Production of Nanobiocontrol Agent in Molasses and Tofu Liquid Waste Media by Streptomyces sp. TT10

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Abstract. The objective of this study has processed the waste of agriculture into high economic value materials using fermentation technology by Streptomyces sp. TT10 in the form of new materials in Nano size. In this study, anti-phytopathogenic agents would be produced using fermentation technology in the form of antifungal that fought fungi that often infected strawberries. The research series consisted of the fermentation process for seven days, 30°C, pH 7 on molasses and tofu liquid waste medium. The broth of fermentation was extracted using ethyl acetate. Concentrated ethyl acetate extracts were ground using a shaker mill PPF-UG to obtain the size up to 100 nm. Furthermore, Nano sizes were characterized using (particle size analyzer) PSA and (scanning electron microscope) SEM. The anti-phytopathogenic agent was analyzed by agar diffusion and microdilution. The results showed that activity of ethyl acetate extract of Streptomyces sp. TT10 was increased up to 80% after milling process, against pathogenic strawberry that is Penicillium sp., Aspergillus sp., Rhodotorula sp., Kluyveromyces sp., Candida krusei, Candida pseudotropicalis and Fusarium oxysporum with inhibitory diameter (mm), minimum inhibition concentrations (MIC) (ppm) and the minimum fungicidal concentration (MFC) (ppm) value (%) are respectively (82.85;50;50), (81.29;50;50), (82.85;50;50), (83.87;50;50), (74.07;50;50), (79.95;50;50), and (83.33; 50; 50).

Keywords: molasses, tofu liquid waste, fermentation technology, nanotechnology, pathogenic strawberry

1. Introduction
The need for fruits as a source of vitamins and minerals has become an urgent matter for current conditions. Tropical fruits provide many benefits for surviving of creatures in the world. Increased production of tropical fruits is very dependent on how to handle from where it grows to distribution in
the field. Maintaining tropical fruits from pathogen is an effort to maintain the quality of tropical fruits [1].

Based on this, it is necessary to look for agents in the form of secondary metabolites of *Streptomyces sp*. TT10 to overcome diseases in tropical fruits (case study: strawberry fruits). *Streptomyces sp.* was known as a producer of secondary metabolites such as antimicrobial, antiviral and anti-fungi [2-7]. *Streptomyces sp.* TT10 is isolated from Indonesian volcanic soils.

The agricultural industry produces primary and secondary products as renewable resources that support other important industrial sectors outside agriculture. Agricultural secondary products that are not used in the industry still contain high nutritional value to produce secondary metabolites in the form of antifungal, antibacterial, antiviral and antioxidant. These metabolites can be applied as biological control agents in general having an inhibitory mechanism against pathogens through its metabolites [8].

The objective of this study was processed the waste of food and agricultural become high economic value materials using fermentation technology and find the formula of anti-pathogenic for tropical fruits (case studies of strawberry fruit) in the form of new materials in Nano size from *Streptomyces sp.* strain TT10.

Nanoparticles are microscopic particles that have the same size as atoms to molecules. Nanoparticles are ultrafine particles with a length greater than 0.001 micrometers (1 nanometer) and smaller than 0.1 micrometers (100 nanometers) (ASTM). In general, nanoparticles are also known as collections of atoms that bind to each other with a diameter of less than 100 nm [9]. Nanoparticles have very small sizes or even invisible, but have a very large influence on living things and the environment. These particles help the development of technology, improve health and well-being, and have helped maintain environmental stability [10,11].

Nanoparticles have a large surface area due to the small geometry size. This large surface area causes nanoparticles to become highly reactive and easily bind to each other to form larger particles to become a macro size. To get nanoparticles, it can be through bottom up and top down methods. The bottom up method is how to form nanoparticles by making the smallest atoms or molecules. This method is generally used by nature to form nanoparticles. The top down method is how to form nanoparticles with mechanical equipment, in general with the milling process. In the process, the material will be reduced to Nano size [12,13].

One method to get Nano scale material is to use high energy milling method, which is a technique that uses collision energy between crushing balls and rotating chamber walls in a certain way. The ball in the chamber will collide with each other which causes the particle to break to reach Nano size [14].

There are several variables that must be considered to get the desired particle size, such as: type of grinder, duration of milling, temperature of grinder, type of material to be milled, number and type of grinding media material, ratio of mass of grinding and powder media, and frequency [15].

In this study Nanobiocontrol agents will be produced based on fermentation technology and nanotechnology through self-assembly or biosynthesis and top down (milling process) method in the form of new material anti-fungi that can fight pathogenic strawberries fruits.

2. Experimental method

2.1. Microorganisms

2.1.1. Secondary metabolite producer. *Streptomyces sp.* TT10 is used as a producer of anti-pathogenic metabolite regenerated on potatoes dextrose agar (PDA) media Difco.

