of pretreatment would have a good impact on fermentable sugar yield. One obstacle generally encountered in *scale up* for biological processes which involve microorganic activity is the lengthy processing time. Maximum fermentable sugar yield was obtained within 16 days. It indicates that the duration of pretreatment was influence on the fermentable sugar. If the pretreatment time was extended, there will be a degradation of cellulose and hemicellulose were significant. It causes to be the fermentable sugar yield obtained are low. The fermentable sugar yield was significantly increased at day 16 for the concentration of *co-factor* Mn^{2+} of 0.5%. Allegedly on day 16 of incubation, It has occurred the damage morphological structure of lignin as in studied Eka-Sari et al. (2014). If It has occured the damage of the morphological structure of lignin, then it is exactly time to the stopped of the biological pretreatment and then done the enzymatic hydrolysis. It aims to get the optimum lignin degradation and open access for cellulase enzyme to hydrolized cellulose, and prevent the degradation of cellulose and hemicellulose. These resultssimilar to the results of the study by Isroi et al. (2011), which showed that the addition of Mn^{2+} can increase digestibility of Oil Palm Empty Fruit Bunches to 55 % during 21 days of incubation with the *Pleurotus floridanus*, decreasing when the incubation time was extended to 42 days .

If the effects of the addition of concentrations of *co-factor* Mn^{2+} with concentration 0.5% of dry weight on the growth of the fungus and substrate degradation of lignin, cellulose and hemicellulose are evaluated on the 16nd

days incubation, then the profile of the changes to the fungal biomass and substrate degradation will be evident, as seen in Figure 3.



(a) (b)

Figure 3. Changes in the concentration of fungus (a) and Substrate Degradation (b) on the 16nd day Incubation

Figure 3 shows in the pretreatment of water hyacinth with fungus, differences in the fungal biomass and substrate degradation occur when varying the concentrations of additions of *co-factor* Mn^{2+}. Changes to the fungal biomass are relatively stable, which suggests that the addition of *co-factor* Mn^{2+} has less influence on the increase in the concentration of the fungus. This is in contrast to lignin, where the higher the concentration of co-factor added, the more significant the increase in lignin degradation. It proves that the addition of *co-factor* Mn^{2+} can increase the production and activity of enzymes and ligninolytic production [Isroi et al. 2011]. This phenomenon is different from the degradation of cellulose and hemicellulose, where experimental results show that the degradation of cellulose and hemicellulose decreases with the addition of *co-factor* Mn^{2+} in the pretreatment process.

As explained earlier, the fungal activity to degrade lignocellulose, so the fungus will be produce ligninolytic and hydrolytic enzymes. Ligninolytic enzymes to degrade lignin, and hydrolytic enzymes to degrade cellulose and hemicellulose. If the addition of *co-factor* Mn^{2+} can increase the production of enzymes ligninolytic, then the possibility of the production of hydrolytic enzymes is inhibited. If the production of hydrolytic enzymes decreases, so the potential for degradation of cellulose and hemicellulose will decrease too. Presumably, this is the cause of the degradation of cellulose and hemicellulose to be decreased and increased lignin degradation after adding of *co-factor* Mn^{2+}. The decreased of cellulose and hemicellulose degradation will be beneficial, because it can control the loss of cellulose and hemicellulose during the pretreatment process, so it can also increase the recovery yield of glucose in the enzymatic hydrolysis process.

The concentration of fungi and substrate degradation with the addition of varying concentrations of *co-factor* Mn^{2+} can be seen in Tabel 1.

Table 1. Concentration of Fungus and Substrate Degradation at Various of co-factor Mn2+ on the 16 Day Incubation

Concentration of *co-factor* Mn^{2+}	Concentration of Fungus	Reduction of Substrate					
(g/g DW)	(g/g DW)	Lignin		Cellulose		Hemicellulose	
		(% w/w)	g	(% w/w)	g	(% w/w)	g
0	0.0703	24.39	0.1462	9.06	0.1856	8.35	0.2179
0.05%	0.0746	27.08	0.1624	6.75	0.1383	5.57	0.1453
0.10%	0.0813	29.88	0.1792	4.44	0.0909	3.72	0.0971
0.50%	0.0793	42.45	0.2545	2.91	0.0596	2.71	0.0707
1%	0.0794	46.61	0.2795	1.63	0.0335	1.26	0.0328

Table 1 shows the changes in concentrations of fungi, the degradation of lignin, cellulose and hemicellulose caused by the addition of various concentrations of *co-factor* Mn^{2+}. Without the addition of a *co-factor* Mn^{2+}, the

concentration of fungus reached 0.0703 g/g DW. If the *co-factor* Mn^{2+} was added at 0.1%, the increase of fungus was 0.011 g/gDW. Furthermore, the concentration of fungi remains relatively stable even when the concentration of *co-factor* Mn^{2+} is increased 10 fold to 1%. This suggests that the addition of *co-factor* Mn^{2+} has virtually no effect on fungal growth. This was in contrast with the lignin degradation, which increased significantly when *co-factor* Mn^{2+} was added at 0.5% and the degradation of lignin doubled to 42. 45%. But if the co-factor is increased 2 fold, the increase in the lignin degradation was only about 4.16%, suggesting that the addition of *co-factor* Mn^{2+} by a corresponding amount is required in order to show significant improvement.

