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Cellulase Activity and Glucose Production by *Bacillus Cereus* Mono-culture and Co-culture Utilizing Palm Kernel Cake (PKC) under Solid State Fermentation

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Abstract. The cellulolytic degradation by *Bacillus* sp. and its mixed culture have been investigated. This study focused on the cellulase activity produced by *Bacillus* mono-culture and co-culture utilizing the palm kernel cake as a substrate. Two types of inocula used for the solid state fermentation of PKC were *Bacillus* mono-culture and *Bacillus-Trichoderma* co-culture; *Trichoderma* sp. is a fungal species known to degrade cellulose. When grown on PKC, the culture supernatant of this isolate was found to possess cellulolytic activity, as demonstrated from the glucose production. For the 2nd seeding *Bacillus* mono-culture, the highest cellulase activity of 3.69 FPU/ml was obtained on the defatted PKC and 25.4 % increase of enzyme activity can be achieved. The maximum cellulase activity by 2nd seeding *Bacillus*-Trichoderma co-culture, 2.738 FPU/ml was attained on the defatted PKC and 28% increase in cellulase activity can be gained. However, raw PKC scored higher cellulase activity about 31.5% compared to defatted PKC. Conversely, 1st seeding *Bacillus* mono-culture possessed comparatively lower cellulase activity to the uninoculated PKC but its co-culture registered an improvement in the production of cellulase activity. *Bacillus* monoculture was unable to break down cellulose effectively in both seedings if compared to co-culture. Co-culture of *Bacillus* sp. and *Trichoderma* sp. increased the capacity of Bacillus cellulolytic activity. *Trichoderma* sp. exerts a protagonistic effect on the cellulolytic capability of *Bacillus* sp. The degradation of the cellulose compound to simpler glucose units by these microorganisms has made PKC a good alternative for cellulose production from industrial point of view since pure commercialized cellulose is too expensive to be used as substrate.

Keywords: Cellulase activity, Bacillus mono-culture, Bacillus-Trichoderma co-culture, 1st seeding, 2nd seeding, Solid state fermentation, Raw palm kernel cake (PKC), Defatted palm kernel cake (PKC).

1. Introduction

In the last few decades, many efforts have been spent in the study of enzymes with cellulolytic activity as potential sources in obtaining energy from an abundant and renewable cellulose especially the cellulase system of the fungus *Trichoderma* sp. It has been known that cellulases from Trichoderma sp. can be effectively hydrolyze crystalline cellulose [1]. Among the bacteria species, *Bacillus* species produce a variety of extracellular polysaccharide hydrolyzing enzymes [2] and it has been the most commonly used for enzyme production by utilizing oil cakes. Enzymes are produced industrially either by submerged fermentation (SmF) or solid state fermentation (SSF) [3]. Unfortunately, most *in vitro* studies have focused on activities from fungal monocultures compared to syntrophic co-cultures of fungi and methanogens [4]. Furthermore, production of enzymes from microorganisms is one way of reducing the cost of enzyme production of industrial scale. Another approach to reduce the cost of cellulase production is the use of cheap and easily available substrates which in this case, the PKC, act as a substrate. The present study focused on the cellulase activity by *Bacillus* sp. mono-culture and co-culture (with *Trichoderma* sp.) on raw and defatted PKC as a substrate. Additionally, the comparison between the two methods of growing *Bacillus sp*. was also being tested.

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2. Materials and Method

2.1. Sampling and Preparation of Palm Kernel Cake (PKC)

Freshly produced PKC was obtained from a local palm kernel mill. The PKC was divided into two portions. A portion was immediately stored at 4^{o}C in a refrigerator until later use and labeled as raw PKC. Another portion was defatted using Soxhlet technique with hexane as solvent and extraction carried out for 8 hours. The residual hexane was then removed and the defatted PKC was stored at 4°C until ready for use.

2.2. Production of Bacillus

The microbes (*Bacillus* sp.) from isolate B1 are grown on slant test-tubes of nutrient agar media for 24 hours according to the method outlined by Choi and Reynard [5].

