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Screening and Isolation of Effective Microbes for Decomposition Process among Different Organic Waste

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Abstract: ***Organic waste is material that is biodegradable and comes from either plant or animal. It can be decomposed over time by microbes into methane, carbon dioxide, water, and other simple organic molecules. Microorganism especially bacteria and fungus are able to decompose cellulose by an enzyme known as cellulase. Since plants are made up from cellulose, this study is focused on isolating and identifying the microbes that are responsible in decomposing the organic waste by screening for the microorganisms that are able to produce cellulose enzyme. In this experiment, the bacteria were isolated from three different samples, which are; compost product, decayed wood and empty fruit bunch mix with oil palm frond. The samples were collected from the fresh decomposing organic waste. The samples undergo serial dilution, screening the ability of the microorganism to solubilize phosphate by using National Botanical Research Institute's Phosphate media (NBRIP) and screening the ability of the microorganism to produce cellulose enzyme by using Carboxymethyl Cellulose Media (CMC) treated with Congo Red and NaCl for confirmation of the targeted effective microbes. The result shows that the targeted effective microbes will produce the halo zone or clear zone formation after being treated with Red Congo and NaCl solution on CMC media and producing a halo zone on NBRIP media. In this experiment, we manage to isolate and identify the bacteria that is responsible in producing cellulase enzyme and solubilizing phosphate that is known as Streptomyces sanglieri. Application of effective microbes will promote the plant uptake due to its ability in converting the nutrient into available form to the plant.***

Keywords: ***Cellulose producing bacteria, effective microorganism, phosphate solubilizing bacteria.***

I. INTRODUCTION

Organic waste is the biodegradable material that comes from either plant or animal [1]. It can be decomposed over time by microorganisms like bacteria and fungus. Carbon dioxide, water and methane are the major by-product from this decomposition process [2]. The cellulose and hemicellulose are carbohydrates that can be breakdown by enzymes and acids and then fermented to some usable product like ethanol renewable electricity, fuels and biomass-based product [3].

Some of the wastes can be decomposed even if it just left for a period of time. This process happened due to the presence of microorganisms that responsible for the decomposition in the soil [4]. The microbial population of soils is comprised of 5 noteworthy gatherings including bacteria, actinomycetes, parasites, and green growth protozoa. Among these gatherings, bacteria are the most significant organism for deteriorating waste [5]. These microbial are able to degrade the waste for their own food and also make it into available forms of food or nutrients for the nearby plants [6]. That's why the plants are able to survive when there is some waste that gives the nutrients to the plants. It is because each of the living things have the cellulase that is able to degrade the cellulose to convert the food into available form [7]. As an example, the empty fruit bunch is used to supply nutrients to the plants. However, these empty fruit bunch cannot be used as fertilizer or soil amendment if it is not decomposed by the bacteria. Bacteria will break down (or decompose) dead organisms, animal waste, and plant litter to obtain nutrients. These microbes don't just eat this nature's waste, but they recycle it. The process of decomposition releases the nutrient such as carbon, nitrogen, and phosphorus that readily to be uptake by plants. The microbes survive in soil by getting the food from the plants, and in the same time breaking down the cellulose of the plants which produce some elements that is needed by the plants [8]. It is just a life cycle of the living things that depend on others. The microbial population in the soil improve the soil fertility by upgrading the environmental nitrogen fixation, delivering phytohormones, upgrading root surface regions to encourage nutrients uptake, and as an assembling and solubilizing inaccessible organic and inorganic nutrients [9]. The plant comprises of cellulose that act as the plant's primary building material [10]. Cellulose will undergo either chemical or enzyme hydrolysis to form soluble sugar. These sugars, in turn, can be used by microbes in the production of biofuels, single cell protein, methane or other oxidation products [11]. In reality, the sophisticated cellulose degradation technology for recycling paper, carton, sawdust and more is extremely developed [12].

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This study focuses on identifying the number of effective microbes by varying the sample of organic waste (compost product, decayed wood and oil palm fronds mix with empty fruit bunch) that theoretically will speed up the decomposition process and at the same time were able to solubilize the phosphate by applying this microbe as booster in composting process. Analysis for (Phosphate Solubilizing Bacteria) PSB was performed by treating the grown bacteria in NBRIP media [13]. Then, the isolates with positive result for NBRIP plate will be further tested on (Carboxymethyl Cellulose) CMC media for identifying the ability of the isolate to produce cellulase enzyme. Lastly, the selected bacteria that were grown in CMC will be treated with Congo Red and NaCl solution to confirm that the bacteria were truly effective microbes [14]. The effective microbes will produce the halo zone formation around the bacteria.

