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Performance of β -glucosidase produced by *Ganoderma Lucidum* using waste substrate as carbon source

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ABSTRACT

Oil palm empty fruit bunch (OPEFB) was used for the production of β -glucosidase by *Ganoderma lucidum* in submerged fermentation. The activity of β -glucosidase had shown an improvement by going from pH 4 to 6 and declined towards alkaline pH. The highest activity (47.75 U/mL) was obtained at pH 6 with 2.0 mm size of substrate's particle. The smaller the particles size, the higher surface area of substrate which provides more space for hyphae to colonize. Optimum substrate concentration was obtained at 6.0 g/L (43.99 U/mL). The highest β -glucosidase activity occurred at 27 °C (53.37 U/mL). The nutrient content in composted OPEFB provides a relevant reason that should be considered in the performance of high activity β -glucosidase enzyme. Therefore, the results of this study can be used to produce high activity β -glucosidase using cheap and economical substrate (compost OPEFB).

INTRODUCTION

Cellulose is a microfibrils structure which strengthens the frame of cell wall. It is generally known that cellulose can be converted enzymatically into glucose by cellulase such as β -glucosidase. Cellulase has a great potential in industrial sector which can be used in textile, paper and poultry industry. In future, β -glucosidase is believed to be one of the most potential enzymes that lead to the production of bioethanol [4]. The enzymatic breakdown of substrates by β -glucosidase brings the most promising technology for the conversion of lignocellulosic waste biomass. Oil palm empty fruit bunch (OPEFB) is an inexpensive waste of lignocellulosic biomass from oil palm processing [18,21]. More than fifteen million tons of OPEFB was generated by the oil palm mills in Malaysia [33]. One of the alternative ways to reduce the biomass in the oil palm mills is to decompose the OPEFB by microbial composting [4]. Throughout the composting process, the material undergoes the process of mineralization and humification of organic matter. Composting is safe to be used in agriculture especially in fertilization process. However, low value end product is produced at this level [5]. Based on our present knowledge, very few studies were made to utilize this compost OPEFB. The use of compost OPEFB can reduce the cost for enzyme production because it is naturally produced organically.

It was generally known that white root fungal of *Basidiomycetes* has good ability to grow on lignocellulosic substrate using hydrolytic enzymes. Wood decaying fungi, *Ganoderma lucidum* (polyporaceae or ganodermaceae of an ascomycota) [2,43] was employed in this study due to its rapid mycelia growth once it enters the substrate cell wall. The mycelium yield and enzyme production was depending on the

substrate and condition using submerged fermentation [44]. Previously, a few reports were found mentioning about β -glucosidase activity using soybean (0.15 U/mL), and soy meal (4.74 U/mL). Both substrates were utilized by *Ganoderma lucidum*. However, all activity that has been reported was quite low and the substrate used was costly. Therefore, a new study was conducted to increase the performance of β -glucosidase using more readily and available waste substrate especially to fulfill the interest of Malaysian industry.

In the present study, the performance of β -glucosidase using compost OPEFB (without addition any macro nutrient), which was optimized by type of substrate, substrate concentration, size of substrate particle, temperature, and rotation per minute (rpm) had been investigated. In addition, a literature comparison was performed in this study. With regard to efficiency of the overall process, this study focused on biological compost OPEFB by its environmental friendly aspect. Therefore this study was conducted to investigate the possibility of using compost oil palm empty fruit bunch (OPEFB) for β -glucosidase production by *Ganoderma lucidum* as one of the cheapest and economical sources for enzyme production and also to study the environmental factors affecting the production of β -glucosidase produced by *Ganoderma lucidum*.

MATERIALS AND METHODOLOGY

Organism

Ganoderma lucidum was maintained in slant agar of potato dextrose agar medium (PDA) supplement with 1% *Penicillin streptomycin* antibiotics [18].

