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Microbial Delignification of Corn Stover by *Phanerochaete chrysosporium* in Solid State Fermentation

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Abstract : Delignification of corn stover by white rot fungus Phanerochaete chrysosporium in solid state fermentation was evaluated for improving subsequent enzymatic hydrolysis. Corn stover has major components of lignocellulosic material which have large potential for the development future products. Corn stover consists of cellulose (36,81%), hemicelluloses (27,01%), lignin (15,70%), ash (6,04%) and other material (14,44%). Some white-rot fungi has used to lignin degradation. The most commonly utilized fungus is the white-rot fungus Phanerochaete chrysosporium. The purpose of this research is to determine temperature effect of incubation and mold inoculum volume to find the best condition in the biodelignification process of corn stover using Response Surface Methodology. The experimental result shows that significant factor in biodelignification is temperature. The best condition from corn stover biodelignification are temperature of 30oC and 10 ml mold inoculum. Under this condition, produce lignin removal 11,73 %, α-cellulose removal 24,59 % and hemicelllulose removal 32,44 %.

Keywords *- biodelignification, corn stover, Phanerochaete chrysosporium, lignin content, solid state fermentation*

I. INTRODUCTION

Lignocellulosic materials including agricultural wastes, forestry residues, grasses and woody materials have great potential for bio-fuel production. It is rich in carbohydrates (55 – 75% dry basis) and widely available [1]. Availability of abundant lignocellulose source can be used as raw material for alternative energy. Utilization of lignocellulose source as raw material for alternative energy assessed more potential than starch or sugars source. Because it can reduce competition of land use and prevent of *food-feed-fuel conflicts.*

Corn is important food crops in Indonesia. In Indonesia, corn production on 2015 is 20.666.702 ton. Its increase than corn production on 2014 (19.008.426 ton) and 2013 (18.511.853) [2]. Corn stover, as the most abundant agricultural residue, has the greatest potential to be used for ethanol production. Corn crop consist of 50% trunk (DB), 20% leaf (DB), 20% corncob (DB), and 10% corn husks (DB). Corn stover contain of cellulose (36,81%), hemicellulose (27,01%), lignin (15,70%), ash (6,04%) and others (14,44%) [3].

Lignocellulose consists of lignin, carbohydrates such as cellulose and hemicellulose, pectin, proteins, ash, salt and minerals [4]. Cellulose protected by the combination of hemicellulose and lignin. The complex structure of lignocellulose, how ever is highly resistant to enzymatic hydrolysis, resulting in low cellulose conversion. Therefore, a pretreatment process is needed to reduce the biomass recalcitrance by breaking lignin seals and disrupting the crystalline structure of cellulose for improving enzymatic hydrolysis. To facilitate the enzymatic hydrolysis of lignocellulose, different pretreatment technologies have been developed to remove lignin from corn stover, including physical, chemical, physic – chemical and biological ones [5,6,7,8,9,10].

Chemical pretreatment is expensive, corrosive and possibly toxic to workers. As an alternative to chemical pretreatments, the use of lignolytic white-rot fungi (WRF) for converting lignocellulosic materials to more digestible feedstuffs has been investigated. The feasibility of fungal pretreatment for improving enzymatic digestibility of various biomass feedstocks, such as corn stover [11, 12], wheat straw [13], rice straw [14], cotton stalks [15], and woody biomass [16], has been reported.

Recently, the biological pretreatment of lignocellulosic biomass using solid state fermentation (SSF) by microorganism is intensive researched [17,18,19]. Microbial pretreatment of lignocellulosic biomass by solid state fermentation is considered to be an environmentally friendly process with some advantages. There are no use of severe chemicals, reduced energy input, no requirement for pressurized and corrosion-resistant reactors, no waste stream generated and reduced or no inhibitor to fermentation [11].

The ability of several white rot fungi species to selectively degrade the lignin component of wood has great potential for applications in industrial processing lignocellulosic materials to produce cellulose. Several white rot fungi, such as *Phanerochaete chrysosporium* [11,14,15,20], *Pleurotus ostreatus* [16,21], *Coriolus versicolor* [22], *Cyathus stercoreus* [11], and *Ceriporiopsis subvermispora* [23], have been studied for pretreatment of a wide range of biomass feedstocks. Although delignification is of significance to reduce the

biomass recalcitrance, white rot fungi with a high selectivity for lignin degradation over cellulose loss, as aforementioned and discussed in the later sections, aremost important to fungal pretreatment for biofuel production.

