Isolation and Identification of Effective Microorganisms from Water Hyacinth Biofertilizer

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Abstract. The use of microorganisms to promote growth and increase crop production has begun to be studied. Microorganisms that isolated from biofertilizers vary according to the source of organic matter. One of the biofertilizer quality determinants is the type and number of populations of microorganisms. This study aims to know the type and abundance of microorganisms, as well as nutrient content in water hyacinth biofertilizers. The experiment was conducted at the Greenhouse and Laboratory of Food Crops Cultivation, Payakumbuh State Agricultural Polytechnic, Limapuluh Kota Regency, West Sumatra from March to July 2019. The experimental stages included (1) Manufacture water hyacinth biofertilizers, the experiments using Completely Randomized Design (CRD) with four measurements of water hyacinth treatment in biofertilizer, A = 25\%, B = 50\%, C = 75\%, and D = 100\% and five replications, (2) Isolation of microorganisms from biofertilizers using pure plate and streak plate methods. Eleven bacterial species and two fungal species were identified using molecular analysis based on 16S rRNA gene fragments. Effective microorganisms that play a role in the degradation of biofertilizers are Pseudomonas aeruginosa, Bacillus subtilis, Trichoderma asperellum, and Trichoderma harzianum. Biofertilizers contain C-organic 3.61-3.92\%, N 0.166-0.278\%, C/N 14.10-21.75, P2O5 0.347-0.729\%, K2O 1.422-2.090\%, and Ca 0.140-0.259\%.

1. Introduction
Biofertilizer is a fertilizer product that is formulated to contain one or more microorganisms that can improve plant nutrient status either by replacing soil nutrients and or by increasing the availability of nutrients for plants and or by increasing the relationship of plants with bacteria [1]. Biofertilizers increase the rate of nutrient mineralization which is insoluble in the soil through N and P fixation [2] and can replace chemical fertilizers [3].

Several microorganisms genera have been isolated and identified to have the ability as biofertilizers, including genera of Bacillus bacteria such as Bacillus subtilis [4] and Pseudomonas aeruginosa including P. solvent groups Trichoderma fungi such as Trichoderma asperellum [4]. The potential of microorganisms as biological fertilizer is due to its ability to stimulate plant growth through nutrients N, P, and K which are dissolved by these microorganisms.

The role of microorganisms as plant growth stimulators (PGPR) and potential plant disease suppressors in the development of sustainable crop production systems [5]. PGPR inhabits the roots of biofertilizers, including genera of Bacillus bacteria such as Bacillus subtilis [4] and Pseudomonas aeruginosa including P. solvent groups Trichoderma fungi such as Trichoderma asperellum [4]. The potential of microorganisms as biological fertilizer is due to its ability to stimulate plant growth through nutrients N, P, and K which are dissolved by these microorganisms.

The role of microorganisms as plant growth stimulators (PGPR) and potential plant disease suppressors in the development of sustainable crop production systems [5]. PGPR inhabits the roots of plants and provides positive effects ranging from the mechanism of direct influence to indirect effects. PGPR can support plant health by increasing soil fertility, nutrient availability and absorption [6]. The
role of biological fertilizers will be optical if the microorganisms in it are mutually synergistic and do not inhibit each other's growth, thus forming a consortium of microorganisms.

The role of beneficial bacteria directly on plants as plant growth stimulants, by the mechanism of nitrogen binding, P and K dissolution, siderofor production, phytohormone production in the form of IAA [7]. The role of saprophytic fungi in the rhizosphere area indirectly is to protect plants from fungal pathogen attacks [8].

Identification and characterization of microorganisms in biofertilizers are important to know the type and number of microorganism populations that can survive and play a role in the decomposition of organic matter, as well as increasing the availability of nutrients for plants. This study aims to determine the name of the genus and the abundance of microorganisms contained in water hyacinth-based biofertilizers.

2. Materials and Methods
The research was carried out in the Greenhouse and Laboratory of the Payakumbuh State Agricultural Polytechnic from March–July 2019.

2.1. Manufacture of Biofertilizer
Making biofertilizers is done at the Greenhouse using a Completely Randomized Design (CRD) with 4 treatments and 5 replications. The treatment is a comparison between cow faeces and water hyacinth: 75% cow faeces and 25% water hyacinth (A), 50% cow faeces and 50% water hyacinth (B), 25% cow faeces and 75% water hyacinth (C), 0% cow faeces and 100% water hyacinth (D), so there are a total of 20 treatments.

Cow faeces is taken that is pure and has not been mixed with soil or sand that comes from a cow pen. Then weigh 750 g, 500 g, and 250 g each with 5 replications. Then put in a plastic jar and air-dried for 24 hours. After 24 hours add 2 liters of distilled water and ferment for 40 days.