2.1.2. Pathogenic Strawberry. The microorganisms used for testing were *Penicillium sp.*, *Aspergillus sp.*, *Rhodotorula sp.*, *Kluyveromyces sp.*, *Candida krusei*, *Candida pseudotropicalis* and *Fusarium oxysporum* are isolated from infected strawberries. All isolates were grown, maintained, and tested in potatoes dextrose agar (PDA) Difco as well as in potatoes dextrose broth (PDB) Difco media.
2.2. Sporulation Process, Fermentation Process and Purification of Fermentation Broth. Sporulation process of *Streptomyces sp.* TT10 was carried out on starch (10 g/L), glucose (10 g/L), tryptone (5 g/L), yeast extract (2.5 g/L) and calcium carbonate (1 g/L) for 72 hours at 30°C, pH 7. Five to ten percent of sporulation media at optimum growth times (hours) are inoculated into fermentation media containing molasses and tofu liquid waste. The biosynthesis process is carried out for seven days, 30°C, pH 7, and 120 rpm. The concentration of glucose and protein was analyzed during the fermentation process. The broth of fermentation and biomass were separated using centrifuge TOMY MX-301 at 9000 rpm, 4°C for 20 minutes. The broth of fermentation is extracted using ethyl acetate organic with a ratio of 1: 1 at 28 °C. Ethyl acetate extract was evaporation using evaporator at 50 rpm, 1 atm, and 50 °C [16-18].

2.3. Milling process

Ethyl acetate extract is converted into Nano particles by milling from 1 to 3 hours in PPF-UG shaker mill with a duration of one hour on and 10 minutes off at 70°C. The mass ratio of grinding and ethyl acetate extract is 10: 1 BPR (ball to powder weight ratio) with a frequency of 500 rpm. The results of milling were analyzed using a particle size analyzer (PSA) HORIBA LB-550 (IK 03 TP 016) and scanning electron microscope (SEM) JEOL JSM IT-300-JAPAN [19-22].

2.4. Testing of Antipathogenic Strawberry Activities

Micro and Nano sized of ethyl acetate extracts of *Streptomyces sp.* TT10 were tested for anti-pathogenic strawberry using diffusion and dilution methods [23].

The diffusion method was carried out in a Petri dish that containing PDA media which had been homogeneously mixed with pathogenic strawberry suspension, then incubated for 72 hours at 30°C. Observations were made by measuring the diameter of the clear barriers in millimeters.

Minimum inhibition concentration (MIC) is determined by a liquid micro dilution method using PDB media in 96 well plates Nunc. Ten different concentrations of fermented extracts were tested for pathogenic strawberries. Stock solutions used for ten different concentrations (ppm): 1000; 500; 250; 125; 62.50; 31.25; 15; 63; 7.8; 3.9; 1.95 then incubated for 72 hours at 30°C. MIC is the lowest concentration that inhibits pathogenic strawberry.

After determining the MIC, the cultures of all wells that were not overgrown with strawberry pathogenic were observed in a petri dish that had been filled with PDA media and then incubated for 72 hours, 30°C. The endpoint of minimum fungicidal concentration (MFC) was defined as the lowest concentration of an antifungal agent which killed > 99.9% of the initial pathogenic strawberry population where no growth was observed. Efficiency calculated by:

\[
[(A-B)/ B] \times 100 \%
\]

Where are GA = zones of diameter (mm) after milling process, MIC (ppm) after milling process and MFC (ppm) after milling process. B = zones of diameter (mm) before milling process, MIC (ppm) before milling process and MFC (ppm) after milling process.

3. Results and discussion

3.1. Sporulation, Fermentation and Extraction of *Streptomyces sp.* strain TT10

The growth and formation of secondary metabolites in the form of anti-pathogenic strawberry are formed at stationary phase at 38-52 hours and reach optimum at the 50th hour referring to Figure 1.

From pH value was showed *Streptomyces sp.* TT10 produced anti-pathogenic strawberry metabolites at acidic atmosphere. The data of concentration of glucose and protein showed that *Streptomyces sp.* TT 10 required carbon and protein sources for the growth and formation of anti-pathogenic strawberry metabolites that was indicated by a decrease of glucose concentrations from 6.39 mg / ml to 4.12 mg / ml and a decrease of protein concentrations from 0.66 mg / ml to 0.42 mg / ml (Figure 1).

The fermentation liquid which has been separated from the biomass was tested for antifungal
activity against pathogenic strawberry. The table shows the antifungal metabolites was formed from the first day to the seventh day. The highest activity was reached at the third day, and then the activity was decreased [24].

