Utilization of Lignocellulosic Wastes as a Carbon Source for the Production of Bacterial Cellulases under Solid State Fermentation

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***Abstract*—Palm kernel cake (PKC) and vegetable wastes were used as a fermentation substrate for the evaluation of cellulase activity secreted by Bacillus sp. In the current work, PKC and vegetable wastes were used as substrates in order to reduce the cost of cellulase production. The aim of this study was to determine the cellulase activity by Bacillus sp. on lignocellulosic materials mainly on different sizes of PKC and vegetable wastes. Besides that, pH, temperature, and inocula concentrations will also being tested for the optimum reaction of Bacillus sp. on the substrates. The PKC that were used can be divided into two types which are raw PKC and defatted PKC that had undergone Soxhlet oil removal. The results shown raw PKC, defatted PKC and vegetable wastes substrates have highest cellulase activity at 2.65 FPU/ml, 7.73 FPU/ml and 85.48 FPU/ml respectively. The optimum pH and incubation temperature for cellulase activity of Bacillus sp. was pH 4 and 50°C respectively. Bacillus concentrations of 2 x 105 cells/ml and 2 x 106 cells/ml, inoculated on PKCs and vegetable wastes produced the highest bacterial cellulases respectively. PKC with the particle size of 500µm was the most effective in producing higher cellulase activity likewise for larger particle size, of 1mm for vegetable wastes. From this study, Bacillus sp. holds the potential of converting lignocellulosic materials into products of commercial and industrial values such as glucose and other biofuels.**

***Index Terms*—Biofield, cellulase activity, palm kernel cake, vegetable wastes, solid state fermentation.**

I. Introduction

Commercial cellulases are mostly generated by fungi and there have been a plenty work conducted on this. Conversely, there are limited studies on cellulase production from bacteria [1]-[4] Cellulase has been widely used in the industries mainly in textile or pulp and paper industry. In most industries, cellulase contribute to the almost half of the total production costs. The application of biomass lignocelluloses in cellulase production has received attention nowadays as an alternative for the cellulase productions in exchange to the usage of purified cellulosic substrates such as Avicel and Solka Floc or soluble inducers such as lactose and carbomethyl cellulose [5]. Several cellulase production using lignocelluloses as a substrate such as banana fruit stalk, sugarcane bagasse, wheat straw, oil palm empty fruit bunch (OPEFB) and many more [6]-[9]. In this study, we focus on the effects of incubation temperature, pH, substrates particle size and inocula concentrations on cellulase production form *Bacillus* sp. using raw and defatted palm kernel cake and vegetable wastes as the carbon sources.

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II. Materials and Method

A. Production of *Bacillus* sp.

The microbes (*Bacillus* sp.) from isolate B1 are grown on slant nutrient agar for 24 hours. In the first seeding process, the *Bacillus* sp. was inoculated onto the nutrient broth media consisted of 1.3% nutrient broth. The cultures were incubated for 5 days at room temperature, and pH was set to be between 5.0 to 7.5. The second seeding process was carried out by using the non-conventional process (involving aeration). A working stock was prepared comprising of 1.5% (w/v) glucose, 0.15% (w/v) ammonium sulfate, 0.04% (w/v) potassium phosphate, 0.05% (w/v) yeast extract, 0.1% (v/v) *Bacillus* while the remaining purified water. The second seeding process undergoes a fermentation process that was completed in 16 to 22 hours. The working stock was adjusted to pH range of 5.0 to 7.5 using 1N NaOH according to the method outlined by Choi and Reynard [10]. The *Bacillus* sp. cells concentration was determined using a haemocytometer.

B. Sampling and Preparation of Substrates (Palm Kernel Cake and Vegetables Waste)

Freshly produced PKC was obtained from a local palm kernel mill. The fresh samples were divided into two portions. A portion was immediately stored at 4°C for later used and labeled as raw PKC. Another portion was defatted using soxhlet technique with hexane as solvent and extraction was carried out for 8 hour as done by Yan et al. [11]. The residual hexane was then removed and the defatted PKC was stored at 4°C until ready for use. Meanwhile, the vegetable waste was taken from the local market located at Bayan Baru, Pulau Pinang. The vegetable was dried inside the oven at 60°C for 24 hours. Both the PKC and the dried vegetables were grinded and the substrates were screened in a sieve shaker to obtain three mesh sizes (1) 250µm, (2) 500µm and (3) 1mm.