Freshwater hyacinth chopped until smooth. Then weigh 250 g, 500 g, 750 g and 1000 g each with 5 replications. Enter the water hyacinth into the fermentation of cow faeces according to treatment A, B, C, and D. Add bone flour and shell flour as a source of Ca, shallots and molasse as much as 1% in each treatment. Fermentation of organic fertilizer for 30 days.

2.2. Isolation and Identification of Microorganisms
Isolation of microorganisms in fermented biofertilizers was taken from each treatment. Take 5 ml of biofertilizer solution and bring it to the laboratory, then culture on Sodium Agar and Pikovskaya's media. Bacterial isolation was carried out by the pour cup and scratch plates. Furthermore, the solution is taken 1 ml and grown on a Petri dish containing NA agar media. See the development of bacteria on the third day. Bacteria that grow are separated and purified. Then identified the bacteria found. Identification of the characteristics of pure bacterial isolates based on the shape of the colony, edge shape, surface, smooth rough surface, surface color, pigment color, and bacterial body thickness.

The isolate was identified using EzTaxon server molecular analysis [9] based on 16S rRNA gene sequence data. The bacterial genome DNA extraction was carried out using the GES method [10]. Amplification of 16S rDNA fragments was carried out using GoTaq (Promega) with a pair of general primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [11]. The sequencing result data is then processed with the Bioedit program [12].

A pure culture scratch from Petri dish was transferred to a PCR tube and 50 µl nuclease-free water was added and boiled for 10 minutes [13], the resulting solution would then be used as a DNA template for PCR. Make a PCR mixed solution consisting of a 16S 0.5 µl Primary Forward reagent, a 0.5 µl Reverse 16S Primary Primer, a 12.5 µl GoTaq Green Master Mix, a 1 µl DNA template, and a 10.5 µl Nuclease free water.

The PCR mixture was put into the PCR machine with the following settings: initial denaturation of 95oC for 30 seconds, followed by 30 cycles consisting of denaturation at 95oC for 30 seconds, annealing at 50oC for 30 seconds and elongation of the strand at 72oC for 90 seconds. After 30 cycles ended, proceed with elongation of the final strand at 72oC for 10 minutes. The PCR results were then
sequenced by the Sanger method to determine the sequence of DNA strands and were matched using the Basic Local Alignment Search Tool (BLAST) [14].

2.3. Calculation of Microorganism Population
The bacterial population on NB media was calculated using the serial dilution method from dilutions 10-1, 10-2, 10-3 to 10-6 dilutions. Each dilution was piped 0.1 ml and put into NB media, then incubated at 37°C for 48 hours. The fungus population on THSM media. To calculate the population used the formula: Population density = Number of colonies x 1 / (Dilution Factor) x (CFU/ml)

2.4. Observation
During the fermentation, stirring is done once a week so that the water hyacinth can decompose perfectly. The temperature of biofertilizers is observed every day in the first week and once 3 days in the next 3 weeks.

After 30 days of fermentation, observations of microbial species and species were observed, the number of microbial populations, nutrient content N, P2O5, K2O, Ca, C-organic, and C / N of liquid biofertilizer. Chemical analysis of biofertilizers includes nutrients N (Kjeldahl Method), P (Spectrophotometry), K (Flamephotometry), and C-organic (Loss on Ignition). Nutrient data were analyzed statistically and continued with the DNMRT test.

3. Result and Discussion

Based on biological fertilizers made with basic ingredients of cow faeces and water hyacinth (25%, 50%, 75%, 100%) the results obtained are as shown in Table 1. Found 2 types of bacteria and 2 types of dominant fungi in biofertilizer.

| Microorganism types | Microorganism species       | Homology (%) | Number of colonies (CFU/ml) |
|---------------------|----------------------------|--------------|-----------------------------|
| Bacteria            | a. *Pseudomonas aeruginosa* | 99.0         | 2.8 x 10^8                  |
|                     | b. *Bacillus subtilis*     | 99.7         | 4.3 x 10^7                  |
| Fungi               | a. *Trichoderma asperellum*| 100.0        | 74.25 x 10^4               |
|                     | b. *Trichoderma harzianum* | 100.0        | 23.25 x 10^3               |

Pseudomonas aeruginosa was found as a bacterium with the most dominant population density (2.8 x 108 CFU/ml) in water hyacinth biofertilizer, this is due to P. aeruginosa bacteria having high adaptability and synergy to other microorganisms so that these bacteria can multiply themselves in various conditions. According to [15] liquid biofertilizers containing Pseudomonas spp are N2-inhibiting bacteria applied by soaking seeds or spraying them to plants so that they can increase the population of these bacteria in leaves, stems, and roots of rice plants. Literature [16] stated that P. aeruginosa has the potential as a PGPR with immersion method in seed suspension and coating, and gives the same results with inorganic fertilizer treatment (without P. aeruginosa). The results of [17] research on the use of P. aeruginosa in castor can act as biocontrol agents and synthesis of PGP compounds such as IAA, GA3, ACC deaminase, siderophore, and NH3.

