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# Production of edible fungal (*Rhizopus delemar* CBS 145940*)* biomass from organosolv-pretreated oil palm empty fruit bunch (OPEFB) in submerged fermentation

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**Abstract.** Accumulation of oil palm empty fruit bunches (OPEFB) from palm oil industry poses challenges for the disposal process, which leads to environmental damage. For this reason, valorization of OPEFB fractions to produce edible fungal biomass was carried out in this research. The fungus was *Rhizopus delemar* CBS 145940, which is an edible fungus, Indonesian indigenous, and is favorable for the production of several end products. Organosolv pretreatment was first conducted on OPEFB using ethanol (50%) as the solvent. Enzymatic hydrolysis was then performed using Cellic® Ctec3 on the pretreated-OPEFB fractions. Hydrolyzates from cellulose-rich fraction, slurry (a mixture of cellulose-rich fraction and hemicellulose-rich fraction), and hemicellulose-rich fraction were used as the cultivation media for fungal growth. The corresponding yield of fungal biomass from each medium was 0.62 ± 0.07 g/g glucose; 0.41 ± 0.02; and 0.61 ± 0.13 g/g fermentable sugars, respectively. These results showed that *Rhizopus delemar* CBS 145940 could be grown in all the hydrolyzates from the OPEFB fractions. Nevertheless, in order to obtain higher fungal biomass, supplementation of nutrition was needed.

## 1. Introduction

Oil palm empty fruit bunch (OPEFB) is one of solid wastes from palm oil industry that reached 37 million tons in 2017 in Indonesia [1]. Some of OPEFB from palm oil industry is used for soil fertilizer and fuel for boiler, while some remains as waste to be disposed of or burned. Improper handling of OPEFB such as open dumping and burning cause environmental and sustainability problems such as extensive land requirements and air pollution. When in fact OPEFB is a promising biomass as it contains 59.7% cellulose and 22.1% hemicellulose [2]. Therefore, environmentally friendly and economically viable bioprocess to convert OPEFB to valuable products is of necessary.

As OPEFB is a lignocellulosic material, its bioconversion can be accomplished by three main steps. The first step is pretreatment that has a purpose to enhance the digestibility of cellulose and

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hemicellulose by separating them from lignin. The second step is hydrolysis in order to decompose cellulose and hemicellulose fractions into fermentable sugars. The third step is fermentation to convert the fermentable sugars into various valuable products [3]. In this work, OPEFB that has been pretreated using organosolv pretreatment was hydrolyzed to produce fermentable sugars to be used as a medium for growing edible fungal biomass. Edible fungal cultivation is a promising process that can be done to utilize sugars since it is environmentally friendly and economically feasible due to the minimum need of chemicals, its valuable products, and easy purification of the ultimate product.

Zygomycetous fungus (*Rhizopus delemar* CBS 145940) was used in this work. It is a GRAS (Generally Recognized as Safe) microorganism and is known as a rapid-growing fungus that requires relatively simple nutrients for growth. The biomass of *Rhizopus delemar* has gained interest in chitosan production, single cell protein, and fish meal production due to the amount of amino acids, lipids, protein, and chitin. Furthermore, this fungus is Indonesian indigenous as it is isolated from Indonesian traditional food, tempeh [4; 5].

Hence, the main purpose of this work was to produce edible fungal biomass using *Rhizopus delemar* CBS 145940 in sugar media derived from OPEFB. Specifically, the feasibility or the potency of the fractions from OPEFB after being pretreated and hydrolyzed to be used as a medium for fungal growth was evaluated.

## 2. Material and methods

### *2.1. Material*

*2.1.1. Oil palm empty fruit bunch.* Oil palm empty fruit bunch was obtained from a palm oil industry in Medan, Indonesia. OPEFB was sun-dried to achieve 7% of moisture content. Dried OPEFB was then milled to particle size distribution of 44.24% of >500 μm; 17.96% of 250-500 μm; 23.81% of 100-250 μm; and 13.99% of 63-125 μm.

*2.1.2. The fungus.* The fungus was *Rhizopus delemar* CBS 145940, originally isolated from tempeh inoculum usar in Indonesia and registered in Centraalbureau voor Schimmelcultures, the Netherlands. The fungus was maintained on a potato dextrose agar (PDA) medium containing (in g/L) 20 glucose, 15 agar, and 4 potato infusion. The cultures were aerobically grown at 30 °C for three days, stored at 4 °C, and renewed every month. For the cultivation, spore suspension was obtained by adding 20 mL of sterile milli-Q on the plates using disposable plastic spreader resulting in $5.6 \times 10^8$ spores/L.