C. Solid State Fermentation

Solid state fermentation of *Bacillus* sp. was performed with five grams ground raw PKC as the solid substrate with the addition of two ml of Mandels medium [12]. The Mandel's medium was prepared with the following composition (g/l): (1) urea, 0.3; (2) peptone, 0.75; (3) yeast extract, 0.25; (4) $(NH_4)_2SO_4$, 1.4; (5) KH_2PO_4, 2.0; (6) $CaCl_2$, 0.3; (7) $MgSO_4.7H_2O$, 0.3, and trace elements (mg/l): (1) $FeSO_4.7H_2O$, 5; (2) $MnSO_4.4H_2O$, 1.6; (3) $ZnSO_4.7H_2O$, 1.4, and (4) $CoCl_2.6H_2O$, 20.0. The medium and the trace

elements were autoclaved separately at 1.03 x 10^{-5} Pa, 121°C for 15 min and cooled to room temperature before one ml of sterilized trace elements was added and inoculated with different concentrations of *Bacillus* sp.: $2x10^8$, $2x10^7$, $2x10^6$, and $2x10^5$ cells/ml. The PKC and the inocula are mixed thoroughly and undergo pH adjustment to 6.5. Known volumes of sterile distilled water was added just enough to keep the PKC moist. The entire samples and controls were incubated for five days at ambient temperature. Controls were prepared the same as the other samples except without inoculation with *Bacillus* sp. The entire samples and controls were incubated for five days at 37°C. Then, the test was repeated by using vegetable waste as substrates.

D. Enzyme Extraction

A 20 ml distilled water was added to the five grams of fermented PKC (which had undergone the solid state fermentation earlier) and was swirl until homogenous. All the flasks were vigorously shaken on the rotary shaker at 200 rpm for 30 mins. The solid biomass was separated from the suspension by filtration through Whatman No.1 filter papers. The supernatant was used as the source of crude enzyme preparation. Similar procedure was used to examine the effect of different treated raw and defatted PKCs and also the vegetable waste.

E. Measurement of Enzyme Activity

Measurement of enzyme activity was carried out based on the method of filter paper assay for saccharifying cellulose (FPU Assay) as outlined by Ghose [13]. One ml of 0.05M citrate buffer (pH; 3.0, 4.0, 5.0, 5.5, 6.0, and 7.0.) was added to the test tube containing one Whatman No.1 filter paper strip (1cm x 6cm). After that, 0.5ml of sample solution (supernatant) was added to the citrate buffer solution. The resulting solution was mixed thoroughly and then it was transferred to a water-bath maintained at 30, 40, and 50°C. After 60 minutes (reaction step) the test tubes were removed from the water bath, and 3 ml of DNS solution was added and mixed thoroughly to stop the enzymatic reaction. Tubes were covered and placed in a boiling water bath for 5 min. All the tubes were cooled to room temperature with a cooling water bath. The absorbance was determined at 540 nm against water blank. The reducing sugar was estimated by plotting the absorbance to the glucose standard curve. Enzyme activity was expressed as FPU/ml (Amount of reducing sugar released per ml of filtrate per hour).

F. Statistical Analysis

The data in this experiment were analysed using Analysis of Variance (ANOVA) from Minitab 15 with 95% of confidence interval.

III. Results

A. Effect of Incubation pH on Cellulase Activity by Bacillus sp.

The pH is an essential factor in determining the cell growth and enzyme production. The initial pH of the medium used varied between pH 3.0 to 7.0 (Fig. 1). As shown in Table I, cellulase activity was found best in the medium with an initial pH of 4.0 with the maximum cellulase activity of 0.68 FPU/ml. pH that is lower or higher than pH 4.0 both reduced the cellulase production, exceptional for pH 7.0.

TABLE I: Effect of Incubation pH of Medium on Cellulase Production

pH	Enzyme Activity, FPU/ml
3	0.63
4	0.68
5	0.66
6	0.57
7	0.62

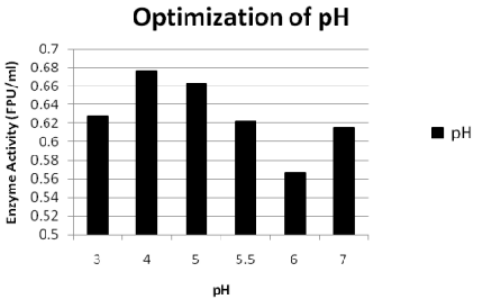

Fig 1. The effect of the incubation pH on cellulase activity by *Bacillus* sp. incubated for 5 days at room temperature (30°C±2).