Phanerochaete chrysosporium were selected to carry out bioconversion process. Based on research result, *Phanerochaete chrysosporium* have the ability to lignin degradation 81,4 % and cellulose degradation 22,3 % with the incubation time for 30 days [24]. On the process of corn stover delignification using *Phanerochaete chrysosporium*, unaccounted for the best incubation temperature and volume of mold inoculum addition for increase lignin degradation and decrease cellulose and hemicellulose degradation. The goal of this research is determine of effects, temperature, and mold inoculum addition to find the best condition in the biodelignification process of corn stover with *Phanerochaete chrysosporium*.

II. MATERIALS AND METHODS

2.1. Materials

Corn stover varieties of Bisi 2, consist of trunk, leaf, corncob, and corn husks. Its collected from Kulon Progo, Yogyakarta. The biomass was firstly ground to pass through a 40 mesh [1] mixed until homogeneous (4,49% corncob, 4,72% corn husks, 83,28% trunk, 7,02 % leaf, and 0,49% minor composition). *Phanerochaete chrysosporium* was provide by LIPI Cibinong, Bogor.

2.2. Methods

2.2.1. Inoculum Preparation

Phanerochaete chrysosporium inoculated on potato dextrose agar (PDA) at 30^{o}C for 7 hours. Then further inoculation of *Phanerochaete chrysosporium* on potato dextrose broth (PDB) at 30^{o}C for 7 hours. Inoculum stok is *Phanerochaete chrysosporium* with added nutrition ($(NH_4)_2SO_4$, $MgSO_4.7H_2O$, KH_2PO_4, yeast extract, and glucose) and homogenized using *waring blender*.

2.2.2. Corn Stover Characterization

Corn stover characterization carried out as a basic calculation of biodelignification analysis. Its consist of water content, extractive content, lignin content, holocellulose content and α-cellulose content.

2.2.3. Substrates Preparation

Corn stover (10 grams, 40 mesh) mixed with aquadesh (aquadesh:corn stover = 5:1), then cooked at 100^{o}C for 1 hour. Cooking result, pressed to separating water and solids. Solids used as substrates for biodelignification.

2.2.4. Biodelignification

10 grams of corn stover have been cooked, put in a glass jam and sterilized at 121^{o}C for 15 minutes. Inoculum stok inoculated in substrates (corn stover) compatible with design experiment. Incubation time of biodelignification is 7 days [11].

Corn stover have been biodelignification, be pondered to knows final weights after biodelignifications process. Then, its sterilized (121^{o}C, 15 minutes) for shut off *Phanerochaete chrysosporium.* To facilitate analysis process, corn stover dried on 50^{o}C for 48 hours. Analysis of biodelignification result consist of water content, extractive content, lignin content, holocellulose content and α-cellulose content

2.2.5. Design Experiment

Design experiment used in this research is two-level factorial design with two factors treatment, there are temperature incubation (x_1) and mold inoculum volume (x_2) (Table 1).

Factorial experimental design to determine the effect of linear from two factor for the desired response is:

$$y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i<j} a_{ij} X_i X_j$$

Description:
y : response of each treatment
a_0, a_i, a_{ij}, a_{ii}: coefficient parameter
X_i: influence of linear main treatment factor
X_iX_j: influence of linear two factors treatment

Factors that influence the response processed using *Response Surface Methodology*. Equation design is:

$$y = a_0 + \sum_{i=1}^{n} a_i X_i + \sum_{i<j}^{n} a_{ij} X_i X_j + \sum_{i=1}^{n} a_{ii} X_i^2$$

Description:
y : response of each treatment
a_0, a_i, a_{ij}, a_{ii}: coefficient parameter
X_i: influence of linear main treatment factor
X_iX_j: influence of linear two factors treatment
X^2: quadratic effect main treatment factor

2.2.6. Statistical Analysis
Statistical analysis and estimation the best conditions of biodelignifications process carried out using software of Design Expert 7 trial version.

