Culturable bacterial abundance in *Volvariella volvacea* cultivation medium and characterization of its bacteria

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Abstract

Straw mushroom (*Volvariella volvacea*) is one of the popular edible fungi in Indonesia. Previous researches showed the correlation among the type of substrate, substrate quality, and its composting process to the microbial community, yield, and biological efficiency. The aim of the research is to analyze the culturable bacteria abundance in straw mushroom cultivation medium, characterize the bacteria in several stages of mushroom cultivation and investigate the interaction between *V. volvacea* with its resident bacteria. Samples were taken from mushroom farmers in Subang and Karawang regencies, Indonesia. The materials for cultivation medium are the mixture of cotton and paddy straw and the pasteurization was performed at 65-70°C for 7 hours. The result shows the abundance of the bacteria in most of the cultivation stages is relatively similar *i.e.* 10^8 CFU/g, except in 15 days after inoculation (DAI), the bacterial abundance is lower *i.e.* 6.24 x 10^7 CFU/g. Twenty-five isolates were obtained and Gram-positive bacteria is the dominant bacteria found in the cultivation medium, especially rod-shaped Gram-positive bacteria. According to co-culture assay there are nine isolates that decrease the growth rate and clearly inhibit mycelial growth. The other 10 isolates have lower inhibitory activity, and 6 isolates have no inhibitory activity to the mycelial growth. C38 isolates have the highest mycelial growth inhibition. It belongs to rod-shaped Gram positive group of bacteria which isolated from the early stage of *V. volvacea* cultivation medium (5 DAI).

Keywords: bacterial diversity, bacterial abundance, *Volvariella volvacea*

Introduction

Straw mushroom (*Volvariella volvacea*) is one of the popular edible fungi consumed in Indonesia. According to The Ministry of Agriculture (2019), straw mushroom production is decreasing 0.97 Ku/Ha from 2016-2017. The decreasing of straw mushroom production caused by several factors, one of them is the cultivation medium. The substrate composition in the cultivation medium and other intrinsic factors such as nitrogen sources, carbon to nitrogen ratio, pH, moisture, minerals and particle size have the significant role in the growth and development of mushroom fruiting bodies. On the other side, the poor nutrient content, inadequately mixed and too compact cultivation medium can lead to poor mycelial growth. According to Zikriyani *et al.* (2018) agricultural wastes can be used as cultivation medium *i.e* paddy straw, cotton, banana leaves. Cotton has the highest biological efficiency among other
materials. Zhang et al. (2019) reported that millet straw also could be utilized as cultivation medium of Agaricus bisporus and the yield is 20 kg/m².

In addition to cultivation medium, the diversity of bacteria has effect on the growth of the mushroom. Bacterial diversity in the substrate is likely related to the environmental condition in the cultivation medium such as acidity, moisture, and nutrient content (Zhang et al. 2018). Edible mushroom mostly cultivated in the microbe-rich medium which leads to the interaction among the bacteria and the fungi both antagonism and mutualism (Deveau et al. 2018). Silva et al. (2009) reported that Agaricus brasiliensis cultivation using sugarcane wastes and paddy straw with 14 days composting period has several dominant bacteria such as Bacillus, Paenibacillus spp. and Enterobacteriaceae group with the abundance up to 3x 10⁸ CFU/g. Generally, the microbial diversity in mushroom cultivation medium is fluctuating, the highest diversity in the pre-composting stage and the lowest diversity is before the compost stockpiled and then gradually increasing in the early spawning stage (Vajaet al. 2010).

Resident bacteria in cultivation medium likely play a significant role in promoting mycelial growth and support fruiting body development. Several species of Bacillus and Pseudomonas able to promote the mushroom growth by producing mushroom growth-promoting factors such as indole acetic acid (IAA), phosphate solubilization, cellulolytic enzyme, and volatile compounds (Zarenejad et al. 2012; Xiang et al. 2017; Febriansyah et al. 2018). Therefore, the aim of the study was to analyze the culturable bacteria abundance in straw mushroom cultivation medium, characterize the bacteria in several stages of mushroom cultivation and investigate the interaction between V. volvacea with its resident bacteria.