B. Effect of Incubation Temperature to the Cellulase Activity of Bacillus sp.

The effects of incubation temperature on the cellulolytic activity were examined. The temperature range studies were 40-60°C (Fig. 2). As shown in Table II, 50°C proved to be the best temperature for the enzyme synthesis in the present study. The results revealed that *Bacillus* sp. grew significantly at the temperature of 50°C with the maximum cellulase activity of about 0.77 FPU/ml respectively. However, the production of enzymatic activity was found to decrease as the temperature increased. At the temperature below 50°C, the production of cellulase activity was low.

TABLE II: Effect of Incubation Temperature on Cellulase Production by Bacillus sp.

Temperature, °C	Enzyme Activity, FPU/ml
40	0.64
50	0.77
60	0.57

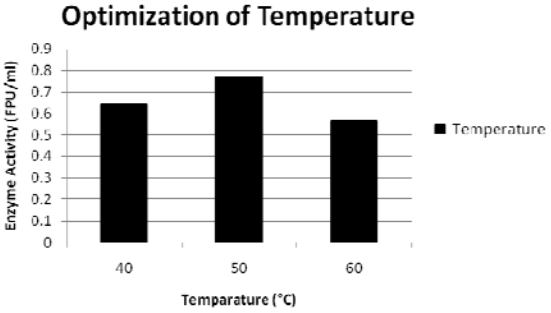

Fig 2. Effect of Incubation temperature on cellulase activity by *Bacillus* sp. incubated at different temperature.

C. Effect Of Inocula Concentrations On Cellulase Activity By Bacillus sp.

The effect of the inocula size based on the spore count was studied. The inocula concentrations were varied from 2 x 10^8, 2 x 10^7, 2 x 10^6, and 2 x 10^5 cells/ml (Table III). As shown in Fig. 3 and 5, when the inocula concentration was higher than 2 x 10^5 cells/ml, the enzyme activity is obviously low when tested on raw and defatted PKCs. Slight variations in the cellulase activity were observed when inoculated with concentrations ranging from 2 x 10^8 to 2 x 10^6 cells/ml,. Maximum cellulase activity was achieved in raw and defatted PKCs when inoculated with 2x10^5 cells /ml. Conversely, vegetable wastes gave the greatest cellulase activity when inoculated with 2 x 10^6 cells/ml (Fig. 7). *Bacillus* sp. as an inocula can yield maximum cellulase activity of 85.48 FPU/ml on vegetable wastes as a substrate.

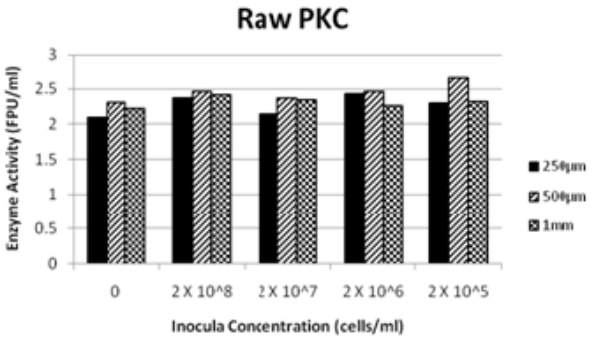

Fig 3. Cellulase activity by *Bacillus* sp. measured on different particle size of Raw PKC using different Inocula Concentration

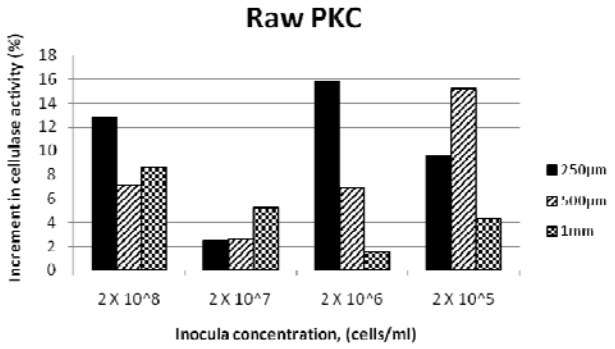

Fig 4. Percentage increment of cellulase activity by *Bacillus* sp. measured on different particle size of raw PKC using different inocula concentration

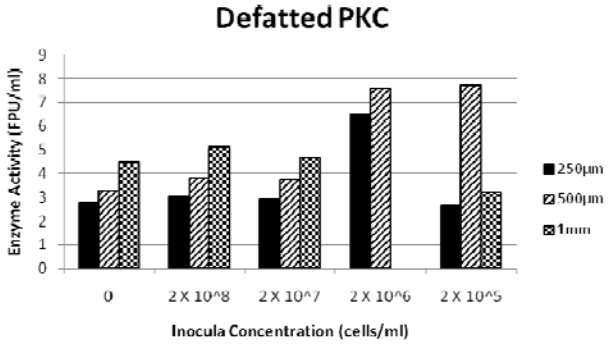

Fig 5. Cellulase activity by *Bacillus* sp. measured on different particle size of defatted PKC using different inocula concentration