2.3. Fungal Cultivation and Inocula Preparation.

A 30% (v/v) mixed vegetable (V8) broth was prepared, pH adjusted to pH 5.0 and autoclaved at 1.03 x 10^5 Pa, 121˚C for 15min. About 1% (wt/v) of *Trichoderma* sp. was inoculated into the sterile liquid V8 media. The inoculated liquid media was incubated for 5 days at 30˚C on a rotary shaker at 150 rev.min^{-1}, where masses of biomas formed. The biomass in the liquid media was filtered and both the biomass and the filtrates collected. The filtrate contained conidia which were used as inocula. The conidia concentration was determined using a haemocytometer.

2.4. Solid State Fermentation (SSF)

SSF of fungus was performed with 5gm ground PKC as the solid substrate. The substrate was autoclaved at 1.03 x 10^{-5} Pa, 121°C for 15min and cooled to room temperature before inoculated with different concentrations of 1st seeding Bacillus mono-culture: $1x10^6$, $2x10^{6,}$, $3x10^6$, $4x10^6$ and $5x10^6$ cells/ml. Another set of substrates were inoculated with different concentrations of 2nd seeding *Bacillus* mono-culture: $1x10^7$, $2x10^7$, $3x10^7$, $4x10^7$ and $5x10^7$ cells/ml. For the co-cultures, the flasks were inoculated with ($1x10^6$ or $1x10^7$ cells/ml) of *Bacillus* sp. suspension together with a different concentrations of *Trichoderma* sp. inocula for each flask. Different concentrations of *Trichoderma* sp. conidia prepared at: $4x10^6$, $8x10^6$, $12x10^6$, $16x10^6$ and $20x10^6$ conidia/ml. The PKC and the inocula are mixed thoroughly and undergo pH adjustment. 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 37°C. Controls were run without inoculation.

2.5. Enzyme Extraction

At the end of the fifth day of fermentation, as much as 20 ml of sterile distilled water was added to 5 g of SSF substrate, the fermented PKC and swirl it until it becomes homogenous. All the flasks were vigorously shaken on the rotary shaker at 200 rev.min^{-1} for 30 minutes. The solid biomass was separated from the suspension by filtration through Whatman no.1 filter paper. The extract was used as the source of crude enzyme.

2.6. Measurement of Enzyme Activity

Measurement of glucose (reducing sugar) and enzyme activity was carried out based on the method of filter paper assay for saccharifying cellulose (FPU Assay) outlined by Ghose [6].

2.7. Statistical Analysis

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

3. Results and Discussion

Palm kernel cake consists of between 15% to 18% protein and 60% to 70% polysaccharides. The majority of the polysaccharides are non starch, comprising of beta- mannans type of hemicelluloses. The solid state fermentation of both raw PKC which still contains residual oil and defatted PKC, having the oil completely removed were tested with Bacillus sp. and combined *Bacillus*-*Trichoderma* sp. for cellulase activity.

3.1. Estimation of Cellulase Activity of Bacillus Mono-culture on Raw and Defatted PKC.

Cellulase activitiy detected in PKC inoculated with *Bacillus sp.* was higher than found in uninoculated PKC. However the concentrations of cells employed have effect on the cellulase activity. The cellulase activity is deciphered from the amount of glucose able to be detected from the SSF. By using 2nd seeding method of growing *Bacillus* sp., the highest cellulase activity of about 3.693 FPU/ml was obtained when using 10^7 cells/ml of *Bacillus* sp. in the defatted PKC sample (substrate). This generated roughly about 25.44% increase in enzyme activity over that of uninoculated defatted PKC as observed in Figure 1.The highest cellulase activity in the raw PKC sample was 3.132 FPU/ml inoculated with10^7 cells/ml of *Bacillus* sp. and the cellulase activity increased to about 11.22 %. The SSF of defatted PKC inoculated with *Bacillus* sp. gave higher cellulase activity than the Raw PKC. However, for *Bacillus* sp. grew on 1st seeding, the cellulase activity measured dropped down almost 3.58% for raw PKC and 13.66% for defatted PKC when compared to the uninoculated PKC.Higher cellulase activity was appraised in defatted PKCs than raw PKC.The compound in the oil itself may affect the enzyme activity in the sample. Removal of oil will make the PKC polysaccharide structure more accessible to fungi to breakdown the digestible biomass for cellulose production [7].