III. RESULTS AND DISCUSSION

3.1. Corn Stover Characterization

Corn stover has the greatest potential lignocellulose. Chemical compounds of corn stover, showed Table 2. Corn stover have a high lignin, holocellulose, hemicellulose, and α – cellulose content. Lignocellulosic or woody biomass is composed of carbohydrate polymers (cellulose and hemicellulose), lignin and a remaining smaller part (extractives, acids, salts and minerals). Holocellulose content (74,62 %) make corn stover to be potential as alternative raw material of bioethanol. Holocellulose consist of hemicellulose and α – cellulose. The cellulose (40,79%) and hemicellulose (33,83 %), are polysaccharides that can be hydrolysed to sugars and eventually be fermented to ethanol. The lignin (23,16 %) cannot be used for ethanol production.
The combination of hemicellulose and lignin provides a protective sheath around the cellulose, which must be modified or removed before efficient hydrolysis of cellulose can occur, and the crystalline structure of cellulose makes it highly insoluble and resistant to attack [25]. Therefore, to economically hydrolyse hemicellulose, more advanced pre-treatment technologies are required than in processing sugar or starch crops. After the cellulose and hemicellulose have been saccharified, the remainder of the ethanol production process is similar to grain-ethanol.

3.2. Biodelignification of Corn Stover

Lignin is chemically difficult to degrade because it is a three dimensional polymer interconnected through diverse carbon–carbon and other bonds that are not hydrolysable under biological conditions. The capability to degrade lignin presented by several White-Rot Fungi is due to their extracellular non-specific and non-stereoselective enzyme system [26] composed mainly by laccase, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Lignin peroxidases are strong oxidants that interact directly with non-phenolic lignin structures to cleave them, but cannot penetrate the small pores in sound lignocellulose. Manganese-dependent peroxidases produce small diffusible strong oxidants that can penetrate the substrate. Ferulic acid and p-coumaric acids that are esterified to hemicellulose sugars constitute another limitation to biodegradation of lignocellulosic walls and thus the feruloyl esterase is another key enzyme in the delignification process. Most of the feruloyl esterases have been shown to act synergistically with cellulases, xylanases and pectinases to break down complex plant cell wall carbohydrates [27]. Schematic of goals of pretreatment on lignocellulosic material represented of Figure 1.

Solid state fermentation be affected some factors, there are particle size, supplement, inoculum, and temperature. Particle size of the substrate is also a major factor affecting the performance of solid state fungal pretreatment. Large particle size can hamper the penetration of fungi into cellulosic biomass and also prevent the diffusion of air, water, and metabolite intermediates into the particles [1]. Particle size of the substrate on this research is 40 mesh. However, the reduced particle size with a decreased size of interparticle channel may adversely affect interparticle gas circulation [25].

Supplementation of inducers, such as Mn^{2+}, H_2O_2, and aromatic compounds, can potentially stimulate secretion of ligninolytic enzymes and lignin degradation on biomass feedstock. Shrestha *et al.* (2008) tested effects of Mn^{2+}, H_2O_2, and veratryl alcohol on lignin degradation of corn fiber by *P. chrysosporium* and found Mn^{2+} addition resulted in the highest lignin degradation [20]. Some studies, however, showed the addition of Mn^{2+} did not improve lignin degradation on cotton stalk by *P. chrysosporium* [15]. Supplement that used in this research are $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, KH_2PO_4, yeast extract, and glucose. Addition of supplemental nitrogen may inhibit lignin degradation while stimulating growth of white rot fungi and consumption of carbohydrates.
Phanerochaete chrysosporium yields spores which enable convenient inoculum preparation and mixing with the substrate. Similar to liquid fermentation, fermented materials in solid state, fresh substrate can be fed to partially replace fermented materials. A minimum level of inoculum is generally required for effective colonization and subsequent delignification. Akhtar *et al.* (1998) tested different levels of inoculum using precolonized wood chips by *P. chrysosporium* for the decay of aspen wood as alternatives to mechanical pulping. The results

showed that a 2–5% inoculation level gave good performance and energy savings while increasing the inoculation level to 20% did not correspondingly increase energy savings for mechanical pulping [29].
In general, white rot ascodiomycetes grow well around 39 °C while white rot basidiomycetes can grow between 15 and 35 °C and their high delignification rate is generally obtained within an optimal temperature range between 25 and 30 °C [25]. The metabolism of white rot fungi generates heat and causes temperature gradients in solid state cultivation. The accumulated heat can kill or inhibit the fungal growth and metabolism. Therefore, in the scale up of solid state cultivation, heat dissipation is one of the key factors to be taken into account in bioreactor design.