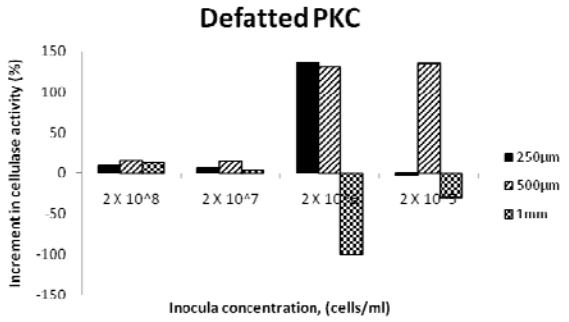

Fig 6. Percentage increment of cellulase activity by *Bacillus* sp. measured on different particle size of defatted PKC using different inocula concentration

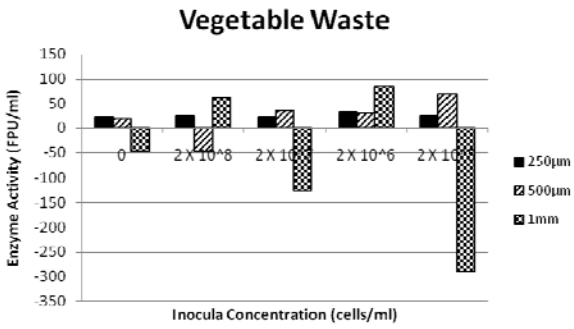

Fig 7. Cellulase activity by *Bacillus* sp. measured on different particle size of vegetable wastes using different inocula concentration.

TABLE III: EFFECT OF INOCULA CONCENTRATIONS AND PARTICLE SIZES ON CELLULASE PRODUCTION MEASURED ON DIFFERENT SUBSTRATES

		Enzyme Activity (FPU/ml)		
Particle Size	Inocula concentration (cells/ml)	Raw PKC	Defatted PKC	Vegetable Wastes
	0	2.09	2.76	22.44
	2 X 10^8	2.36	3.01	25.68
250µm	2 X 10^7	2.14	2.91	21.25
	2 X 10^6	2.42	6.52	33.57
	2 X 10^5	2.29	2.68	24.88
	0	2.3	3.28	20.28
	2 X 10^8	2.46	3.81	-46.84
500µm	2 X 10^7	2.36	3.77	35.55
	2 X 10^6	2.46	7.6	31.58
	2 X 10^5	2.65	7.73	68.42
	0	2.22	4.51	-46.84
	2 X 10^8	2.41	5.12	62.27
1mm	2 X 10^7	2.34	4.69	-124.23
	2 X 10^6	2.26	0.0	85.48
	2 X 10^5	2.32	3.20	-287.93

D. Effect of Particle Sizes to the Cellulase Activity of Bacillus sp.

In this experiment, the particle sizes of substrates used were 250µm, 500µm, and 1mm. Fig. 7, shows the enzyme activity was higher with 1mm particle size of vegetable wastes was used compared to 250µm and 500µm with the maximum yield of 85.48 FPU/ml cellulase activity. Of the various particle sizes examined, 500µm of defatted PKC and raw PKC (refer to Fig.3 and Fig.5) yield higher

cellulase activity at 7.73 FPU/ml and 2.65 FPU/ml correspondingly.

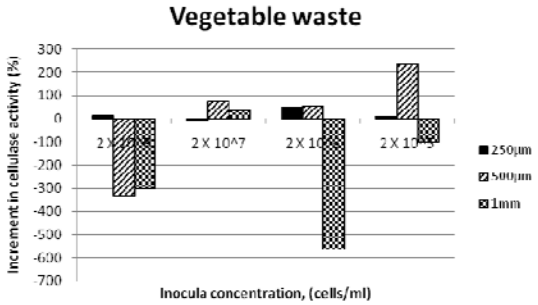

Fig 8. Percentage increment of cellulase activity by *Bacillus* sp. measured on different particle size of vegetable wastes using different inocula concentration.

Based on the statistical analysis, analysis of variance (ANOVA) using Minitab 15, the types and particle size of the substrates does not affect the enzymatic activity of *Bacillus* sp. significantly since its p value is more than 0.05 when tested with 95% confidence level. Whereas, an inocula concentration shows significant result with $p < 0.05$.