### *2.2. Methods*

*2.2.1. Organosolv pretreatment of OPEFB.* Organosolv pretreatment was done using ethanol 50% for 90 minutes at 210ºC. Sulphuric acid of 0.07% was added as a catalyst until a pH 3 was obtained. The solid to liquid ratio was 1:10. Cellulose-rich fraction was obtained through sieving and washed with pretreatment solvent (ethanol 50%). The black liquor was diluted with water to precipitate lignin, which was recovered by centrifugation. The remaining liquid, *i.e*. hemicellulose-rich fraction was evaporated using a rotary evaporator (LABO ROTA 20, Heidolph, Germany) at 110 °C, 40 rpm, and a vacuum pressure of 100 mPa [6].

*2.2.2. Enzymatic hydrolysis.* The cellulase enzyme used was Cellic® Ctec3 (Novozymes, Denmark) with the enzyme activity of 222 FPU/mL. Cellic® Ctec3 enzyme solution was prepared by diluting the enzyme solution 10 times and filtered it with sterilized disposable disc filter with a pore size of 0.2 micron. Diluted enzyme solution was added into the substrate based on the enzyme activity and substrate content. Enzymatic hydrolysis was done in a water bath shaker at 50 °C at 125 rpm for 18-24 hours. The percentage of hydrolysis was calculated using the following formula:

$$\textbf{Percentage} = \frac{glucose\ concentration\ after\ hydrolysis\ (\frac{g}{L})}{maximum\ concentration\ of\ theoritical\ glucose\ concentration\ (15.10)\ (\frac{g}{L})} \times 100\% \quad (1)$$

*2.2.3. Fungal cultivation.* Fungal cultivations were performed in 250 mL cotton-plugged Erlenmeyer flasks containing 50 mL medium with initial pH of 5.5. Spore solution of 20 ml/L was added in each Erlenmeyer flask. Cultivations were carried out in a water bath shaker at 35 °C, 125 rpm for 72 hours under aerobic condition. The media of fungal cultivation were hydrolyzates from the enzymatic hydrolysis of the OPEFB fractions, *i.e.*:

1. Cellulose-rich fraction with glucan content of 13.72 g/L with and without supplementation of nutrition.
2. Slurry (a mixture of cellulose-and hemicellulose-rich fractions) with glucan content of 13.72 g/L and soluble solid of 2.08 g/L with and without supplementation of nutrition.
3. Hemicellulose-rich fraction with 2.08 g/L soluble solid with and without supplementation of nutrition.

Nutritional supplementation for the medium was 5 g/L yeast extract; 10 ml/L trace metals solution; 1 ml/L vitamin solution; 2.25 g/L $MgSO_4.7H_2O$; 3.5 g/L $KH_2PO_4$; 1 g/L $CaCl_2.H_2O$; and 9.656 g/L $NaNO_3$ [7]. Fungal biomass was harvested using a sieve and washed three times with distilled water until clear effluent was obtained. The volume of the final medium was measured using measuring cylinder. For further analysis, the biomass was dried overnight in an oven at 70 °C.

*2.2.4. Analytical methods.* The intial sugars in OPEFB fractions were determined according to the standard method developed by the National Renewable Energy Laboratory (NREL, Denver, Colorado, USA) [8]. Glucose concentration was analyzed using a high performance liquid chromatography (HPLC; Waters 2695, Waters, Milford, USA) equipped with a hydrogen-based column (Aminex HPX-87H, Bio-Rad) operating at 60 °C and 0.6 mL/min of 5 mM $H_2SO_4$ as the eluent using a refractive index (RI) detector (Waters 2414).

The dry weight of fungal biomass was determined by weighting overnight dried biomass using KERN&SOHN GmbH (Germany) scientific balance. All experiments were carried out in duplicate. All intervals and error bars were reported using two standard deviations.

## 3. Result and discussion

Organosolv pretreatment was performed to fractionate OPEFB into cellulose-rich fraction, hemicellulose-rich fraction, as well as lignin-rich fraction. As lignin does not contain any sugars, only the fractions of cellulose and hemicellulose, which were enzymatically hydrolyzed. The fungus was then cultivated in the hydrolyzate media from cellulose-rich fraction, slurry (a mixture of cellulose-and hemicellulose-rich fractions), and hemicellulose-rich fraction. Supplementation of minerals, yeast extract, trace metal solution as well as vitamin solution was conducted in order to know the adequacy of nutrition in the OPEFB-derived media.