Cultivation Medium

Ganoderma lucidum has grown in the PDA medium in a petri-dish, and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized house-developed cutter. The seed cultures grown in a 500 mL flask containing 200 mL of the basal medium (g/L) consist of peptone 5.0, yeast extract 5.0, KH_2PO_4 1.0, K_2HPO_4 1.0, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, and glucose 40.0 [27]. For the first preculture, 200 mL medium with initial medium pH 6.0 was prepared in a 500 mL flask, and then 10 colony of mycelium suspension from petri-dish was inoculated, and followed by 14 day incubation at 27 °C on a rotary shaker (100 rpm) [3].

Fermentation Medium Composition

The medium for fermentation consisted of the following component (g/L); glucose (40.0), peptone (5.0), yeast extract (5.0), KH_2PO_4 (1.0), K_2HPO_4 (1.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), diluted in 1 L distilled water [27]. Composted OPEFB was used as the carbon source instead of glucose for the production of β -glucosidase. The fermentation was incubated for 30 days, and the sampling took place every 5 days. All fermentation experiments were performed at least in triplicate [43].

Fermentation Conditions

Effects of Different Carbon Source

Different carbon source was used to test its effects on β -glucosidase production; compost OPEFB, raw OPEFB, treated OPEFB (chemically), and commercial cellulose. *Ganoderma lucidum* were inoculated and incubated for 30 days at 27 °C. Samples were collected at every 5 days for 30 days, centrifuge at 10,000 rpm for 10 minutes and kept at 4 °C [43].

Effects of pH

Erlenmeyer flask (250 mL) containing 100 mL of modified fermentation media [27] containing 10 g/L compost OPEFB as a substrate were prepared and the pH were adjusted to 4.0, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0 in different flask using pH meter with 1 N HCl and 1 N NaOH. After the pH was adjusted each flask has been autoclaved at 121 °C for 15 minutes. *Ganoderma lucidum* were inoculated and incubated for 30 days at 27 °C. Samples were collected at every 5 days for 30 days, centrifuge at 10,000 rpm for 10 minutes and kept at 4 °C [43].

Effects of Substrate Particle Size

Compost oil palm empty fruit bunch (OPEFB) appears in needle-like strand. The long strands (OPEFB) were ground at different particle size; 0.5 mm, 1.0 mm, 2.0 mm and 5.0 mm using grinder machine (Hsiangtai, Taiwan). Different particle sizes were placed in different 250 mL Erlenmeyer flask containing 100 mL fermentation and 10 g/L of compost OPEFB at different particle size. *Ganoderma lucidum* were inoculated and incubated for 30 days at 27 °C. Samples were collected at every 5 days for 30 days, centrifuge at 10,000 rpm for 10 minutes and kept at 4 °C [43].

Effects of Agitation (Rotation per Minutes, rpm)

Rotation per minutes (rpm) delivers oxygen throughout the experiment to the culture (fungi). Different rpm results in different oxygen transfer and also the growth of the fungi. Each

fermentation cycle was tested with different rpm; 100 rpm and 180 rpm. Substrate used was compost OPEFB (10 g/L) in 100 mL fermentation medium [27]. *Ganoderma lucidum* were inoculated and incubated for 30 days at 27 °C. Samples were then collected at every 5 days for 30 days, centrifuged at 10,000 rpm for 10 minutes and kept at 4 °C [43].

Effects of Substrate Concentration

Each Erlenmeyer flask (250 mL) was added with different concentration of compost OPEFB in 100 mL of fermentation medium [27]. The substrate concentration was divided into 4.0 g/L, 6.0 g/L, 8.0 g/L and 10.0 g/L. Different flask was used for different substrate concentration and at least triplicate was done. *Ganoderma lucidum* were inoculated and incubated for 30 days at 27 °C. Samples were collected at every 5 days for 30 days, centrifuged at 10,000 rpm for 10 minutes and kept at 4 °C [43].

Effects of Temperature

Based on each parameter used, the last parameter was the effects of temperature. Parameter that obtains the highest β -glucosidase concentration was used to proceed to the next parameters. So, different temperature was used in different cycles of fermentation which are 25, 27, 30 and 35 °C. *Ganoderma lucidum* were inoculated and incubated for 30 days at 27 °C. Samples were collected at every 5 days for 30 days, centrifuged at 10,000 rpm for 10 minutes and kept at 4 °C [43].