3.2.1. Influence of Temperature and Mold Inoculum Volume on Lignin Content

Lignin (23,16 %) is present in all lignocellulosic biomass. Therefore, any ethanol production process will have lignin as a residue. It is a large complex polymer of phenylpropane and methoxy groups, a non-carbohydrate polyphenolic substance that encrusts the cell walls and cements the cells together. Data processing result of lignin content have equation:

$$L = 1{,}274 + 0{,}007x_1 + 0{,}060x_2 - 2{,}17\text{x } 10^{-5}\, x_1x_2 - 3\text{x } 10^{-5}x_1^{\,2} - 0{,}005x_2^{\,2}$$

p > F of model is 0,6160, unreal for *L*. Its show that temperature and inoculum volumes are unreal take effect for L. While p > F of lack of fit is 0,2171, means unreal for L. Its show that the equation not enough to describe biodelignification process. Therefore, further data processing is required.
Influence of temperature and inoculum volume for lignin content showed Figure 2. Based on Figure 2, appears that changes of temperature and inoculum volume not influence for lignin content. Its showed on graph tends to be flat on changes of temperature and inoculum volume. Surface shape respons on Figure 2, disposed do not change in another combination with another factors. It is reinforced by p > F chance from analysis of variance result, show that influence of the interaction is unreal. Value of p > F chance interaction of temperature and inoculum volume for lignin content showed on Table 3.

3.2.2. Influence of Temperature and Mold Inoculum Volume on Hemicellulose Content

During hydrolysis, hemicellulose sugars may be degraded to weak acids, furan derivates and phenolics. These compounds inhibit the later fermentation, leading to reduced ethanol yields [25]. Data processing result of hemicellulose content have equation:

$$HE = 2{,}459 - 0{,}050x_1 + 0{,}144x_2 + 0{,}0001x_1x_2 + 0{,}0006x_1^{\,2} - 0{,}01078\, x_2^{\,2}$$

p > F of model is 0,0666 unreal for *HE*. Its show that temperature and inoculum volumes are unreal take effect for *HE*. While p > F of lack of fit is 0,0361, means unreal for HE. Its show that the equation not enough to describe biodelignification process. Therefore, further data processing is required.
Influence of temperature and inoculum volume for hemicellulose content showed Figure 3. Based on Figure 3, appears that changes of temperature and inoculum volume not influence for hemicellulose content. Its showed on graph tends to be flat on changes of temperature and inoculum volume. Although hemicellulose content is increase on temperature change, but the increase is not significant. It is reinforced by p > F chance from analysis of variance result, show that influence of the interaction is unreal. Value of p > F chance interaction of temperature and inoculum volume for hemicellulose content showed on Table 4.

3.2.3. Influence of Temperature and Mold Inoculum Volume on α-Cellulose Content

Cellulose is a key carbohydrate used for producing glucose, which is a major raw material in the synthesis of biofuels and bio-based chemicals by microbial fermentation. In hydrolysis the polysaccharide is broken down to free sugar molecules by the addition of water. The product, glucose, is a six-carbon sugar or hexose.Data processing result of α – cellulose content have equation:

$$\alpha = 1{,}196 + 0{,}034\, x_1 + 0{,}137\, x_2 + 0{,}001\, x_1x_2 - 0{,}0003\, x_1^{\,2} - 0{,}016x_2^{\,2}$$

p > F of model is 0,4054, unreal for α. Its show that temperature and inoculum volumes are unreal take effect for α. While p > F of lack of fit is 0,0234, means unreal for α. Its show that the equation not enough to describe biodelignification process. Therefore, further data processing is required.
Influence of temperature and inoculum volume for lignin content showed Figure 4. Based on Figure 4, appears that changes of temperature and inoculum volume not influence for α - cellulos content. Its showed on graph tends to be flat on changes of temperature and inoculum volume. Surface shape respons on Figure 4, disposed do not change in another combination with another factors. It is reinforced by p > F chance from analysis of variance result, show that influence of the interaction is unreal. Value of p > F chance interaction of temperature and inoculum volume for α - cellulose content showed on Table 5.