II. MATERIAL AND METHODS

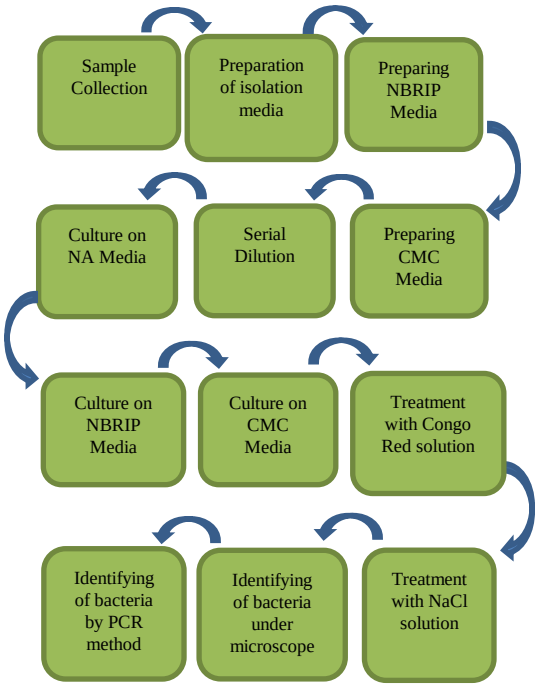

Fig. 1. Flow chart of the experiment

A. Sample collection

Samples were taken from the solid wastes that loose and wet in texture. The samples are compost product (planting media), decayed wood and mixture of oil palm fronds and empty fruit bunch. These samples are commonly found in planting field that is suitable to be treated as nutrient supply for plants.

B. Isolation

The samples were collected from the sampling site were air dried in sterile Petri dishes at room temperature (28 °C) for four hours prior to isolation. Soil samples undergo serial dilution up to 10^{-5} by using Ringer's solution and spread plating were done onto actinomycetes isolation agar, soil extract agar, Luria bertani agar, Nitrogen free media and Nutrient agar. All the isolation plates were incubated at 28 °C up to two weeks. All the isolation media were supplemented with antifungal, cycloheximide (50 µg/ml) to reduce the growth of fungal. All the isolates were purified onto nutrient agar.

All purified bacterial cultures preserved for further testing. All the bacterial cultures were subcultured to fresh medium and stored at low temperature incubation. Before storage, bacteria were allowed to grow by incubating them overnight at 28 °C. There are 2 ways of storage, which are short- and long-term storage that differ in their storage temperatures.

A. Short term storage can only store up to 4 months before the next subculturing. This method needs pure culture to re-streak on non-selective media, NA agar slant at 28 °C.

B. Long term storage isolates can be stored up to several years. Few colonies that are already grown on NA were scrapped and kept into glycerol 20 % (v/v) and kept at -20 °C for long term preservation.

C. Screening for Phosphate Solubilizing Bacteria

Table I: Chemical composition of Phosphate Solubilizing Bacteria Media

Chemical composition	Weight
Glucose (Bio Basic Canada)	10 g
Calcium phosphate (Sigma)	5 g
Magnesium Chloride Hexahydrate (Analar)	5 g
Magnesium Sulfate Heptahydrate (Analar)	0.25 g
Potassium chloride (BDH)	0.2 g
Ammonium sulfate (Univar)	0.1 g

The screening for phosphate solubilizing bacteria was carried out by using National Botanical Research Institute's Phosphate media (NBRIP) [15].

Two strains per plate were stabbed in triplicate using sterile toothpicks. The halo zone formed and colony diameters were measured after 2 weeks of incubation on the NBRIP media plates at 28 °C. The ability of the bacteria to solubilize insoluble phosphate was described by the solubilization index [= the ratio of the total diameter (colony + halo zone) to the colony diameter. All the chemicals in the Table I were mixed and autoclaved at 121 °C for 15 minutes.