β -glucosidase assay

The β -glucosidase was determined by the method as described by Wood and Bhat [42]. β -glucosidase from the fermented *Ganoderma lucidum* with composted OPEFB were extracted at every 5 days intervals, and mycelium was removed by centrifugation (10,000 rpm, 20 min, 4 °C). β -glucosidase activity was routinely assayed by using a 1 mL reaction mixture containing 5 mM p -nitrophenyl- β -D-glucoside (p NP-bG) (Sigma Chemical, St. Louis, MO, USA), 100 mM acetate buffer (pH 5.0) and an appropriate dilution of enzyme preparation. After 30 min of incubation at 50 °C, the reaction stopped by adding 2 mL of 1 M Na_2CO_3 , and the p -nitrophenol release was monitored at 400 nm. One unit of activity was the amount of enzymes that released 1 mmol of p -nitrophenol per minute under the assay condition. The p -nitrophenol released from p -nitrophenyl- β -D-glucoside has been measured using a spectrophotometer (Model Shimadzu, UV-1601 PC). One unit of β -glucosidase activity was defined as 1 mol of p -nitrophenol/min released [16]. Protein content has been determined by the Lowry method using BSA as a standard [45].

RESULTS AND DISCUSSION

Cultivation

In this study, fully grown inoculums was produced in 10 days followed by submerged fermentation in 30 days, unlike in most studies that took place from 3 to 5 month [34] for the *Ganoderma lucidum* to have a basidiocarp growth. *Ganoderma lucidum* agar medium was added with *Penicillin streptomycin* antibiotics to prevent the contamination by surrounding bacteria.

Effects of Carbon Source (from different treatment)

For preliminary study, different carbon source based on OPEFB and β -glucosidase activity has been shown in Figure 1. Compost OPEFB shows the highest activity at 2.91 U/mL followed by treated OPEFB, raw OPEFB and commercial cellulose (180 rpm at 30 °C). Compost OPEFB was reported consist of nutrients;

carbon, nitrogen, phosphorus, zinc, copper, and low amount of heavy metals [4]. It can provides proper condition for the mycelial growth and induce the production of β -glucosidase by *Ganoderma lucidum*. For treated OPEFB it was reported that the chemical treatment on the fiber surface enhanced its mechanical properties [29] and it is unfavourable to be used by *Ganoderma lucidum*. In raw OPEFB, the lignocellulosic linkage was difficult to be breakdown due to the absent of inducer to break it [29]. Baharuddin [4] reported that the structural condition of raw OPEFB viewed under electron microscope shows turgid strand of EFB compared with treated OPEFB. Commercial cellulose shows the lowest β -glucosidase activity due to its sole content of cellulose without accompanied with other mineral sources. Therefore, compost OPEFB was chosen to proceed with the next experiment.

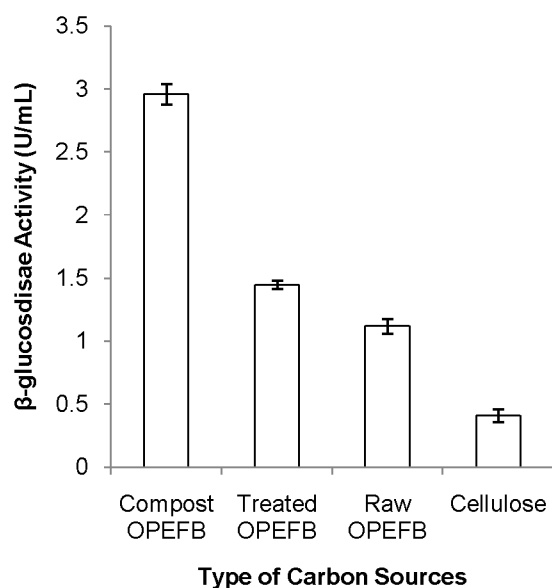


Figure 1: Effects of carbon source (from different treatment) on the production of β -glucosidase activity. Arrow bars indicate the standard deviation of triplicate data.