Figure 1. Growth curve of *Streptomyces sp.* TT10

![Growth curve of Streptomyces sp. TT10](image)

Figure 2. Data of pH, glucose, protein (mg/ml) and zones of inhibition for seven days fermentation of *Streptomyces sp.* TT10

3.2. Testing of Ethyl Acetate Extract of *Streptomyces sp.* TT10

In vitro test on ethyl acetate extract showed the activities of pathogenic strawberry with zones of inhibition (mm): *Penicillium sp.* (35 mm), *Aspergillus sp.* (38.75 mm), *Rhodotorula sp.* (17.5 mm), *Kluyveromyces sp.* (15.5 mm), *Candida krusei* (20.25 mm), *Candida pseudotropicalis* (19.75 mm) and
Fusarium oxysporum (30 mm) with MIC from 31.25 to 3.91 ppm and MFC from 62.5 to 7.81 ppm (Figure 2) [25].

3.3. Mechanical treatment with shaker mill against ethyl acetate extract of Streptomyces sp. TT10

After being milled for three hours, the ethyl acetate extract showed a reduction in size from micro to Nano as shown in Figure 3, 4 and 5. Likewise with the weight of the extract, the longer of milling process, the smaller the extract weight. This is due to the longer of duration of milling process, the reduced particle size will increase. The duration needed to get the desired results varies depending on the type of grinder, milling intensity, ball mass ratio: powder, and milling temperature. The greater the ratio, the less time required for milling because the intensity of the collision will increase. Milling temperatures will increase due to the increase in the intensity of ball collisions. There is a possibility that this increase in temperature will result in cold welding and increasing particle size. The same effect was also shown due to variations in the size of the steel ball. The bigger the size, the greater the impact energy that will occur. The rotation speed of the grinder is directly proportional to the energy that will be produced. The higher the rotation speed, the more energy will be generated. Ball mill grinder rotation speed is limited by critical speed. When the rotation speed is above the critical speed, the steel balls will move around the surface in the grind and will not give a shock load to the powder. Therefore, it is necessary to know the optimal speed to be able to produce the biggest impact energy [26].

![Figure 3. Shaker Mill PPF-UG](image-url)

![Graph showing particle size distribution](image-url)

(a)
Figure 4. Size and Distribution Particles Analysis of Nano biomaterial of Ethyl Acetate Extract of *Streptomyces sp.* TT10 for (a): 1 hour milling; (b): 2 hours milling; (c): 3 hours milling

Figure 5. Nanometer analysis using SEM after 3 hours of grinding
In Figure 6, 7, 8 and Table 1, shows a clear difference in the in-vitro activity of Nano-acetate extract in Nano size was 80% higher inhibiting pathogenic strawberry compared with ethyl acetate extract before and after milling process.

This is caused by smaller size changes which will increase the antifungal effect. High viscosity and wide range of tissue and high efficiency and stability of active substances in the absorption of active ingredients into the tissue enhancements so that it easily penetrates the cell lipid membrane in plant tissues.
Figure 7. (a): MIC and (b): MFC of ethyl acetate extract *Streptomyces sp.* TT10 before and after milling process.

Figure 8. Anti pathogenic activity of *Streptomyces sp.* TT10 against strawberry by diffusion agar methods, (a) nanobiocontrol > 100 nm and (b) nanobiocontrol < 100 nm.

Table 1. Anti pathogenic activity of *Streptomyces sp.* TT10 against strawberries by micro dilution method.

| K+ | K-   | 1.95 | 3.91 | 7.81 | 15.63 | 31.25 | 62.5 | 125  | 250  | 500  | 1000 |
|----|------|------|------|------|-------|-------|------|------|------|------|------|
| ppm| ppm  | ppm  | ppm  | ppm  | ppm   | ppm   | ppm  | ppm  | ppm  | ppm  | ppm  |

*PDB+*: pathogenic strawberries = *Penicillium sp.*, *Aspergillus sp.*, *Rhodotorula sp.*, *Kluyveromyces sp.*, *Candida krusei*, *Candida pseudotropicalis* and *Fusarium oxysporum*.

Milling process will result the newly produced material, it was assessed that the size of material particle decreases efficiently with increased grinding time. Milling process is able to produce until 100% particle without affected the chemical phases. With the optimal average size of 25 nm and a narrow particle size distribution. The spontaneous self-assembling behavior observed in nature that leads to complex, multifunctional, hierarchical structures within biological systems. Recent research undertaken to synthesize hierarchically assembled functional material have underscored the need as
well as the benefit in synergistically combining top down fabrication methods with bottom up self-assembly [27-30]. Nanobiocontrol in the shape of emulsion and powder have been produced by Research Unit for Clean technology (LPTB), Indonesian Institute of Sciences (LIPI).

Figure 9. Laboratory scale of nanobiocontrol in emulsion (a) and powder (b) produced by LIPI.

4. Conclusion
Food and agriculture industry waste, namely tofu and molasses can be used as a medium for the growth of *Streptomyces sp.* TT10 to produce bio-control agents. Ethyl acetate extract of *Streptomyces sp.* TT10 in the shape of new nanoparticles has been shown enhancement to the antifungal activities against pathogenic strawberries fruits. This top-down approach by milling process provides a new outline for the future design of nanostructured of bio-pesticide.

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