### *3.1. Determination of enzyme activity in enzymatic hydrolysis of pretreated OPEFB*

In order to determine the appropriate enzyme activity, which was needed for the enzymatic hydrolysis process, several enzyme activities were evaluated in the enzymatic hydrolysis. In this specific experiment, the substrate used was slurry, which was a mixture of cellulose-rich fraction and hemicellulose-rich fraction.

The results, presented in Figure 1, showed that the percentage of hydrolysis reached a plateau after 18 hours. Using enzyme activities of 10, 15, 20, and 30 FPU/g, the hydrolysis resulted in 36.29 ± 2.84; 55.45 ± 3.72; 82.66 ± 3.09; 94.06 ± 4.71% of hydrolysis, respectively. It can be seen from Figure 1, when the value of enzyme activity increased, the percentage of hydrolysis also showed an increase. However, because the increase in the percentage of hydrolysis from enzyme activity of 20 and 30 FPU/g was small, the enzyme activity was not increased further. Based on these results, 18 hours and enzyme activity of 30 FPU/g were selected as the conditions for the enzymatic hydrolysis of cellulose-rich

fraction, slurry (a mixture of cellulose-rich fraction and hemicellulose-rich fraction), and hemicellulose-rich fraction.

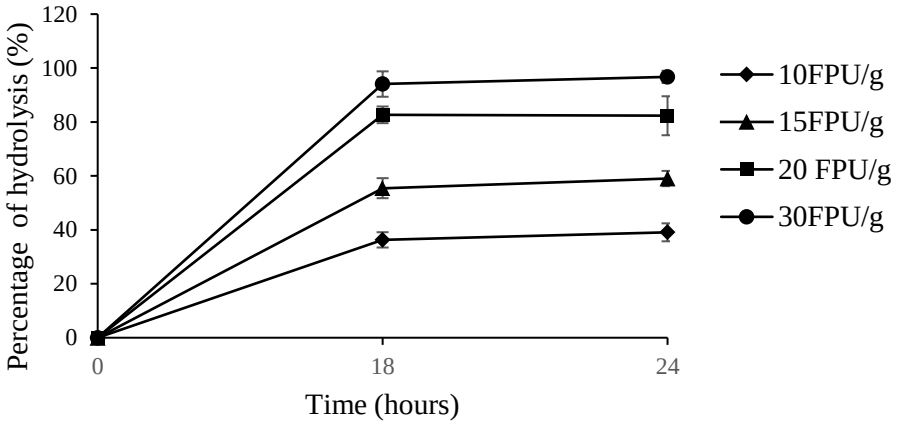


**Figure 1.** Enzymatic hydrolysis with 4 different enzyme activities using slurry as substrate.

When the enzyme activity of 20 FPU/g glucan was used, the percentage of hydrolysis in this study was 82% (Figure 1). This value was higher than the result by Kim *et al*. [9] who carried out enzymatic hydrolysis of OPEFB after hydrothermal pretreatment using the same value of enzyme activity. The higher result of enzymatic hydrolysis in this study may have been caused by the organosolv pretreatment method used. Organosolv pretreatment using ethanol can hydrolyze lignin bonds and lignin-carbohydrate bonds, and therefore lignin and hemicellulose can be disassembled from the lignocellulosic structure [10]. This will result in a higher proportion of cellulose and can contribute to facilitating enzyme accessibility.

### *3.2. Fungal cultivation*

*3.2.1. In hydrolyzate of cellulose-rich fraction.* Figure 2 presents glucose consumption by the fungus in the medium with and without supplementation of nutrition. It is shown that glucose could be consumed completely by the fungus in the medium with supplementation of nutrition. Whereas, glucose was slightly consumed in the medium without supplementation of nutrition with glucose concentration of 10.83 ± 0.19 g/L after 72 hours of cultivation. Accordingly, the biomass yield from fungal cultivation in the medium with supplementation was much higher compared to that of without supplementation (Figure 3). The fungal biomass yields from media with and without supplementation of nutrition were 0.62 ± 0.07 and 0.14 ± 0.01 g/g glucose, respectively.

