Morphological study of microbial and hydrolysis of coconut palm peat (*cocos nucifera*) using worm tea

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***Abstract*-** Coco peat was used as biomass to produce reducing sugar with worm tea, rich with diverse microbial content, to assist sugar hydrolysis biologically. The aim is to determine the morphology of bacteria and ability to breakdown cellulose. Comparison of water and dilute acid pretreatment to produce reducing sugar from coconut peat at different pH was also studied. The bacteria were found to be *Bacillus pumilis, Bacillus subtilis*, *Micrococcus spp*, *Staphylococcus aureus* and *Aspergillus fumigatus*. All of them were investigated for starch hydrolysis test and cellulase clearance zone for breakdown of amylase and cellulase. Sugar yield was recorded the highest for acid pretreatment and worm tea hydrolysis at 7.94 Brix%, pH 9. These results indicated that compost tea has great potential in bioethanol production.

***Index Terms*-** coco peat, worm tea, agar, Gram staining, yeast

I. INTRODUCTION

Soaring petroleum cost and depleting fossil fuel has set researchers to explore on alternative energy resources. Bioethanol (CH_3CH_2OH) or ethyl alcohol, is a liquid biofuel and can be derived from numerous varieties of biomass feedstocks using conversion technologies (Demirbas., 2005). Bioethanol usually derived from agricultural crops such as sugarcane, rice and maize. Unfortunately, usage of current starch-based resource will bring impact to food security due to limited land for agriculture (Chen et al., 2007; Chandel et al., 2007a). Lignocellulosic biomass is inexpensive, renewable and vastly available (Ho et al., 1998). The main substances of lignocellulosic biomasses are cellulose, hemicelluloses, lignin, extractives and ash (Karimi et al., 2006). Conversion of this biomass into glucose and other simple reducing sugars has been considered as a prospective path for bioethanol production (Curreli et al., 1997).

The samples used in this study are worm tea and coco peat from coconut palm (*Cocos nucifera*). Coconut peat or coir is a hard fiber from coconut palms (*Cocos nucifera*) which can be found in tropical countries. There are three types of coir have been used which is the mattress fiber coir, omat fiber coir and bristle fiber coir. The mattress fiber coir is short, thin, fragile and good mulching agent. Omat fiber coir is long and strong. Bristle fiber coir comes from the remaining coir after other coirs were separated. This bristle fiber coir has highest lignin content and the highest density among other two core types in coconut husk. Bristle fiber is used in the manufacture of brushes and brooms (Rajan et al., 2005).

Worm tea is the liquid extract of compost that comes from an infusion of water in compost for a defined period of time. The compost is removed and the remaining liquid is worm tea (Hargreaves, Adl and Warman., 2009). Worm tea consists of different organisms and nutrients, depending on the biomasses applied. There is no 'perfect' compost, but several different types. Compost supply nutrients and beneficial microorganisms, decreases environmental problems related to waste management by reducing them and by killing potentially harmful organisms (Amlinger et al., 2003; Sæbø and Ferrini., 2006; Bess 2000). Besides that, compost tea can be produced through two methods which are aerated system and non aerated system. Aerated system is a continuous system with the addition of air and nutrients such as molasses, rock, dust and so on to increase microbial population density. For non aerated system, the mixture is minimally disturbed after initial mixing (Carballo *et al*., 2008). For this study, worm tea was collected from compost made from biomass such as sugar cane (*Saccharum officinarum*), aloe (*Aloe vera*), banana flower (*Musa acuminate*), pandan leaves (*Pandanus fascicularis*. The objective of this study is to find a sustainable way of sugar yield using worm tea and investigate the ability of a consortium of microbial in worm tea to breakdown cellulose to simple reducing sugar.

II. MATERIALS AND METHODS

2.1 Raw materials

Worm tea was kindly donated by a local company and coco peat was bought from a local company. Coco peat was washed thoroughly with tap water, autoclaved (121˚C, 1 atm for 15mins) and dried (80˚C for 72hr) prior to grinding to approximately 3cm.

2.2 Morphological studies

Three types of agar; Nutrient Agar, Potato Dextrose Agar, and Czapek Dox Bengal Rose were prepared according to the standard Preparation of Culture Media (APHA, 1999). Microorganisms were grown via spread plate method and all plates were incubated at 37^{o}C for 24 hours, except Czapex Dox Bengal Rose Agar (7days). Gram staining was applied and morphology of microorganisms were studied under a light microscope (Microscope Millenium-LMS Series2000) (Maier, 2009). Starch Hydrolysis Test and Cellulose Clearance Zone were applied to discovered microorganisms.