Effects of pH

Table 1 shows the activity of β -glucosidase obtained during biosynthesis process using *G. lucidum* in different initial pH medium. The activity increased from pH 4.0 to 6.0 and decreased after that as the biosynthesis of β -glucosidase had greatly influenced by pH 6.0, especially in acidic condition [3]. Similar pattern was also reported by a few researchers [15,31,36]. The highest activity, 47.75 U/mL was obtained at pH 6.0, which is in agreement with Tangnu et al [40]. Each microorganism possesses a specific pH range for its growth and activity. Filamentous fungi exhibit good growth over a broad range of pH, with an optimal range of 3.8-6.0 [16].

The pH below 5.0 and above 8.0 was not favorable for growth and sporulation of fungi; resulting a decrease in β -glucosidase production at extreme acidic and alkaline conditions. In contrary, cellulase production by *Clostridium acetobutylicum*, was found to be optimum at pH 9.0 [37]. The likely other reasons for the result was probably because of in composted-OPEFB, perhaps

cellulose, urea carbohydrates and their derivatives were presented in an adequate amount favorable for growth of *Ganoderma lucidum*. Cellobiose and urea was considered to have the ability to promote the production of β -glucosidases [13] as cellobiose became an active inducer in the synthesis of β -glucosidase by promoting the specific proliferation incultures [39]. Enzyme formation can be induced by various carbohydrates and their derivatives, including lactose, sophorose, xylobiose, D-xylose, and L-sorbose [20].

The presence of urea at the beginning of cultivation was believed to reduce the production of the metabolites or derivatives that in turn, halted the reduction in pH of fermentation media and promote the production of β -glucosidase [9]. The high activity obtained might due to more macro and microelement available in composted OPEFB as one of the important factors affecting biodegradation processes is the presence of metals in the substrate that favorable for the growth of *Ganoderma lucidum* and synthesis of enzyme. In addition to that, previous researchers reported high amount of zinc, manganese, copper and boron in the composted-OPEFB [21,41]. Generally, the biogenic metals related to copper, and manganese involve in ligninolysis. Zinc caused a significant increase in the production of two major cellulolytic enzymes (CMCase and β -glucosidase) as well as cadmium [6]. The activity of cellulose and hemicellulose-degrading enzymes were also related to the presence of Cu, Mn, and Pb. Copper and cadmium were also found to significantly increase laccase activity in liquid cultures of *P. ostreatus*, and manganese was responsible for MnP induction in liquid cultures of several white-rot fungi [38]. Currently, the range of technically applicable substrates is still limited since most of the carbon sources are too expensive for industrial fermentations; however, by using composted-OPEFB, this problem can be overcome. Based on the result obtained, the β -glucosidase can be regulated easily by controlling the initial medium pH. Thus, pH 6.0 is the best pH using composted-OPEFB for the synthesis of β -glucosidase by *Ganoderma lucidum*.

Effects of Substrate Particle Size

β -glucosidase activity produced from this experiment was considered high compared to other studies (>10.0 U/ml). This might due to high ability of *Ganoderma lucidum* to produce the hydrolytic enzymes when supplied with rich natural nutrient. It was suggested that different particle size could give huge impact on cellulase production during the growth of fungi [22]. In this study substrate 2.0 mm shows the highest activity of β -glucosidase (47.75 U/mL) compared to 0.5 mm (4.9 U/ml). Substrate with particle size, 0.5 mm shows a close β -glucosidase activity (4.9 U/ml) with a previous study (4.2 U/ml) [28]. However, with bigger particle size (2.0 mm) the activity increased 3.76 fold higher as compared to Md Shah et al. [28]. The differences were strain of fungi used and pretreatment of OPEFB.

It has been discussed that varies in particle size of substrates have resulted due to poor activity of β -glucosidase in the shake flask fermentation [22,30]. Most researchers claimed that the optimum fungal growth and cellulases production contributed by 0.4-0.6 mm sized particles [8,22,30].