As an additional consideration for development towards *scale up*, *co-factor* Mn^{2+} is a compound form of inorganic salts which is not cheap, so in using it as a supplement to increase the degradation of lignin in the pretreatment of lignocellulosic material, benefits must be evaluated correctly. Additions can be carried out at appropriate concentrations to produce expected increases in lignin degradation. For pretreatment of water hyacinth, concentrations of *co-factor* Mn^{2+} can be chosen corresponding to 0.5%.

3.2 Fermentable Sugar Yield

In evaluation of the effects of the addition of *co-factor* Mn^{2+} on the degradation of lignin, the results showed that optimal lignin degradation occurred with the addition of 0.5%. *co-factor* Mn^{2+} Once the water hyacinth has undergone pretreatment, an enzymatic hydrolysis process is carried out using cellulase enzymes to produce sugar fermentation. Obtaining sugar fermentation by the addition of different concentrations of *co-factor* Mn^{2+} can be seen in Figure 4.

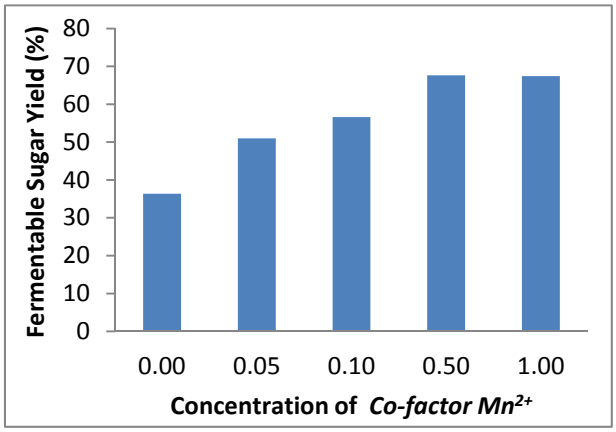


Figure 4. Fermentable Sugar Yield at Various Concentrations of Additions of *Co-factor* Mn^{2+}

Figure 4 shows an increase in fermentable sugar yield with increasing concentrations of the addition of *Co-factor* Mn^{2+}. Without the addition of *Co-factor* Mn^{2+}, so fermentable sugar yield obtained 36.37%. If added *Co-factor* Mn^{2+} at a concentration of 0.005%, the yield of fermentable sugar reached 50.99%. If the concentration of *Co-factor* Mn^{2+} was added 2 fold at 0.1%, so an increase in fermentable sugar yield reached 56.62%. The highest fermentable sugar yield reached 67.66 % at concentration of *co-factor* Mn^{2+} of 0.5 % at 16 days of pretreatment. Whereas when the concentration of *co-factor* Mn^{2+} was added 2 fold at 1 % , the fermentable sugar yield decreased to 67.46 %. This suggests that higher concentrations of added *co-factor* Mn^{2+} may increase the fermentable sugar yield, but if the concentration of the addition of *co-factor* Mn^{2+} is too high, the fermentable sugar yield will stagnant. Addition of *co-factor* Mn^{2+} at concentrations that correspond to approximately 0.5 % can increase lignin degradation and the fermentable sugar yield, but the addition of more higher concentration will no affect with lignin degradation and fermentable sugar yield.

It indicated that there was a maximum concentration that can increased the degradation of lignin, as required by the fungus in activities to degrade of water hyacinth. If the appropriate concentration required to stimulate the activity of ligninolytic enzymes, it will have an impact on increasing the degradation of lignin. but if the concentration of *Co-factor* Mn^{2+} more higher, and excessive than required then it will not affect the increase in lignin degradation. In biological pretreatment on the water hyacinth using *Phanerochaete chrysosporium,* indicating that the concentration of 0.5% of the dry weight was an appropriate concentration to stimulate the activity of ligninolytic enzymes to degrade lignin. Therefore, it is necessary to studi the mechanism of molecular competition of *Co-factor* Mn^{2+} , and the reaction changes on Mn^{2+} into Mn^{3+} involving *MnP* enzyme that has not been studied in this research.

4. Conclusion

The addition of *co-factor* Mn^{2+} can increase the degradation of lignin, but also can control cellulose and hemicelluloses degradation during biological pretreatment of water Hyacinth using *Phanerochaete Chrysosporium*. In general, the higher the concentration addition of Mn^{2+}, it will also increase the lignin degradation, but if it has reached the optimum concentration to stimulate enzyme activity ligninolytic, then more higer of Mn^{2+} has no effect on the lignin degradation. The optimum concentration for the addition of Mn^{2+} in biological pretreatment of water hyacinth is a concentration of 0.5% of dry weight. The addition of Mn^{2+} of 0.5% of the dry weight showed an increase in the lignin degradation from 24.39% to 42.45%, and a decrease of cellulose degradation from 9.06% to 2.91% and hemicellulose from 8.35% to 1.26% during 16 days of incubation. Increased yield of glucose occurred significantly from 36.36% to 67.66%.

Acknowledgement

We thank the Indonesian Directorate General of Higher Education (DP2M DIKTI) for financial support of this work through the research Grant for doctoral students "Doctoral Dissertation Grant 2010", Gadjah Mada University Yogyakarta, and we are grateful to all participants of the Sultan Ageng Tirtayasa University, Cilegon Banten for all their support of this work.

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