2.1.1 Starch hydrolysis test

Starch hydrolysis test was done according to methods presented in Microbiological Applications Lab Manual. Plates were divided into four sections and 0.1ml of each inoculum was poured in the holes bore using the Durham tube. After 24 hours incubation, lugol iodine was added to detect presence of amylase enzyme.

2.1.2 CMC clearance zone (CCZ)

CMC agar was prepared according to recipe by Kim et al., 2012. The plates were divided into four sections on 0.1ml of pure inoculums were poured on each hole. After incubation for 48 hours, CMC agar plates were flooded with 1 % congo red and allowed to stand for 15 mins at room temperature. 10ml of 1M NaCl was thoroughly used for counterstaining the plates. The plates were examined for presence of clear zones around the colony, which indicates cellulose hydrolysis (Irfan et al., 2012). The diameter of the visible halo zone for both tests was measured in millimeter.

2.3 Pretreatments

2.3.1 Acid pretreatment

1.0M Sulfuric acid (H_2SO_4) was added to 20g of coco peat. The ratio between biomass and sulfuric acid was 1:10 w/v. The mixture was autoclaved and washed till neutral.

2.3.2 Water pretreatment

A total of 240ml of distilled water was added to a beaker containing 20g (1:12 w/v) of biomass (coco peat). The samples were autoclaved (Vertical High Pressure Steam Sterilizer (Sakura)/ASV2403) for 15 minutes at 121ºC, 1 atm. The biomass were washed before proceeding to the next stage.

2.5 Experimental setup

A total of eight reactors was set up in Erlenmeyer flasks (250ml), as shown in Table 2.1.

Reactor	Pretreatment	pH
R1	Acid pretreatment + worm tea	5
R2	Acid pretreatment + worm tea	9
R3	Acid pretreatment + distilled water	5
R4	Acid pretreatment + distilled water	9
R5	water pretreatment + worm tea	5
R6	water pretreatment + worm tea	9
R7	water pretreatment + distilled water	5
R8	water pretreatment + distilled water	9

Table 2.1 Reactors for sugar hydrolysis

Worm tea was added in the ratio of 1:12. 1M of Sodium Hydroxide (NaOH) was added drop by drop to alter the pH. The conical flasks were kept in incubated shaker (160rpm, 50°C). Aliquots were withdrawn every 24 hours, centrifuged (Universal 32 Centrifuge, Hettich ZENTRIFUGEN) at 4000rpm for 10mins and tested for sugar content for 10 days time period.

2.6 Analysis

pH of the hydrolysate was tested using a calibrated pH meter (HACH, sension 3). Sugar content was evaluated using Refractometer PAL-1 (ATAGO).

III. RESULTS AND DISCUSSIONS

3.1 Consortium of Microorganisms in Worm Tea

CHNS analyses yield that carbon 0.25%, Hydrogen 3.93%, Nitrogen 0.02% and sulphur 0.48%, suggesting the presence of live microorganism. Four different bacteria and fungi were screened from microbial grown on Nutrient Agar, Potato Dextrose Agar and Czapek Dox Agar. Bengal Rose was added to Czapek Dox to inhibit growth of microorganisms, allowing fungi to generate. Gram staining was performed and under light microscope with 100x magnification, all organisms were gram negative. The grayish green fungi were stained with lactophenol blue had both Uni and Bi sporangium. Thus, we conclude the fungi present had been of *the Aspergillus fumigatus family*. No further studies were made on fungi as author was keen on bacteria. The microorganisms were further studied on their morphological. The bacteria discovered were cocci and bacilli and all of them were purple, proving no Gram negative bacteria in compost tea.

Based from catalase test, 103 and 104 proved to be catalase positive. Biochemical tests using API kits and manual calculations using chart provided revealed that all four bacteria are 101-*Bacillus subtilis*, 102- *Bacillus pumilis*, 103- *Micrococcus spp*, 104- *Staphylococcus aureus* and *Aspergillus fumigatus.*

To prove more on microbial activity, certain tests were performed. Starch hydrolysis test was performed on each pure culture to identify the organisms that are able to produce amylase enzyme, breaking down starch. All bacteria were able to, it was just the matter of speed of activity. The same method was implied to fungi, incubated for 7 days, however fungi did not show any clearance zone. Table 3.1 shows microbial activity, or effectiveness of the microorganisms in releasing amylase. The CMC agar test was also performed to evaluate effectiveness of the microorganisms in breaking down cellulose, as coco peat is inedible item, containing cellulose and not edible starch. For our achievement, the bacteria showed cellulolytic activity with clear surroundings. The results were summarized in Table 3.2. Based from the results, it is found that worm tea is an effective microorganism (EM). EM consists of mixed cultures of beneficial and naturally-occurring microorganisms such as phototrophic bacteria, lactic acid bacteria and yeast that can be used as inoculants to increase the microbial diversity of soils and plant. EM contains selected species of microorganisms that are mutually compatible with one another and coexist in liquid culture. The uniqueness of microorganisms and their often unpredictable nature, given a specific set of environmental and cultural conditions, has made them likely candidates for solving for solving particularly difficult problems in the life sciences and other fields as well. If used appropriately, EM can significantly increase the beneficial effects of these practices (Higa and Wididana, 1991b).