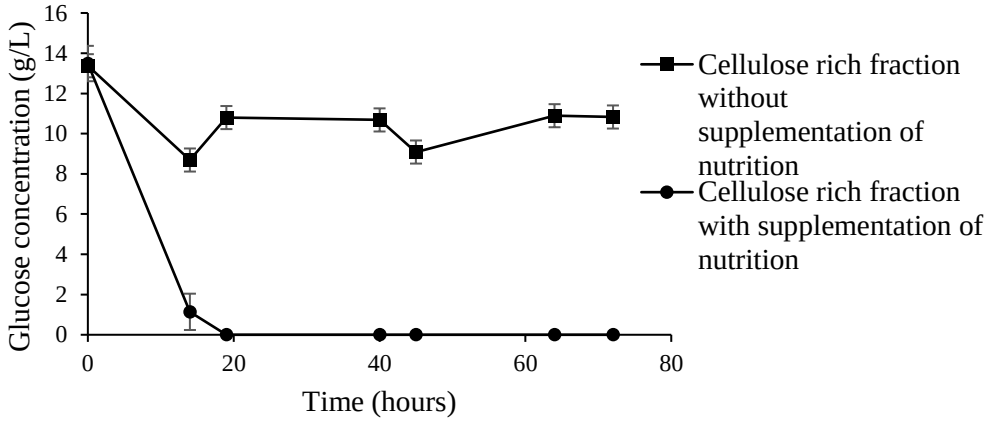


**Figure 2.** Glucose consumption by *Rhizopus delemar* CBS 145940 in submerged fermentation in the medium derived from cellulose-rich fraction.

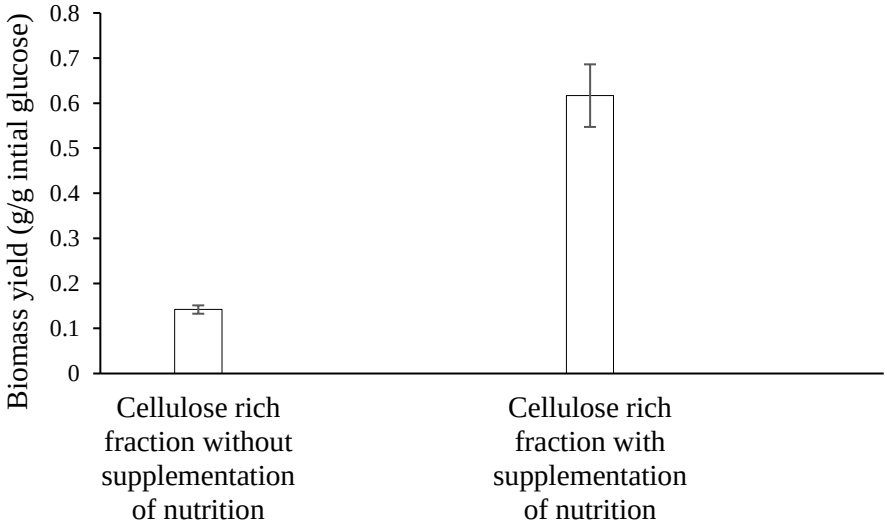


**Figure 3.** Biomass yield from fungal cultivation using the medium derived from cellulose-rich fraction.

Cellulose-rich fraction had a high glucose concentration with the absence of other hexoses and pentoses. This provided a relatively sufficient carbon source for the fungus to grow without having high osmotic pressure. In addition, small amounts of total protein were detected in OPEFB. Therefore, as cellulose-rich fraction is the pulp fraction of OPEFB, this fraction may have contained protein, which could have been consumed and used as the nitrogen source by the fungus.

*3.2.2. In hydrolyzate of hemicellulose-rich fraction.* Glucose consumption by *Rhizopus delemar* CBS 145940 in the medium derived from hemicellulose-rich fraction with and without supplementation of nutrition is presented in Figure 4. Glucose was totally consumed within 14 hours in the medium with supplementation, whereas, total glucose consumption was achieved 64 hours in the medium without supplementation.