Materials and methods

Sampling

The sample was taken in straw mushroom farmers in Jatisari- Karawang and Patokbeusi- Subang, West Java, Indonesia. The medium used in the cultivation is the mixture of cotton and paddy straw which pasteurized at 65- 70°C for 7 hours. Generally, the mushroom farmers using a traditional mushroom cultivation house called kumbung that has been built in a certain way. There are several cultivation stages of the sample taken and each stage was taken 3 replicates (Table 1).

| Sample code | Cultivation stages |
|-------------|-------------------|
| C           | 5 days after inoculation (early stage) |
| D           | 15 days after inoculation (first harvesting) |
| E           | 17 days after inoculation (second harvesting) |
| A           | Completely harvested |
| B           | Completely harvested (very late stage/ medium already dry) |

The abundance measurement of bacteria from mushroom cultivation medium

The abundance of bacteria was measured using the total plate count method. Samples were taken from the top of bulk cultivation medium in every cultivation stage available in the mushroom houses. One g of cultivation medium was diluted into 9 ml sterile distillate water and homogenized. One milliliter of the suspension was then transferred into 9 ml sterile distilled water to get 10² dilution. The similar procedure was repeated so that obtained 10⁶ dilution. As much as 100 µL suspension from 10³- 10⁶ dilution was poured and spread into nutrient agar (NA) medium with 3 replicates then incubated at 28°C for 24 hours.
Isolation of bacteria

Several different colonies of bacteria were taken from the total plate count and purified in NA medium and incubated at 28°C for 24 hours. The observation of cell morphology and Gram differentiation was performed through the Gram staining method. Gram staining was conducted using Gram Stain-Kit (HiMedia Laboratories- India) which utilizing crystal violet, iodine Gram and safranin. The detail of the procedure was following the manual provided by manufacturers. The stained bacterial cells were then observed under light microscope Olympus BX-53 (Olympus- Japan) with 1000x magnification.

Co-culture of bacteria and *Volvariella volvacea* isolates

The co-culture method was according to Oh & Lim (2017) with few modifications. Each of those bacteria which were successfully isolated from cultivation medium was co-cultured with *V. volvacea* in an 80 mm petri dish contained potato dextrose agar (PDA) medium. The *V. volvacea* mycelia were grown in PDA medium and incubated at 30°C for 1 week. The *V. volvacea* isolate was taken using cork borer (5 mm diameter) and placed on PDA medium in 80 mm petri dish. The bacterial isolates were then inoculated onto similar PDA medium by streaking with a sterile 1 µL inoculating loop along a 30 mm line with 20 mm distance away from the *V. volvacea*. The average of diameter increase was measured and compared with control which contains *V. volvacea* without bacterial isolates. The mycelial growth inhibition was measured using formula as follows:

\[
\text{Mycelial growth inhibition} = \frac{Rc - Ri}{Rc} \times 100\%
\]

Rc = Mycelial growth of control (*V. volvacea* with no bacterial inoculation)

Ri = Mycelial growth of *V. volvacea* co-cultured with bacteria

(Narayanasamy 2013)

Statistical significance on the index of inhibition was measured using ANOVA single factor and continued using the least significant differences (LSD) with 5% level of significance (α= 0.05).

Results

According to the results, the abundance of culturable bacteria is relatively similar. The abundance of culturable bacteria was around 10⁸ CFU/g (Figure 1). The highest bacterial abundance was in 5 days after inoculation and the lowest was in 15 days after inoculation. The sample in 15 days after inoculation is comparably different among the others. This causal agent of microbial dynamics in the cultivation medium needs to be further investigated by analysing the nutritional content of the medium.