3.2 Hydrolysis of coco peat

Two types of pretreatment were employed; water pretreatment and dilute acid pretreatment and average results were calculated. Both pretreatments were then continued with hydrolysis using water and hydrolysis using compost tea, with coco peat as substrate. In this study, the effect of pH was evaluated. Graph 3.1 summarizes the initial and final value of reducing sugar from each reactor. Hydrolysis was done for 14 days. Interestingly, the level of sugar increased on day 8 for all the reactors except R5, R6, and R8 and thereafter remained constant till day 11. The results were collected at day 8. On day 2, almost all reactors have a slight drop in the reading. This may be due to the organisms in the compost tea adapting to the new feed. Sugar level increased by hydrolysis period. Microorganisms feed on coco peat, breaking down hemicelluloses into reducing sugars. This is also because hydrolysis of carbohydrate or lignocellulosic materials by microbial activity occurs and simultaneous conversion of cellulose to sugar to ethanol by microorganisms. The pH had a sharp effect on the hydrolysis. At alkaline pH, the yield is shown to be higher than acidic condition. Effect of temperature was not taken into consideration as all studies were done at room temperature and microorganisms functions well at room temperature. R2, with acid pretreatment reached maximum sugar concentration of 7.94Brix %. This could be an indicator that all the cellulose and hemicelluloses has been depolymerised to reducing sugar. R5 and R6 (water pretreatment) show less reducing sugar yield. The same trend was observed for R7 and R8. When observed clearly, it can be concluded that coco peat for these four reactors was treated with only water. The lignin has not been degraded, therefore, the hemicelluloses was not readily accessible for microorganisms to convert them into desirable products.

IV. CONCLUSION

In this work, we demonstrated that acid pretreatment is a suitable process to produce sugar from coco peat. Although it is not common to use compost tea as microorganism source, in our work, the isolates proved to be an excellent cellulose breakdown agent. Yet, it takes longer period for the process of breaking down and releasing the desired product. The best treatment is using dilute acid pretreatment of coco peat and using compost tea for enzymatic hydrolysis.

APPENDIX

Table 3.1: Amylase hydrolysis on solid media (Starch agar)

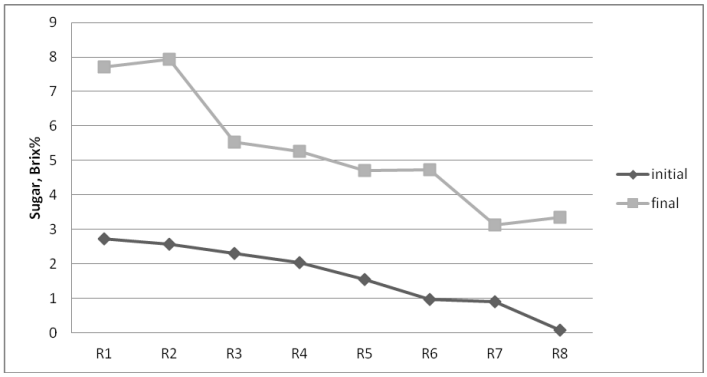
Isolates	Amylase	Diameter of clear Zone on agar (mm)	Amylolytic Activity
101 Bacteria	+	22.1	+++
102 "	+	17.6	+++
103 "	+	11.3	+
104 "	+	14.0	++
Fungi	-	-	ND

+: Positive, -: Negative +++, high activity, ++, moderate activity, +, low activity, ND: Not Detected.
+: low activity (< 11.5mm).
++: moderate activity (11.5mm- 20mm)
+++: high activity (> 20mm).

Table 3.2: Cellulase hydrolysis on solid media (CarboxyMethylCellulose agar)

Isolates Code	Cellulase	Diameter of clear Zone on agar (mm)	Cellulolytic Activity
101 Bacteria	+	19.2	+++
102 "	+	16.6	+++
103 "	+	17.1	+++
104 "	+	13.8	++
Fungi	+	1.4	+

+: Positive, -: Negative +++, high activity, ++, moderate activity, +, low activity, ND: Not Detected.
+: low activity (< 7.5mm).
++: moderate activity (7.6mm- 15mm)
+++: high activity (> 15mm).



Graph 3.1: Initial and final value of reducing sugar, Brix% of different reactors.

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