3.3. The Best Condition of Biodelignification Process

Determination the best condition of biodelignification process, based on lignin content, hemicellulose content, and α – cellulose content on corn stover which has been in biodelignification. Desired conditions are

minimum lignin content, maximum hemicellulose and α – cellulose content. The best condition of biodelignification process can be estimated using equation of lignin, hemicellulose and α – cellulose contents. However, on this equation, there are several factors is not influence, so suitability of equation with actual condition have increase, and estimated value produced more closer with actual value. By eliminating variables that have no effect, resulted equation which have include influence variables. The equation are:

$L = 4{,}662$

$HE = 1{,}720 + 0{,}126\ x_1 + 0{,}274\ x_1^2$

$\alpha = 15{,}883$

Based on the equation, temperature and inoculum volume are unreal for lignin and α – cellulose content on biodelignification process. At hemicellulose content, only temperature that have influence on biodelignification process, while inoculum volume that have not influence on hemicellulose content. Estimated the best conditions using equations changed get condition of biodelignification that showed Table 6.

On estimated of the best conditions, lignin content on corn stover after biodelignification is 1,671 gram, hemicellulose content is 1,868 gram, and α – cellulose content is 2,514 gram. Baseline weight of corn stover before biodelignifications process is 10 gram (dry basis). Decrease of lignin, hemicellulose, and α – cellulose content showed Table 7.

Based on Table 7, can be known that lignin decrease on this research lower than lignin decrease on Fadillah *et al.*, (2008) and Dong *et al* (2013). However, in this condition produce decrease of hemicellulose and α – cellulose fewer than Fadillah *et al.*, (2008) and Dong *et al* (2013).

Based on research result of Fadilah *et al.*, (2008), *Phanerochaete chrysosporium* have the ability to lignin degradation 81,4 % and cellulose degradation 22,3 % with the incubation time for 30 days [24]. And *P. chrysosporium* PC2 was the most efficient strain for SCB degradation, which removed 73.5%, 67.0%, 88.6% and 93.4% of total SCB, cellulose, hemicelluloses and lignin, respectively in 12 weeks [30].

IV. FIGURES AND TABLES

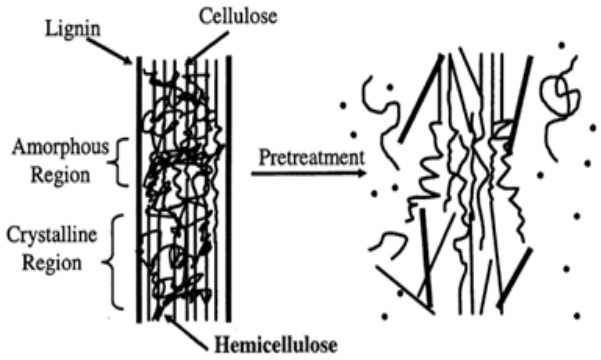

Figure 1. Schematic of goals of pretreatment on lignocellulosic material [28]