There are total of 25 different bacterial isolates obtained from the samples. These isolates are characterized by the cell shape as well as Gram differentiation. Based on the cell shapes, most isolates which successfully isolated are rod-shaped bacteria and based on the Gram differentiation the Gram-positive bacteria is higher than Gram-negative bacteria (Table 2; Figure 2). These isolates will be identified and further characterized in further study.
Figure 1. The total plate count of bacteria from straw mushroom cultivation medium at several cultivation stages

Table 2. Characteristics of bacterial isolates from straw mushroom cultivation medium at several cultivation stages

| Mushroom cultivation stage | Isolate code | Cell shape | Gram stain |
|----------------------------|--------------|------------|------------|
| Early stages (5 DAI)       | C2.1         | Basil      | Positive   |
|                            | C2.2         | Basil      | Positive   |
|                            | C2.5         | Coccus     | Negative   |
|                            | C2.6         | Coccus     | Positive   |
|                            | C3.3         | Basil      | Positive   |
|                            | C3.4         | Basil      | Positive   |
|                            | C3.7         | Basil      | Positive   |
|                            | C3.8         | Basil      | Positive   |
| First harvesting (15 DAI)  | D1.1         | Coccus     | Positive   |
|                            | D2.5         | Basil      | Negative   |
|                            | D2.6         | Coccus     | Negative   |
|                            | D3.3         | Basil      | Positive   |
|                            | D3.4         | Coccus     | Negative   |
| Second harvesting (17 DAI) | E1.1         | Basil      | Positive   |
|                            | E1.2         | Basil      | Negative   |
|                            | E1.3         | Coccus     | Negative   |
|                            | E3.4         | Coccus     | Negative   |
| Completely harvested (late stage) | A1.1 | Coccus | Negative |
|                            | A1.2         | Coccus     | Positive   |
|                            | A2.3         | Basil      | Positive   |
|                            | A3.4         | Basil      | Positive   |
| Completely harvested (very late stage) | B1.1 | Basil | Positive |
|                            | B1.2         | Coccus     | Negative   |
|                            | B2.3         | Coccus     | Positive   |
|                            | B3.4         | Basil      | Negative   |
Figure 2. The grouping of 25 successfully isolates (a) based on the cell shapes (b) based on the Gram differentiation.

The isolates then co-cultured in vitro with *V. volvacea* to analyse the interaction both of mutualism and antagonism between bacterial isolates and *V. volvacea*. According to the co-culture results, most of isolates decreased the growth rate of the *V. volvacea* (Figure 3, Table S1). As mentioned in figure 3, there are nine isolates that significantly decreasing the mycelial growth rate. The remaining 16 isolates also decreasing growth rate with lower activity (Table S1). The highest growth rate is control (without inoculation of bacterial isolates). Further characterization shows that 9 isolates significantly inhibit the mycelial growth of *V. volvacea* i.e C3.8, C2.2, D3.3, D2.6, E3.4, D3.4, A3.4, A1.1 and C3.7 respectively (Figure 5) with isolate C3.8 produce the highest inhibitory activity (Figure 4). Moreover, there are 10 isolates insignificantly inhibit the mycelial growth of *V. volvacea* and 6 isolates with no inhibitory activity (Figure 5).

Figure 3. Radial growth rate of *V. volvacea* co-cultured with bacterial isolates.
Figure 4. The inhibition zone of isolate C3.8 against *Volvariella volvacea*

![Image of inhibition zone]

Figure 5. The mycelia growth inhibition index of *V. volvacea* co-cultured with bacterial isolates after 7 days of incubation

### Discussion

The monitoring of bacteria in *V. volvacea* cultivation medium was conducted to understand the bacterial abundance in different cultivation stages of *V. volvacea*. Meanwhile, isolation of its bacteria was conducted to investigate further the role of these bacteria, whether it promotes or inhibits the growth of *V. volvacea*. Generally, the microorganisms in cultivation medium strongly influence the growth of the mushroom even in some cases are required for the growth and development of fruiting body of cultivated mushrooms (Carrasco & Preston 2020).