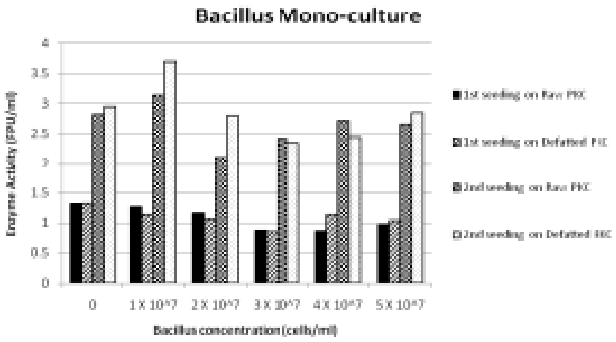

Fig. 1: Cellulase activity of raw and defatted palm kernel cake solid state fermentation (PKC SSF) on mono-culture of two different *Bacillus* sp. seeding with different concentrations

3.2. Estimation of Cellulase Activity of *Bacillus-Trichoderma* Co-culture on Raw and Defatted PKC

Based on Figure 2, the *Bacillus-Trichoderma* co-culture using 2nd seeding method of growing *Bacillus* sp., the maximum cellulase activity of 2.738 FPU/ml was achieved when defatted PKC was used as substrate. The result was obtained after using 8 x 10^6 spores/ml of *Trichoderma* sp.. Likewise, 28.1% more of cellulase activity can be gained when applying the co-culture to the defatted PKC. This demonstrates higher cellulase activity can be generated from the defatted PKC by using lesser amount of the co-culture suspensions as a source of microbes. On the other hand, 2.562 FPU/ml of enzyme activity was achieved using *Bacillus-Trichoderma* co-culture, with 20 x 10^6 spores/ml of *Trichoderma* sp. on raw PKC as a substrate. It recorded lower enzyme activity when compared to the defatted PKC. However, greater cellulase activity was registered in raw PKC when the co-culture was used achieving an increase of 31.5%. Nevertheless, 1st seeding *Bacillus* sp. yielded cellulase activity lower for both PKCs in comparison to 2nd seeding *Bacillus* sp. The cellulase activity determined was 1.424 FPU/ml for raw PKC and 1.748 FPU/ml for defatted PKC with percentage garnered up to 26.58% for raw PKC and 20.8% for defatted PKC. The cellulase activity determined in the SSF inoculated simultaneously with the bacteria and fungus was higher than single culture inoculation. This might be due to the possible interactions between the fungi and bacteria with both possessing the natural capabilty to breakdown cellulose. Synergism can occur when different agents are applied together, and cell wall degrading enzymes (CWDEs) produced by fungi can increase the efficacy of bacteria [8]. The result was supported by [9] who obtained higher cellulase activity from co-culture of fungus maybe due to the secretion of cellulase components (isoenzymatic forms) under common habitat acting synergistically on the cellulose resulting more production of cellulases. Antifungal properties of some bacteria, which possessed the inhibitory effects, were the main factor affecting the potential of *Trichoderma*

sp. in ecosystem colonization [10]. Bacterial strains can slow down the growth of *Trichoderma* by production of volatile organic compounds [11] or by releasing antibiotics [12].

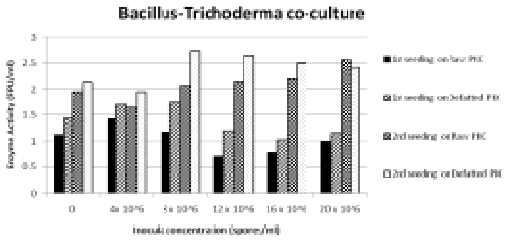

Fig. 2: Cellulase activity of raw and defatted palm kernel cake solid state fermentation (PKC SSF) on co-culture of two different *Bacillus* sp. seeding with different concentrations

3.3. Relationship between the Enzyme Activity and Glucose Concentration by both Bacillus Mono-culture and Co-culture using Raw and Defatted PKC