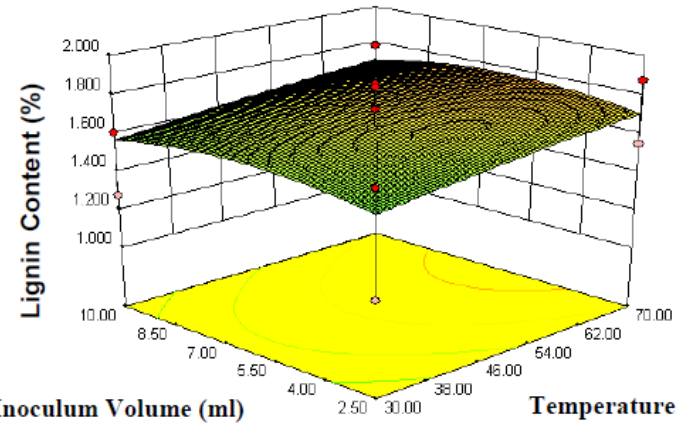

Figure 2. Influence of temperature and inoculum volume for lignin content

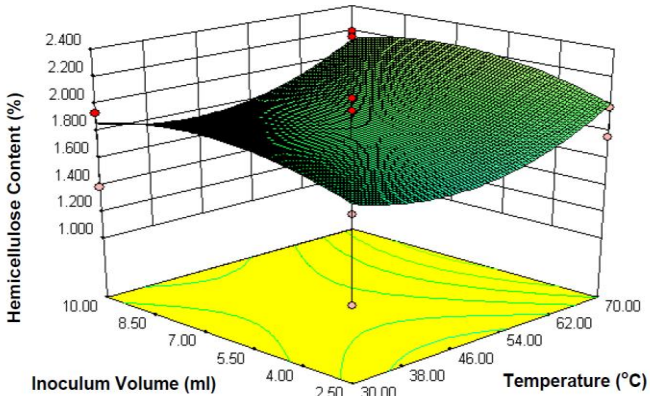

Figure 3. Influence of temperature and inoculum volume for hemicellulose content

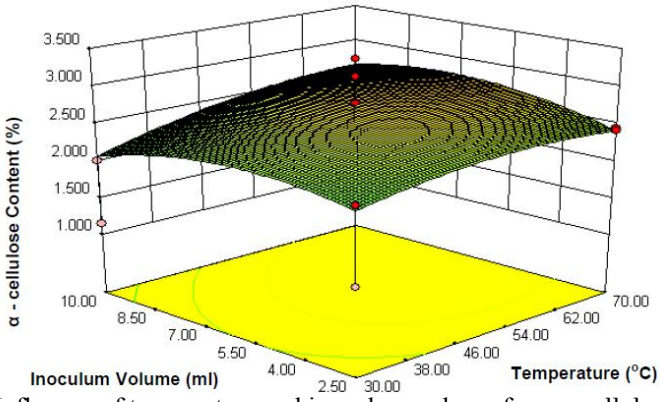

Figure 4. Influence of temperature and inoculum volume for α – cellulose content

Table 1. Low and high values on treatment

Treatment	Low Value	High Value
Mold Inoculum Volume (ml)	2,5	10
Temperature (^{o}C)	30	70

Table 2. Chemical compounds of corn stover

Characteristic	Component (%)
Water content	14,21
Extractive content	4,74
Lignin content	23,16
Holocellulose content	74,62
Hemicellulose content	33,83
α – Cellulose content	40,79

Table 3. p > F chance interaction of temperature and inoculum volume for lignin content

Source	p > F Chance	Description
x_1x_2	0,9804	Unreal
x_1^2	0,8370	Unreal
x_2^2	0,2640	Unreal

Table 4. p > F chance interaction of temperature and inoculum volume for hemicellulose content

Source	p > F Chance	Description
x_1x_2	0,9406	Unreal
x_1^2	0,0484	Unreal
x_2^2	0,1602	Unreal

Table 5. p > F chance interaction of temperature and inoculum volume for α – cellulose content

Source	p > F Chance	Description
x_1x_2	0,5460	Unreal
x_1^2	0,3763	Unreal
x_2^2	0,1392	Unreal

Table 6. Estimated the best conditions of biodelignifications process

Treatments	
Temperature (^oC)	Volume Inoculum (ml)
30	10

Table 7. Decrease of lignin, hemicellulose, and α – cellulose content

No	Component	Baseline Weight (gram)	Final Weight (gram)	Decrease Content (%)
1.	Lignin	1,893	1,671	11,73
2.	Hemicellulose	2,765	1,868	32,44
3.	α – cellulose	3,334	2,514	24,59

V. CONCLUSION

White rot fungi may serve as a good producer of extracellular enzymes including oxidative enzymes and polysaccharide-degrading enzymes. The result show that temperature and inoculum volume are not influence for lignin and α – cellulose content in biodelignification process. But, temperature is influence for hemicellulose content in biodelignification process, while inoculum volume is not influence.

From the equation, the nbest condition of corn stover biodelignification process are temperature 30oC and inoculum volume 10 ml for 7 days incubation. On this condition, produce decrease of lignin 11,73%, decrease of hemicellulose 32,44% and decrease of α – cellulose 24,59%.

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