According to Carrasco *et al.* (2018) there are two main groups of cultivation medium *i.e* fermented-pasteurized substrates and a mixture of steam-sterilized raw materials of the substrates. The cultivation of *V. volvacea* is conducted in fermented-pasteurized substrates. In this study the fungi are cultivated in the mixture of paddy straw and cotton which previously composted and pasteurized. The composting process has a significant effect on shaping the microbial diversity in the cultivated medium. The result of this study showed that Gram-positive bacteria is more dominant than the negative one (Figure 2). It is probably caused by the cellulose content of the substrate which becomes the main carbon source for mushroom...
growth. This result is in line with the Zhang et al. (2014) which stated that in straw mushroom medium compost production, Actinobacteria and Clostridia are the predominant bacteria which play a significant role in cellulose degradation. In the case of Agaricus bisporus cultivation, the diverse population of bacteria and fungi are involved composting process through the bioconversion of the raw materials into compost in the thermophilic composting process. These microorganisms utilizing the accessible carbon sources, releasing ammonia, assimilate cellulose and hemicellulose into the compost which becomes primary nutrition for the cultivated fungi (Kertesz & Thai 2018).

The microbial diversity and abundance in the cultivated medium also vary from the initial process of composting until the pasteurization. According to Silva (2009) the bacterial abundance in the A. brasiliensis mostly 10⁸ CFU/g, however, there are the differences in every composting process. The bacterial abundance is highest in the early composting process and gradually decreases until the pasteurization process. However, bacterial diversity is relatively increases especially for Actinomycetes group. In this research the bacterial abundances also vary and mostly10⁸ CFU/g, however it needs to be investigated since the initial process of raw material composting process. The fluctuation of bacterial abundance in early stage (5 DAI) and 15 DAI was allegedly caused by the physicochemical changes of the substrates due to the mushroom growth stage. However, it needs further investigation about the physicochemical characteristics of the cultivation medium in each mushroom growth stage. According to Zhang et al. (2018), the observed OTU (operational taxon unit) in the hyphal stage of Ganoderma lucidum cultivation is higher than in budding stage, then gradually increased in elongation stages and mature stages. The change of relative abundance is likely attributable to the colonization of bacteria and changes in physicochemical changes of the substrate. Instead of bacterial abundance, total microbial diversity also needs to be investigated to gain a deeper understanding of the changes in microbial community structure in each growth stage.

The bacteria in the cultivation medium have different effects on the growth of V. volvacea. According to in vitro screening result by using the co-culture method, there are no bacteria which clearly promote the growth of V. volvacea. Most of the isolates are decreasing the mycelial growth rate of V. volvacea (Figure 3) whether it clearly shows inhibition zone or not. There are nine isolates have significant inhibition level of V. volvacea with C3.8 has the highest inhibition level. On the other hand, there are 6 isolates which have no inhibitory activity against V. volvacea. However, the screening is only carried out in vitro on the rich medium, so that is not clearly known whether the bacteria have mushroom growth-promoting activity or not. There are several characteristics of mushroom growth-promoting bacteria such as able to grow in the presence of 1-octen-3-ol, inorganic phosphate solubilization activity and produce siderophore (Zarenejad et al. 2012). Therefore, the 6 isolates need to be further characterized for the growth promoting activity.