D. Screening for cellulase producing bacteria

Table II: Chemical composition of Phosphate Solubilizing Bacteria Media

Chemical composition	Weight/ Volume
Monopotassium phosphate (Analar)	1.0 g
Magnesium Sulfate Heptahydrate (Analar)	0.5 g
Sodium chloride (System)	0.5 g
Iron (II) Sulfate Heptahydrate (Univar)	0.01 g
Maganese Sulfate Heptahydrate (Analar)	0.01 g
Ammonium nitrate (Analar)	0.3 g
Carboxy methyl cellulose (Sigma)	10.0 g
Agar (Difco)	12.0 g
Distilled water	1000 ml

Screening for cellulase producing bacteria was performed according to Kasana, [16]. All the chemicals in the Table II were mixed and autoclaved at 121 °C for 15 minutes. All the isolates were grown in nutrient broth and incubated at 28 °C for three days. 5 microliters of grown culture were spot platted on CMC agar and incubated at 28 °C for 3 days. After 3 days, all the plates were flooded with 0.1 % Congo Red for 15 minutes and then followed by 1M NaCl for 15 minutes.

Production of cellulase was indicated by the formation of halo zone around the colony.

E. Gram staining

Bacterial smear was prepared on the glass slide and heat fixed. The fixed slide was flooded with crystal violet for 1 minute. After 1 minute, the crystal violet was rinsed using distilled water. Then, the slide was flooded with iodine for 1 minute. After 1 minute, the iodine was rinsed off with distilled water. The slide was then flooded with acetone for 3 seconds. The acetone was rinsed off using distilled water. For the counterstain, the slide was flooded with safranin for 30 seconds. Safranin was rinsed off with distilled water prior to the observation under the light microscope [17].

F. Identification of bacteria based on 16S rRNA gene sequencing

DNA extraction

Pure cultures of purified isolates grown on NA at 28 °C were used for total genomic DNA extraction was done using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany). Bacterial biomass was suspended in 180 µl pre-lysis buffer (Buffer T1) supplemented with lysozyme (20 mg/ml) and incubated for one hour at 37 °C. Suspensions were then incubated for two hours at 56 °C after the addition of 25 µl Proteinase K (20 mg/ml). Complete lysis of the suspension was obtained after the addition of 200 µl lysis buffer (Buffer B3) and incubation at 70 °C for 10 minutes. Binding condition of the lysates was adjusted with addition of 200 µl molecular graded ethanol (Merck, Germany). Pure genomic DNA was eluted from the binding silica membrane after filtration and washing. Concentration of the genomic DNA was determined using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, United States of America). Genomic DNA was also examined for integrity and quality with 1.0 % (w/v) agarose gel electrophoresis, ran for 30 mins at 100 Volt in SB buffer. The genomic DNA was stored at -20 °C until further use.

16S rRNA Gene Amplification, Sequencing and Analysis

Selected representative isolates were subjected to 16S rRNA gene amplification for the purpose of identification. 16S rRNA gene fragment amplifications was carried out using SwiftTM Maxi Thermal Cycler with a final volume of 50 µl containing 50 ng of genomic DNA, 10 µl of 5X Green GoTaq Flexi Buffer, 1 µl of 10 µM of each primer and 0.15 ul of Go Taq Flexi DNA Polymerase (Promega, United States of America).

The expected 1500 bp amplified 16S rRNA gene fragment was purified using a NucleoSpin Gel and PCR Clean-Up kit (Machrey-Nagel, Germany) according to standard protocol in the manual. The purified products were examined with 1.2 % (w/v) agarose gel electrophoresis, ran for 60 minutes at 100 Volt in SB buffer. Concentration of the purified product was determined with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). Sequencing of the amplified products was carried out by commercial sequencing provider 1st BASE (BASE Life Science Holdings, Malaysia) using BigDye® Terminator chemistry on the Applied Biosystem 3730xl DNA analyzer.

The 16S rRNA gene sequences were visually checked using Sequence Scanner version 1.0 (Applied Biosystem) and at least 1400 bp were aligned with the comparative sequences of reference for the type-strains retrieved from the GenBank database through the EzTaxon server using MEGA6.0.

III. RESULT AND DISCUSSION

A. Gram stain

Generally, there were two types of cell morphology observed under light microscope, which are rod and coccus. From 150 isolates, 70 isolates were gram stain negative and rod shape, only 3 isolates were gram negative coccus. A total of 50 isolates were gram positive rod and 27 isolates were gram positive cocci. Fig. 2 are the photos of each type of cell morphology observed under light microscope after gram staining procedure.