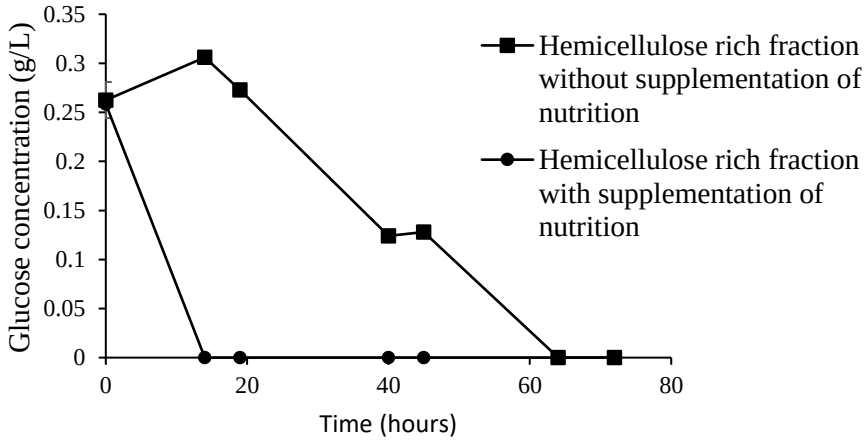


**Figure 4.** Glucose consumption by *Rhizopus delemar* CBS 145940 in submerged fermentation in the medium derived from hemicellulose-rich fraction.

Fungal biomass yields are shown in Figure 5. Fungal cultivation in the medium without supplementation of nutrition did not produce any fungal biomass during the time course of fermentation, whereas fungal cultivation in the medium with nutritional supplementation resulted in 0.61 ± 0.13 g/g fermentable sugars. No fungal biomass obtained from hemicellulose-rich fraction without nutritional supplementation was possibly caused by the low glucose concentration in the medium. The low concentration of glucose in hemicellulose-rich fraction and the absence of nutrition could have been insufficient for the fungus to grow and to produce biomass.

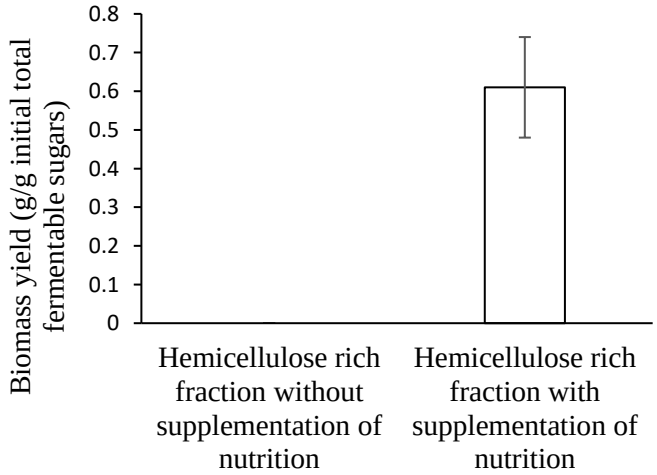


**Figure 5.** Biomass yield from fungal cultivation using the medium derived from hemicellulose-rich fraction.

Hemicellulose is a class of sugar polymer, which contains hexoses such as mannose, galactose, glucose and pentoses such as xylose and arabinose [11]. It was reported that hexoses (mannose, galactose, and glucose) and pentoses (xylose and arabinose) could be consumed by a zygomycetous fungus, *Mucor indicus* to produce fungal biomass [7]. However, it was only glucose consumption, which

was analyzed in this work. Therefore, the biomass yield was then calculated based on the total initial fermentable sugars, and not just glucose.

*3.2.3. In slurry (mixed hydrolyzate of hemicellulose-and cellulose-rich fractions).* Glucose consumption during cultivation in this medium is depicted in Figure 6. The fungus needed 40 hours to totally consume glucose in the medium with supplementation of nutrition. Whereas, glucose concentration was only reduced to 12.55 g/L, and remained constant after 14 hours of cultivation in the medium without supplementation.