P. aeruginosa is one of the genera Pseudomonas which is used as a biomaterial in the biosorption of Copper (Cu), Cadmium (Cd), and Plumbum (Pb) metals [18]. The social biology aspect of P. aeruginosa is having the ability to interact and influence other bacteria, fungi, and multicellular organisms in various biological contexts [19].

In the water hyacinth biofertilizer, Bacillus subtilis includes bacteria with a dominant population density (4.3 x 107 CFU/ml), because most aquatic plants are suitable habitats for the growth of Bacillus spp. According to [20] the water hyacinth species of Bacillus spp is a native microorganism of this plant. Bacillus genera are among the biological control of bacterial agents because many Bacillus species can maintain plant health. Antibiotics produced by these bacteria are effective in controlling plant pathogens.
and the diseases they cause [21]. The same opinion by [22] that the role of Bacillus sp is to inhibit the growth of pathogenic microorganisms. Research by [23] B. subtilis acts as an induction agent for rice resistance to leaf blight.

B. subtilis is one of the bacteria used as a biofertilizer. Some Bacillus genera can bind N from the air, dissolve P and K, produce growth stimulants, and suppress the growth of pathogens [3]. In the process of fermentation, Bacillus species play a major role in degrading cellulose, so it is called cellulolytic bacteria. According to [20] that the more variations of bacteria that work in synergy, the more enzymes involved in the fermentation process and speed up fermentation.

Bacteria from the genera Pseudomonas and Bacillus have the potential to bind N nutrients, increase the solubility of P and K (biofertilizer), produce IAA compounds as growth stimulants (biostimulants), and suppress the growth of pathogens (biocontrol). Pseudomonas and Bacillus live in synergy with one another and interact with other microorganisms. Literature [7] emphasized that bacteria that promote plant growth are bacteria that can increase plant growth and protect plants from disease through various mechanisms.

Besides bacteria, in the biofertilizer found fungi from the genera Trichoderma, especially T. asperellum and T. harzianum. There are similarities between the discovery of B. subtilis and T. asperellum in this biofertilizer with solid biofertilizers made from water hyacinth from previous studies [4]. Trichoderma is a dominant fungus compared to other fungi because Trichoderma spp is very adaptive to the environment and its growth rate is faster than other fungi. Genera Trichoderma spp is well known as a biocontrol agent, including T. asperellum strain GDFS1009. The researcher [24] found several strains of T. asperellum are antagonistic to Fusarium disease in tomato plants. According to [25] Trichoderma is an opportunistic fungus of saprophytes found in the rhizosphere of plants. Some Trichoderma strains function as biocontrols also can produce compounds such as hormones that can stimulate plant growth.

T. asperellum strain GDFS1009 produces chitinase, glucanase, protease, and xylanase. Xylanase specifically secreted by this fungus is a compound that plays a role in inducing plant resistance [26]. The [27] isolated fungi in mangrove sediments and succeeded in identifying the dominant fungi, namely T. asperellum, T. harzianum, and T. longibrachiatum. All Trichoderma strains showed biocontrol actions with an average growth rate of 0.1207 cm/hour or 4 times greater than the growth rate of Fusarium spp of only 0.031 cm/hour through nutrition and space competition. Added by [26] that the mechanism of Trichoderma spp biocontrol includes competition and mycoparasitism followed by stimulation of plant resistance and immunity against pathogens.

Trichoderma is an antifungal fungus that inhibits the growth of pathogenic bacteria, fungi, and yeast [28]. However, the characteristics and antifungal chitinase activity of the Trichoderma species are relatively different. Chitinase T. asperellum strain PQ 34 can inhibit the growth of 2 S. rolfsii strains and 9 mycology Colletotrichum sp [29].

Generally, biological products use Trichoderma genera including T. harzianum (83%) combined with streptomycetes (such as B. subtilis, P. aeruginosa), endomicrocorization and ectomycorrhizae into a compound [30]. According to [28] T. harzianum can improve soil nutrition through the decomposition and biodegradation of organic matter. The addition of T. harzianum in biological products is to improve the protective properties of plants or increase the biological activity of chitosan [30].