Table 1: β -glucosidase activity comparison between different pH with different carbon source

Reference	[15]	This Study	[30]
Carbon Source-g, (Activity)	Soymeal-10g, (U/ml)	Compost OPEFB-8g, (U/ml)	Soybean extract- 40g (U/ml)
pH 4.0	-	0.56±0.06	0.08
pH 5.0	4.74	7.11±0.04	0.10
pH 6.0	6.96	47.75±1.07	0.15
pH 7.0	-	0.55±1.01	-
pH 8.0	-	0.51±0.91	-

Table 2: Effects of different substrate's particle sizes on the production β -glucosidase

	Particle size (mm)					
OPEFB	0.25-0.30	0.42-0.60	0.5	0.84-1.0	2.0	5.0-10.0
1	0.09	0.115	-	0.10	-	0.08
2	-	-	4.2	-	8.6	-
3	-	-	4.9±0.81	3.0±0.02	47.76±1.07	2.21 ±0.12

OPEFB 1- (chemical pretreated) *Botryosphaeria sp.*, Unit (U/g), reference [21]

OPEFB 2-(chemical pretreated), *Chaetomium globosum*, Unit (U/ml), reference [28]

OPEFB 3-(microbial composted), *Ganoderma lucidum*, Unit (U/ml), This study

Another factor that may also contribute to a significant impact on the production of β -glucosidase is substrate inter-porosity. Generally accepted that in a very small particle, the surface area for growth is big, however, the inter-particle porosity is less [8,22,30]. Meanwhile, in larger particle size, the porosity increase but the surface area becomes less. These two opposing factors, decrease in surface area and increase in porosity, probably interact to determine the values corresponding to optimum growth and enzyme production [32]. Based on the morphology of fungi with rigid hyphae network, and the cytoplasm that moves through the hyphae [23], a few hypotheses can be made for explanation. The ability of filamentous fungi to colonize and penetrate into the substrate can also be a good indicator in the synthesis of hydrolytic enzymes [17]. The hyphae networks have no particular pattern or structure, and the mechanisms related to this process has been an interest for over a century [23].

If the particle is too small (<0.4 mm) lumps will formed and the substrate function will become similar to a big particle with low surface area. The condition will become worse due to low inter-porosity available, which results in less hyphae growth. Therefore, fewer enzymes are synthesized [36]. With assumption that no shear stress involved in a small particle, high surface area of substrate provides more locations for hyphae colonization, as suggested that small particles can stimulate greater growth [32]. For a bigger particle, the high porosity structure available caused more penetration of hyphae into the inter-particle pores of the substrate [22]. Moreover, in this condition the hyphae that growth in the pores was exposed to a less shear stress from movement of the liquid mediums. The existence of special micro environment may provide a good static condition that close to the natural

habitat of *Ganoderma lucidum* for the growth of hyphae throughout the whole surface of substrate provided by being supplied with rich macronutrient, micronutrient and mineral from composted OPEFB.

When a larger particle substrate supplied in the fermentation media, a network of aerial hyphae grows into the inter-particle space (inter-particle pore) along with low fungal growth on the surface of the substrate particle and therefore, decreased the production of resulted enzymes [22]. Therefore, the physical properties of substrate, nutrient, strain of fungi and fermentation condition are very important for the efficient growth of hyphae, and they may provide a relevant variable that should be considered in the production of β -glucosidase enzyme. In this analysis, the substrate particle size of 2.0 mm was chosen for the production of β -glucosidase by *Ganoderma lucidum*.

Effects of Rotation per Minutes (rpm)

For *Ganoderma sp.* at rpm more than 100, lower β -glucosidase activity was observed (Table 3). For *Aspergillus sp.*, at 180 rpm, the more promising results can be obtained if compared to analysis at 200 rpm and if the type of substrate is not to be considered (with an assumption all other conditions was constant). Better activity can be obtained with lower rpm (180) that gave 51.15 fold higher than the activity for higher rpm (200). In this case, it seems that *Aspergillus sp.* need higher rpm compared to *Ganoderma sp.* It is because different fungi strains may have a different level of tolerance to the shear stress, and in the movement of liquid medium sufficient oxygen transfer rate can be provided for the hyphae growth.