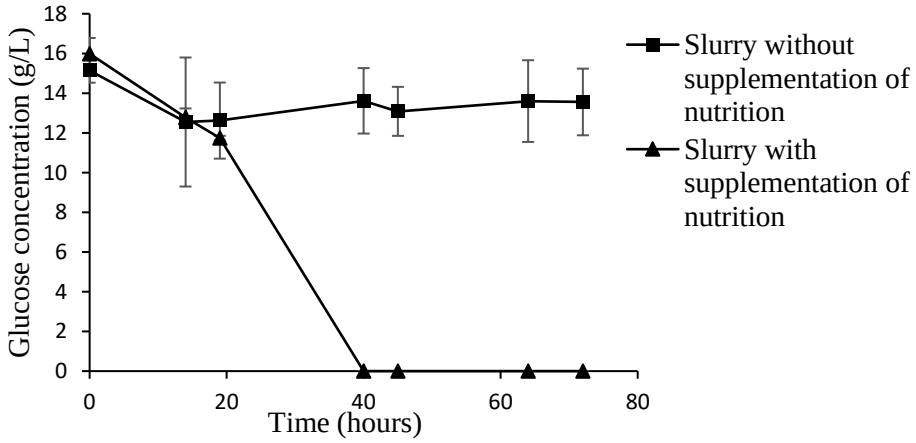


**Figure 6.** Glucose consumption by *Rhizopus delemar* CBS 145940 in submerged fermentation in slurry medium**.**

The results shown in Figure 6 indicate that nutritional supplementation was needed by the fungus to grow and produce biomass. The fungal biomass yields in the medium with supplementation was 0.41 ± 0.02 g/g fermentable sugars and it was only 0.02 ± 0.02 g/g fermentable sugars in the medium without nutritional supplementation (Figure 7). Of the three media derived from the pretreated-OPEFB fractions, in particular the media with nutritional supplementation, the biomass yield obtained from this slurry medium was the lowest. This fact is clearly supported with the results of glucose consumption. Glucose consumption rate by the fungus in slurry medium was slower compared to that of in the cellulose-rich fraction. It took 40 hours for the fungus to completely consumed glucose, whereas, it took only 19 hours in the medium of cellulose-rich fraction. The delay of glucose consumption in slurry medium was possibly caused by its composition, which was a mixture of cellulose and hemicellulose-rich fraction. It was reported that with all hexoses present in a medium, the total consumption of sugars was delayed for more than 24 hours because of the higher osmotic pressure due to a higher sugar content [7].

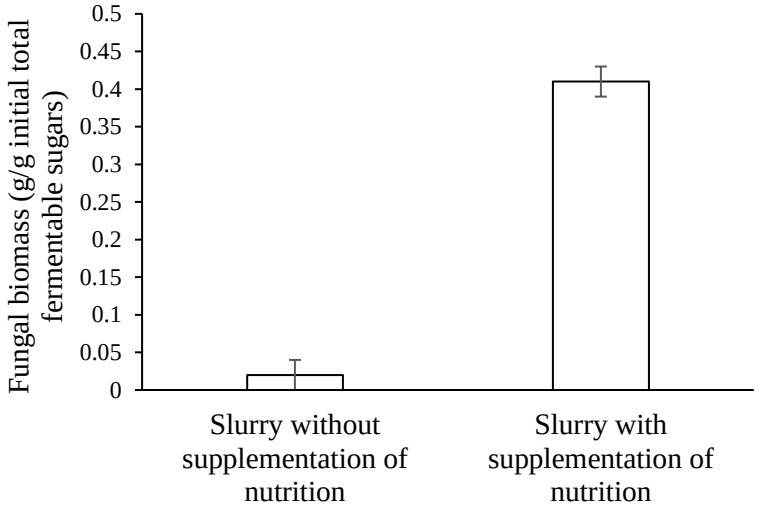


**Figure 7.** Biomass yield from fungal cultivation in slurry medium.

The results of fungal submerged cultivation in the media derived from the fractions of organosolv-pretreated OPEFB showed that supplementation of nutrition was essential to obtain high yield of fungal biomass. The biomass yields from all the media with supplementation of nutrition were much higher compared to the ones from the media without supplementation. Supplementation of media provided some inorganic salts and nitrogen needed for the fungus to grow. Nitrogen is required to synthesize amino acids, purines, pyrimidines, enzyme cofactors, and other substances [12]. The effectiveness of the medium with nutritional supplementation was also due to the addition of trace metals and yeast extract, which increased the rate of fungal growth and biomass yield [7].

## 4. Conclusion

Hydrolyzates of cellulose and hemicellulose fractions of OPEFB could be used as media to grow *Rhizopus delemar*. In all fractions, supplementation of nutrition was essential to obtain higher fungal biomass by providing minerals and yeast extract. The biomass yield from fungal cultivation in the hydrolyzate of cellulose-rich fraction was the highest. The cellulose-rich fraction had a high glucose concentration in the absence of other sugars. In the slurry medium, which contained high concentration of glucose, the fungal biomass yield was lower. Since hemicellulose could contain other types of sugars except glucose, there may have been osmotic pressure that inhibited fungal growth during cultivation.

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