Both bacteria and fungi in Table 1 are effective microorganisms, because of the success of these bacteria and fungi to survive, adapt, and work together to decompose water hyacinth.

Biofertilizers with different sizes of water hyacinth will produce different nutrient strains. Nutrient content Liquid biofertilizer shows low N nutrient, medium-very high P nutrient, low K nutrient, low Ca nutrient, and low organic C, but C/N is at 10-20 which is close to C/N soil. Nutrient content in water hyacinth biofertilizers can be seen in Tables 2 and 3 below.
Table 2. Nutrient content of C-organic, Nitrogen, and C/N in biofertilizer with different doses of water hyacinth

| Water hyacinth doses | C-organic (%) | Nitrogen (%) | C/N   |
|----------------------|---------------|--------------|-------|
| Water hyacinth 25%   | 3.61 a        | 0.166 c      | 21.75 a |
| Water hyacinth 50%   | 3.75 a        | 0.175 c      | 21.43 a |
| Water hyacinth 75%   | 3.78 a        | 0.243 b      | 15.56 a |
| Water hyacinth 100%  | 3.92 a        | 0.278 a      | 14.10 a |

*The numbers in the column followed by the same small letters are not significantly different at 5% level of DNMRT test*

The total C-organic and N content of biofertilizers increases with increasing amounts of water hyacinth. The total N-content of biofertilizer is low due to microorganisms still utilizing existing N, N from the air cannot be bound because the fermentation container is in a closed condition. The C/N ratio of biofertilizers is quite good between 14.10–21.75 which is close to the C/N ratio of soil. Bacteria have a role in binding N from the air, but the fertilizer fermentation container inhibits this mechanism. The role of bacteria is very dominant as a decomposer of organic material derived from cow dung and water hyacinth.

Statement of [17] that P. aeruginosa produces PGPR which can synthesize NH3, HCN, and siderophore compounds that increase nitrogen accumulation in plants. According to [3] biosorption mechanism by B. subtilis by releasing extracellular polymeric substances (EPS) to bind heavy metals.

Table 3. Nutrient content P, K, and Ca of biofertilizer with different doses of water hyacinth

| Water hyacinth doses | P2O5 (%) | K2O (%) | Ca (%) |
|----------------------|----------|---------|--------|
| Water hyacinth 25%   | 0.347 d  | 1.422 d | 0.140 d |
| Water hyacinth 50%   | 0.528 c  | 1.553 c | 0.218 b |
| Water hyacinth 75%   | 0.729 a  | 1.786 b | 0.259 a |
| Water hyacinth 100%  | 0.627 b  | 2.090 a | 0.164 c |

*The numbers in the column followed by the same small letters are not significantly different at 5% level of DNMRT test*

The P2O5 and K2O content increase with increasing water hyacinth dose. Bacteria P. aeruginosa and B. subtilis can dissolve P and K compounds which are bound to organic matter into minerals. In this experiment, the K2O content of liquid biofertilizer was 1.422–2.090%, higher than the hyacinth solid fertilizer of 1.48–1.78% [4]. The [31] reported that most of the Bacillus spp genera can be used as biofertilizer because of the ability of these bacteria to dissolve phosphate, even certain species can dissolve Zn micronutrients [3] and Si.

Nutrient K is the main nutrient with the greatest composition of biofertilizer compared to other nutrients, this may occur due to the presence of solvent bacteria K. Statement of [32] that several species of Bacillus bacteria can dissolve silicate minerals and release K nutrients in the form organic and inorganic acids. So that biofertilizers that contain solvent K bacteria (BPK) can be used as a substitute for chemical fertilizers.

The highest Ca nutrient is obtained at the rate of water hyacinth 50% -75%. According to [3] phosphate solubilizing bacteria dissolve the calcium phosphate complex by releasing organic acids and acidifying the surrounding environment, so P nutrient solubility is also affected.

Overall microorganisms found in biofertilizers play a role in contributing N, P, and K nutrients. The mechanism of bacteria in dissolving several major nutrients indicates their role in stimulating plant growth [32]. The characteristics of beneficial bacterial inoculants will efficiently improve interactions between soil, plants, and microbes [7]. The provision of biofertilizers is an effective way to supplement crop nutrient needs [2]. Statement of [17] the use of P. aeruginosa in plants can save 50% use of inorganic NPK fertilizer.
4. Conclusions
Pseudomonas aeruginosa, Bacillus subtilis, Trichoderma asperellum, and Trichoderma harzianum are dominant and effective microorganisms in water hyacinth biofertilizer. These bacteria and fungi synergize and interact with one another so that they survive in a consortium of microorganisms. These microorganisms have the potential as biofertilizers, biocontrols, and biostimulants for soils and plants.

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