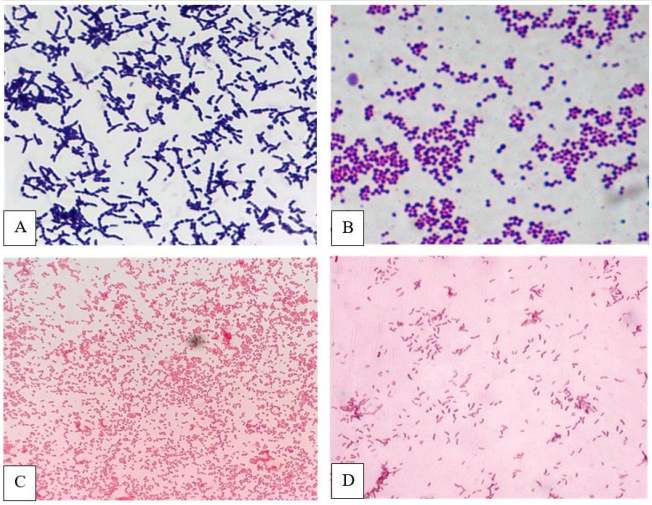

Fig. 2. Different types of cell morphology of the isolated strains. (A) Strain which is gram positive rod-shape; (B) strain which is gram positive cocci; (C) strain which is gram negative cocci; (D) strain which is gram negative rod.

B. Screening for phosphate solubilizing bacteria

In this experiment, a total of 150 of bacterial strains was isolated and purified from the rhizosphere of oil palm. All isolates were tested for their phosphate solubilizing activity using NBRIP medium supplemented with 1.5 % (w/v) Bacto-agar. The phosphate solubilization indexes of the isolates were varied from 0.9 to 6.5. Solubilization index is calculated by total diameter (colony + halo zone)/colony diameter. The isolate SN40 showed the highest phosphate solubilization with index (6.5). Total of 22 isolates out of 150 (14.67 %) are able to solubilize phosphate.

Formation of halo zone around the colony indicates positive result, while no changes in the medium indicate negative result (Fig. 3).

Phosphate solubilization ability of all oil palm rhizosphere isolates was further evaluated in a NBRIP liquid broth medium. All isolates showed consistent results in solubilizing phosphate for both methods; either liquid broth or agar.

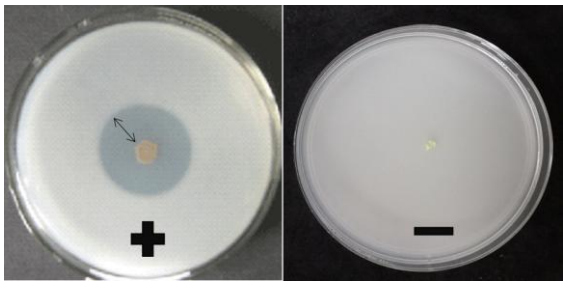

Fig. 3. Screening for phosphate solubilizing bacteria using

free NBRIP medium. Halo zone development (left) on the media showed positive result of phosphate solubilizing test. No halo zone development (right) showed negative result of phosphate solubilizing activity.

Fig. 4. Percentage of phosphate solubilizing bacteria

Based on Fig. 4, isolated bacteria from compost product is at the top for effectiveness in solubilizing phosphate. The observation from compost product shows 53 % which is the highest rate among the other samples. It means the bacterial that contain in the compost product is more effective in solubilizing phosphate as compared to other samples. The bacteria that was isolated from sample of decayed wood is about 44 % which is the second highest. However, the lowest rate for solubilizing phosphate bacteria sample is from the oil palm frond mix with empty fruit bunch that is about 40 %. It is a little bit less than the sample isolated from decayed wood.

Regarding this observation, the content of bacteria in these three samples are different which the different population of bacteria that was grown in NA media can detect. The sample of compost product is 30 plates, decayed wood is 27 plates and for oil palm frond + empty fruit bunch is 15 plates. That's why the sample of compost product has the highest percentage rate of bacteria in solubilize phosphate. Besides that, the different number of plates that show the positive reaction is due to different rate of contamination during the process of culturing the bacteria.