Table 3: Effect of rotation per minute (rpm) to the production of β -Glucosidase from various strains

Strains	rpm	Temp. (°C)	Substrate (%)	Activity (U/ml)	Substrate type	Working volume	References
<i>Ganoderma</i>	100	27	10.0	47.75± 1.07	Compost OPEFB*	100 ml in 250 ml	This Study
<i>Ganoderma</i>	180	27	5.0	9.56± 0.19	Compost OPEFB*	100 ml in 250 ml	This Study
<i>Ganoderma</i>	130	25	3.0	0.84	Soybean extracts	-	[30]
<i>Aspergillus</i>	200	30	10.0	6.10	Treated OPEFB*	100 ml in 500 ml	[35]

*OPEFB, oil palm empty fruit bunch

The yield of the mycelium (*Ganoderma* sp.) increases when the shaking frequency increased from 50 to 100 rpm [43]. It is supposed that a higher rpm implies a better oxygen transfer in the fermenting medium. By implying some limiting factor such as limiting the amount of oxygen transfer to the culture, it would tend to produce the interest product more efficiently. In addition, they suggested the fact that the biomass yields which was lower above 100 rpm could attribute to a detrimental effect to increase shear stress on the mycelium. It was concluded that the *Ganoderma* sp. is an obligate aerobe, with the optimum rotating speed around 100 rpm [43]. With the combination of all these factors (particle size and rpm), it is possible for fungi to have an efficient colonization on the substrate and therefore, hydrolytic enzyme can be synthesized and higher activity of β -glucosidase can be obtained. The production of hydrolytic enzyme from external hyphae associated with colonized fungi root that requires efficient penetration mechanism by the external hyphae [14] as noted that the "hyphae have a range of senses." They can respond to substrate surfaces, physical environmental, gravity, and even electrical fields [23]. Even though more pores are available, however, in some cases poor enzyme production was also related to less surface area.

Effects of Substrate Concentration

The activity of β -glucosidase obtained using different weight of OPEFB by *Ganoderma lucidum* at particle size 2.0 mm is shown in Figure 2. Different weight of substrates gave different β -glucosidase activities with the optimum weight of 8 g (53.37 U/mL). Although 10.0 g/L of substrate were the highest, however, the result was only 47.75 U/mL. This is due to the optimum carbon source 10 g supplied to the fungus create convenient environment for the fungus to live. In the compost form of OPEFB, it may consist of excess nutrient supplied at log OPEFB regarding the growth and function of fungi as limiting factor to the production of β -glucosidase. Compost OPEFB consists of holocellulose (cellulose and hemicellulose) and lignin with a high potential to be the substrate for the production of high value added bioproducts such as sugar, ethanol, gas and others [4]. As for 4.0 and 6.0 g/L substrate, the amount of carbon sources supplied was not enough for the fungus to produce more β -glucosidase. Therefore, 8.0 g/L of substrate concentration of compost OPEFB is the best concentration for the production of β -glucosidase enzyme.

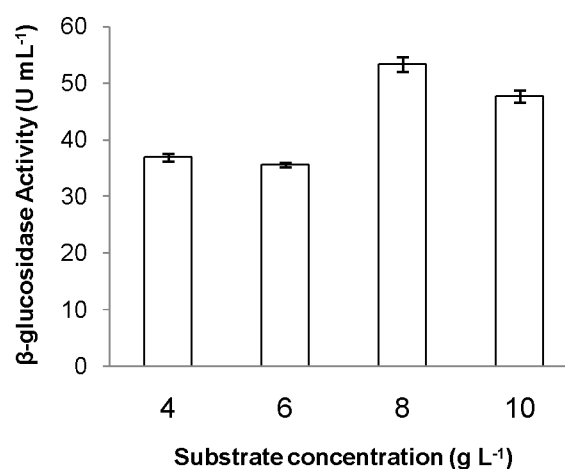


Figure 2: Effect of different substrate concentration (OPEFB) g/L the production of β -glucosidase by *Ganoderma lucidum*. The distinct letter shows significant differences within each substrate concentration of OPEFB ($p < 0.05$). Arrow bars indicate the standard deviation of triplicate data.