Based on the figures, the enzyme activity measured for the mono-culture and co-culture reveal opposing outcome. Reference[13] obtained similar findings when conducting a test for the cellulose utilization by some cellulolytic fungal mono-culture and co-cultures. They stated that co-cultures were more effective in substrate saccharification, which ranged between 85~88% compared to the 62~67% saccharification shown by the monocultures. Among the cellulolytic enzymes, FPase (Filter Paper Cellulase) activity was significantly higher in all the co- cultures than in their respective monocultures. The glucose concentration has a linear relationship with the enzymatic activity at higher concentration of microorganism in both mono-culture and co-culture. The increasing number of the microorganism will increase the requirements for food and energy sources. Sattam [14] stated that at higher inoculums sizes, the growth rate increased drastically and finally resulting in a condition of substrate and oxygen limitation, after a certain period of cultivation time. At this stage, the concentration of the substrate was lower and concentration of waste products increased gradually and as a result, the number of cells dropped rapidly after achieving the maximum growth due to cell lysis. The glucose concentration graph pattern can be observed in Figures 3 and 4. Compared to the monoculture of anaerobic fungi, an increase in cellulose digestion rate was observed in co-cultures by several researchers [15].

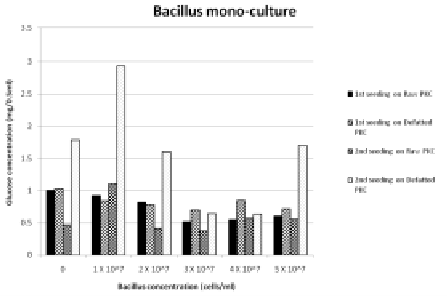

Fig. 3: Glucose Concentration of raw and defatted palm kernel cake solid state fermentation (PKC SSF) on mono-culture of two different *Bacillus* sp. seeding with different concentrations

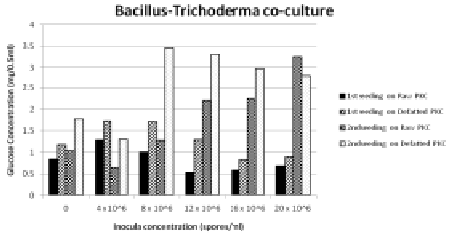

Fig. 4: Glucose Concentration of raw and defatted palm kernel cake solid state fermentation (PKC SSF) on co-culture of two different *Bacillus* sp. seeding with different concentrations

3.4. Enhancement of Cellulase Activity by *Bacillus* Co-culture by using *Bacillus* sp. that had Undergo 2nd Seeding Process

Based on Figure 2, cellulase activities measured on SSF of both raw PKC and defatted PKC using 2nd seeding *Bacillus* sp. were higher compared to the cellulase activity measured on SSF inoculated with 1st seeding *Bacillus* sp. The cellulase activity measured for the *Bacillus* sp. from 2nd seeding and 1st seeding show opposite results. Mono-cultures inoculated with 1st seeding *Bacillus* sp, displays a drop in cellulase activity by about 3.58% on raw PKC and 13.66% on defatted PKC compared to the untreated PKC. The difference in the cellulase activity between both seedings of bacillus was 14.8% for raw PKC and 39.1% for defatted PKC. A higher cellulase activity was evident from *Bacillus* sp. grew from 2nd seeding method for co-culture by about 4.92% for raw PKC and 7.3% for defatted PKC. Based from the statistical analysis using analysis of variance (ANOVA) provided by Minitab 15, all treatments were significant with 95% confidence level.

4. Conclusion

From the results, an improvement in cellulase activity has been demonstrated by *Bacillus* sp. inoculated on PKC which resulted in more cellulose production. It is further established that the cellulase activity when both *Bacillus* sp. and *Trichoderma* sp. were used as co-inocula, the cellulase activity was enhanced and *Trichoderma* sp. increased the capacity of *Bacillus* sp. cellulolytic activity. The degradation of the cellulose compound to simpler glucose units by these microorganisms has made it suitable product is rich in energy for the use of ruminant feeding. Since pure commercialized cellulose is too expensive to be used as substrate, PKC maybe a good alternative for cellulose production from industrial point of view.

5. Acknowledgement

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