C. Screening for cellulose producing bacteria

All 150 isolates were tested for their capability to produce cellulase enzyme. Only 2 isolates are able to produce cellulose (SN45 and SA10). Formation of halo zone indicates positive reaction and no halo zone form indicate no cellulase production by the isolate.

From all the isolates, only SA10 has multifunction characteristic, which are phosphate solubilizer and cellulase producing bacteria.

From the screening result, isolate SA10 was chosen for 16S rRNA nucleotide sequence analysis. The nucleotide sequence was identified by the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon-e server: http://eztaxon-e.ezbiocloud.net. Strain SA10 was closely related to *Streptomyces sanglieri.*

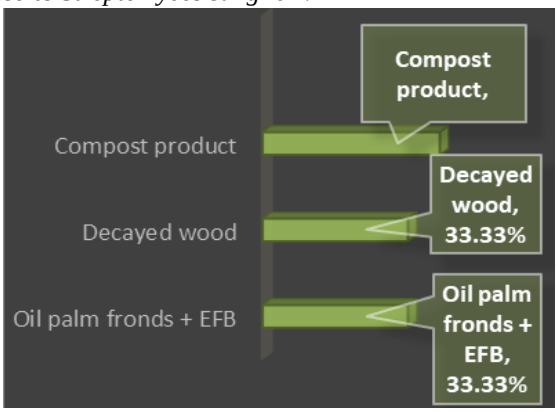

Fig. 5. The proportion of cellulose producing bacteria

Based on Fig. 5, the highest percentage of bacteria ability to degrade the cellulose was the compost product as compared to the other samples. The bacteria that isolated from compost product reach about 40.63 % in decomposing cellulose. Different with sample of decayed wood and oil palm frond mix with empty fruit bunch, these two sample shared a similar percentage of bacteria ability in decomposing cellulose. The percentage for these two samples was about 33.33 %, which shows the number of bacteria that was able to solubilize phosphate, and reacted to cellulose.

In overall, these three samples have bacteria that are able to degrade cellulose and also able to solubilize phosphate but different in its amount. Applying the effective microbes into pure organic waste surely will speed up the decomposition process and promote the nutrient uptake by plant especially phosphorus. The ability of the effective microbes in solubilizing phosphate will increase the root functioning production that is most needed by a new plant.

The sample demonstrates favorable results on the bacteria that form the halo zone. The cultured sample was isolated from the compost product which means the bacteria contained in that compost product was able to solubilize phosphate and reacted to cellulose for decomposition process. The halo zone was about 5.7 cm of diameter. The larger the size of halo zone formation, the higher the rate of effectiveness of the microbes.

D. Microbial Identification

After identifying the most efficient bacteria that can solve phosphate and degrade cellulose, the bacteria were identified and characterized. The grown bacteria in both plate of NBRIP and CMC will be identified under the microscope.

The result observed on the microscope will be carried out using PCR method for confirmation. Based on the DNA sequencing method, by PCR, the studied sample matches 100% to *Streptomyces sanglieri*. *Strepyomyces* are environmentally filamentous Gram positive, commercially valuable bacteria. Actinomycetes are known to generate a broad range of biologically active compounds, particularly those belonging to the genus *Streptomyces and Micromonospora* are prevalent genera from actinomycetes are able to solubilize phosphate.

IV. CONCLUSION

As a conclusion, the organic waste containing bacteria are able in solubilizing phosphate and degrades cellulose. The effective bacteria are grown on both plates that are NBRIP and CMC media. This study shows that NBRIP media helps in finding the phosphate solubilizing bacteria and CMC media helps in finding the cellulase-producing bacteria. Based on the observation, the bacteria which was inoculated from compost product is better in the ability of solubilizing phosphate and degrading cellulose as compared to bacteria from decayed wood and oil palm frond mix with empty fruit bunch. On the other hand, studies on organic waste can be used for screening and isolation of effective microbes into large amount. The higher number of effective microbes applied, the higher rate of rapid decomposition process and crop productivity to be achieved.

In order to make the screening and isolating process of effective microbes, precaution is important to ensure that desired result will be obtained. It is because, handling the bacteria is quite hard as compared to fungi. There are many factors that can lead the result to being contaminated. Further research relating to multifunctional use of the effective microbes must be done. Thus, application of *Streptomyces sanglieri* and other effective microbes can be practiced widely to the waste especially organic waste.

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