Effects of Temperature

The synthesis of β -glucosidase by *G. lucidum* in different temperatures was shown in Figure 3. The enzyme production can be significantly affected by temperature. From 27 to 35 °C, it was shown that the higher the temperature the lower the ability of *G. lucidum* in synthesizing the β -glucosidase. *G. lucidum* is basidiomycetes, a wood-rotting fungi which was originated from temperate country and classified under mesothermic microorganism that exhibits unique relationship with the temperature [34] and grows around 25 to 40 °C [10]. Other researchers gave a lower optimum temperature for basidiomycetes at region 25 to 30 °C [26]. At high temperatures, the activity was going down due to damaged mycelia cell. It was reported that high temperature can give lethal effect to the fungi due to major disrupt of cell wall and the membrane cuticle waxes [37].

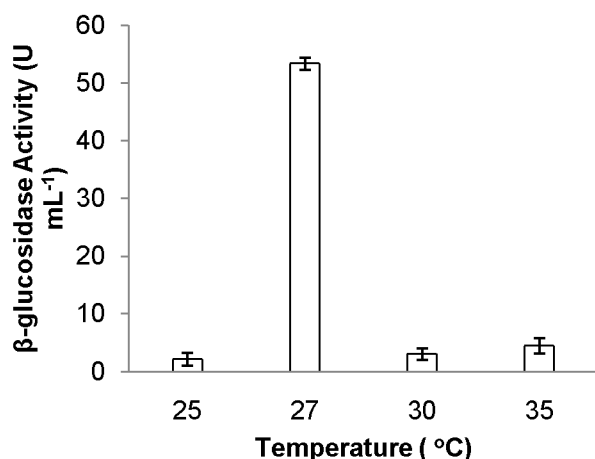


Figure 3: Effect of various temperatures on the synthesis of β -glucosidase by *Ganoderma lucidum*. The distinct letter shows significant differences within each temperature ($p < 0.05$). Arrow bars indicate the standard deviation of triplicate data.

CONCLUSION

There are no specific reports on cellulase production from compost OPEFB. In this study, we developed a method for utilizing compost OPEFB as a sole carbon source for producing β -glucosidase from *Ganoderma lucidum*, without any addition of extra nutrient, chemical pretreatment of OPEFB, or addition of supplements. OPEFB was first compost and then used as the carbon source for a *Ganoderma lucidum* culture. This compost OPEFB improved significantly in the production of β -glucosidase, which was higher in the cellulase activity level obtained compared to other literature review. Perhaps, agricultural waste utilized in this study is naturally rich in carbon content and other vital nutrients essential for fungal growth [19] produced by microflora during composting process. By using this process, a significant increase of enzyme activity has been produced, and therefore increases the economical product yields or decrease the amount of required enzyme needed to reach the level of conversion given. From the economic viewpoint, compost OPEFB may be superior as compared to other carbon sources. Compost OPEFB can be continuously carried out under mild conditions and only requires POME as the reaction matrix; thus, it is a relatively eco-friendly treatment. These results suggested that OPEFB may be superior to other commercial substrate as a carbon source for the production of cellulases by *Ganoderma lucidum*. The development of this technology provides potential consolidated bio-processing means at lower cost for β -glucosidase production from industrial by-product of OPEFB. Furthermore, the high amount of β -glucosidase enzyme will lower the hydrolysis process of OPEFB. Therefore, lower cost will be needed eventually without needing to produce high amount of FPase and also CMCase enzyme. It can be concluded that compost OPEFB (using POME) proved to be an excellent source for the β -glucosidase enzymes production for better usage and also more economical.

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