A previous study by Jemsi & Aryantha (2017) reported that Bacillus cereus– the rod-shaped, Gram-positive bacteria–isolated from the growth medium of V. volvacea able to increase the growth of V. volvacea up to 300% while there are two isolates also found exhibit the inhibitory of V. volvacea. Kertesz & Thai (2018) also summarized that several strains of Pseudomonas and Bacillus have mushroom growth-promoting ability in Agaricus cultivation. The mushroom growth-promoting ability is not limited by Gram determination, instead of Gram-positive Bacillus several Gram-negative strains such as Pseudomonas putida UW4 and Bradyrhizobium japonicum Hn03 also able to stimulate the mushroom growth (Chen et al. 2013; Zhu et al. 2013). Bacterial-fungal interaction can produce various range of interaction, from antagonism to mutualism. Identification of the isolates must be conducted to determine clear name of the isolates and the role of it isolates in the V. volvacea cultivation. The further study of mushroom inhibition will be conducted for the future research to identify the
compound which responsible for the mushroom growth inhibition. Moreover, the 6 isolates with no in vitro inhibitory activity will be further characterized for the growth-promoting ability.

Conflict of interest
The authors state no conflict of interest from this manuscript.

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Author contributions
All authors have reviewed the final version of the manuscript and approved it for publication. MR designed the study, conducted sampling and lab work; IS designed the study and planed the sampling site; IS and RRE analysed the data, wrote and reviewed the paper. MR is the main contributor to this manuscript.

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Supplementary table 1 (Table S1)

Radial growth rate of *V. volvacea* co-cultured with bacterial isolates

| Isolates | V. volvacea radial growth diameter (mm) |
|----------|----------------------------------------|
|          | Day 1     | Day 2     | Day 3     | Day 4     | Day 5     |
| C3.8     | 7.50      | 11.75     | 28.08     | 37.50     | 40.00     |
| C2.2     | 8.50      | 11.00     | 30.00     | 43.25     | 57.75     |
| D3.3     | 8.50      | 10.00     | 24.00     | 39.50     | 58.00     |
| D2.6     | 10.00     | 12.50     | 40.33     | 50.75     | 58.50     |
| E3.4     | 10.00     | 12.50     | 36.67     | 50.00     | 60.00     |
| A3.4     | 7.50      | 11.00     | 33.00     | 47.75     | 61.75     |
| D3.4     | 9.50      | 10.50     | 31.17     | 44.00     | 61.75     |
| A1.1     | 8.50      | 12.25     | 38.00     | 48.75     | 62.50     |
| C3.7     | 9.50      | 11.50     | 34.00     | 49.50     | 63.67     |
| B3.4     | 11.00     | 13.75     | 25.25     | 38.00     | 67.50     |
| B2.3     | 9.50      | 11.00     | 32.33     | 45.83     | 67.50     |
| E1.2     | 7.25      | 11.50     | 36.33     | 49.50     | 67.50     |
| D2.5     | 6.50      | 11.50     | 36.00     | 50.25     | 71.00     |
| C2.1     | 10.00     | 11.00     | 28.50     | 42.08     | 71.75     |
| A1.2     | 7.50      | 10.50     | 36.17     | 51.50     | 73.00     |
| D1.1     | 10.50     | 12.00     | 34.50     | 49.00     | 74.25     |
| B1.2     | 7.50      | 11.50     | 41.17     | 52.75     | 76.00     |
| C.25     | 10.50     | 14.50     | 48.50     | 64.00     | 77.50     |
| C3.3     | 9.00      | 10.50     | 34.00     | 46.25     | 77.50     |
| Control  | 0.00      | 16.00     | 57.50     | 75.00     | 80.00     |
| C.26     | 8.50      | 14.00     | 30.75     | 47.50     | 80.00     |
| E1.1     | 6.00      | 10.75     | 41.67     | 63.50     | 80.00     |
| A1.3     | 4.50      | 11.50     | 36.00     | 51.75     | 80.00     |
| C3.4     | 9.50      | 13.75     | 40.33     | 49.00     | 80.00     |
| E1.3     | 7.50      | 11.50     | 53.50     | 64.25     | 80.00     |
| B1.1     | 9.50      | 11.00     | 40.25     | 58.75     | 80.00     |