IV. Discussions

A. Effect of Incubation pH on the Cellulase Activity

The pH growth range and pH growth optimum vary according to different microorganisms. Microorganisms often grow over wide range of pH. Yet, extreme pH can damage microorganisms by distracting the plasma membrane as well as inhibiting the enzymatic activity and membrane transport proteins [14]. The influence of pH on in-house *Bacillus* sp. is shown in Fig. 1. As the pH of the medium increase, the cellulase activity generated by the *Bacillus* sp. also increased until pH 4.0. Further increase beyond pH 4.0 resulted in lower cellulase activity achieved. Suitable pH is required for the enzyme to maintain the three dimensional shape of the active site. Changes in the pH might alter the ionic bonding of enzyme that contributed to the functional shape of the enzyme [15].

B. Effect of Incubation Temperature on the Cellulase Activity

Temperature sensitivity is one of the most important factors affecting the growth of microorganisms [14]. In this experiment, we aim to seek the optimal temperature cellulase activity. The results revealed that the cellulase activity tend to increase with temperature reaching optimum at 50°C (Fig.2). Further increase in temperature to 60 °C reduced the cellulase activity even much lower as compared with low temperature reaction. Extreme temperature reaction can lead to the modification of the active site enzyme and reduced available sites for the reaction process.

C. Effect of Particle Sizes on the Cellulase Production

The cellulase activity of *Bacillus* sp. was at its highest when the test was done on 500µm PKCs. Meanwhile, vegetable wastes sized 1mm, could produce more cellulase activity compare to other particle sizes vis 250µm and 500µm. The disadvantages of the smaller particle sizes is that its low porosity characteristic will resulted in lack of inter-particle space. This will resulted in difficulty in bacterial respiration and will end up yielding low enzymatic activity [16]. Fan *et al*. [17] suggested that the increase in cellulase production did not solely depend on the amount of cellulose in the medium but may also depend on other factors such as pore size, surface area, cellulose crystallinity [18], substitution reactions with lignin-carbonium ion and depolymerization of lignin [19]. Ray *et al.* [20] obtained higher cellulase production from smaller OPEFB fibre size (2mm rather than 10mm) and it may be attributable to the decrease in particle size caused by the impact energy transfer of hammer milling. Reducing lignocellulose length greatly increased the surface area and reduced the diffusion path of reactants for enzymatic hydrolysis.

D. Effect of Inocula Sizes on the Cellulase Production

Inocula size plays an important role in achieving maximum bacterial growth and enzyme production by *Bacillus* sp. in a solid state fermentation. In this experiment, the cellulase activity increased when the low inocula sizes were used. The cellulase activity started to increase until 2 x 10^6 cells/ml of inocula were used before it gradually dropped when higher concentration was used. Reference [21], [22] elucidated that cellulase production by *Bacillus* sp. increased gradually up to 3% inoculums size but decreased thereafter even with the increment of the inocula sizes. Similar findings was reported by Sarkar *et al.* [23] whom achieved the maximum cellulase activity at 2% inocula sizes before it gradually decreased with the increase in inocula sizes.

Reference [24]-[26] reported that the fungal growth was increased as the inocula size increased until it achieved the optimal inocula size then, the growth decreased sharply by the increase of the inocula size over the optimal one. It was proven that after the optimal growth, an increase in inocula size will lead to the low enzyme productivity due to the fast growing rate of the microorganism that will increase the competition for the nutrient and space. This at the same time will affect the stationary phase length which in the end affects the enzyme productivity.

E. Comparison of Cellulase Production Using Different Carbon Sources

A comparison of cellulase production using PKCs and vegetable wastes is given in Table 6. As can be seen, vegetable wastes are better substrates for growth and cellulase production compared to PKCs. The maximum cellulase activity obtained from the fermentation of *Bacillus* sp. on vegetable wastes shows increment of more than 100% cellulase activity compare to PKCs. It was about 12 times more than those obtained from fermentation of PKCs.

Overall, a differential effect of the carbon sources on cellulase production was observed, this finding is in agreement with [9], [27], who demonstrated that the production of cellulase is influenced by the carbon source used. High cost of substrates can be a limiting factor in enzyme production.

V. Conclusions

PKC and vegetable wastes were found to be suitable as substrates for cellulase production by *Bacillus* sp. in solid state fermentation, with substantial enhancement in growth and enzyme production. It was also an alternative to reduce

the costs for enzyme productions. PKCs with particle size of 500μm produced maximum activity when inoculated with 2 x 10^5 cells/ml of *Bacillus* sp. while vegetable wastes sized 1mm yield its maximum activity during an inoculation with 2 x 10^6 cells/ml of *Bacillus* sp.

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