

Research Article

Screening and Identification of Mushrooms Growth Promoting Bacteria on Straw Mushrooms (*Volvariella volvacea*)

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ABSTRACT

This research aimed to identify the indigenous Mushroom Growth Promoting Bacteria (MGPB) bacteria that can increase the growth of *Volvariella volvacea*. The research began by isolating indigenous MGPB from planting media of straw mushrooms in Karawang, Indonesia. The screening was performed to select bacterial isolates that can promote the highest growth of mushrooms by dual culture method on PDA media. There were 10 of the 58 highest bacterial isolates that have a positive effect on the vegetative growth of mushrooms. The 23K bacterial isolate was the most significant increase in mycelium growth compared to other isolate and bacteria-induced controls. A bacterial isolate 25K by gene analysis was identified by 16S rRNA (518F primer (5'- CCA-GCA-GCC-GCG-GTA-ATA-CG -3') and 800R primer (5'- TAC-CAG-GGT-ATC-TAA-TCC -3')). The result from gene analysis shows that there are ~1550 base pairs products. BLAST analysis and phylogenetic tree adjustment results show that the closest diversity of this bacterial isolate 25K is *Bacillus thuringiensis* serovar *konkukian str.* 92-27 (equality value = 99%).

Keywords: *Bacillus thuringiensis*, BLAST analysis, Indigenous bacteria, MGPB, *Volvariella volvacea*

INTRODUCTION

The factor of mushroom growth besides nutrient content on planting medium is an environmental factor. Environmental factors that are important for the growth and formation of fruit mushrooms body are temperature, moisture, light, and oxygen (Sukendro et al. 2001). All these environmental factors also support the growth of bacteria on the media of mushroom planting which helpful as a promoter of mycelium mushroom growth called MGPB (Mushrooms Growth Promoting Bacteria). Bacteria as MGPB can be found in the cover layer of planting medium which has been composted (Zeranejad et al. 2012).

The previous study reported that MGPB bacteria can induce the growth and productivity of fungi. Young et al. (2013) reported that bacteria in the growing medium and mycelium of *Agaricus blazei* can induce the

growth and productivity of the fungus. It was reported that bacteria that can induce *A. blazei* growth is *Actinobacteria* that present in the planting medium. However, there is a gap to look for MGPB that can increase the growth of *Volvariella volvacea* mycelium. *V. volvacea*, locally called straw mushroom is one of the high nutritional food mushrooms especially in the protein content. Straw mushrooms also have some good mineral content such as potassium and high phosphorus, coupled with the high enough riboflavin and thiamine, make the mushrooms more desirable and needed by consumers today ([Haq et al. 2011](#)).

Microbes become an important part of the growth of fungi. Research on the effect of bacteria on mushroom productivity has been widely practiced. Zeranejad et al. ([2012](#)) isolated and identified bacteria that can induce *Agaricus bisporus* mushrooms production. In this previous study, they found two strains of bacteria that can induce mushroom production. One of the identified strains of molecularly is *Pseudomonas putida*. A Previous study about the increasing production of straw mushrooms with the help of bacteria has been done by Payapanon et al. ([2011](#)), in this previous study found that the bacteria which contribute to increase the nutrition in the composting phase are *Paenibacillus* dan *Bacillus* sp. Another previous study by FAMILONI et al. ([2018](#)) shows that the bacteria were taken from the planting medium and oyster mushrooms fruit body (*Pleurotus ostreatus*) obtains some isolates that can give effect to the growth of thick fungal colonies. They found that there are *Pseudomonas putida*, *Streptomyces* spp., *Trichoderma* spp., *Penicillium italicum*; and others using random amplified polymorphic DNA analysis (RAPD) with 10 primers. However, there is a gap to identify the indigenous MGPB that can induce the growth of *Volvariella volvacea* using DNA sequence 16S rRNA gene analysis.

Based on the research gap in the background, this study aims to identify the indigenous MGPB that can induce the growth of *Volvariella volvacea* using DNA sequence 16S rRNA gene analysis.

MATERIALS AND METHODS

Screening of selected bacteria

The process of obtaining indigenous bacteria that can induce the mushroom growth began with a sampling of mushrooms substrate from four mushroom producing regions in Indonesia including Karawang, Cikampek, Subang, and Sukabumi. The substrate collection as a sample of harvesting and post-harvest was put into a sterile container so that no contamination from the outer bacteria. Bacterial isolation was performed by serial dilution method. After 24 hours, the grown and separated bacterial colonies were duplicated to be isolated by the four ways method. Then the pure isolate was separated into the NA medium inclined in the test tube ([Cappuccino & Sherman 2005](#)). The pure isolates were tested for their ability by the dual culture method. This method has been done to select bacteria that can increase the mycelia growth. This method used PDA in the Petri dish, and then bacteria cultured in the four sides of the medium which was about 4 cm with pieces of mycelium in the middle then incubated in an incubator with a temperature of 35°C. According to Chang & Miles ([2004](#)), the method of tissue culture of straw mushrooms can be incubated at the temperature of 30-35°C. The selected bacteria were the fastest bacteria to reach the edge of the Petri dish, so it can proceed to the next step.

Identification of selected bacteria

The identification was used the DNA sequence 16S rRNA method. DNA isolation in this research was performed by obtaining one selected bacterial

ose aged 24 hours into Eppendorf and resuspended with 100 µl Deion. The sample was heated at 96°C for 1 minute then incubated at -22°C for 3 minutes. The steps were repeated three times. Then the sample was centrifuged at 14,000 rpm for 5 minutes and the supernatant from the centrifugation was used as a template in the PCR (Baker et al. 2003; Araujo et al. 2001; Yuwono 2006). The amplification step of encoding gene 16S rRNA was used PCR kits with the composition including dH₂O 16,9 µl, 10 mM dNTP (dNTP mix) 0.50 µl, 5x KAPA2G buffer DNA polymerase 5 µl, *forward primers* 518F (5'- CCAGCAGCCGCGGTAATACG -3') and *reverse primers* 800R (5'- TACCAGGGTATCTAATCC -3') forward, and then added the KAPA2G robust (5U/ µl) 0,10 µl. The PCR condition used is pre-denaturation at 95°C, 5 minutes; the denaturation step is 95°C, 15 seconds; the annealing step is at 54°C, 90 seconds; and the elongation step is at 72°C, 60 seconds with the PCR process that consists of 25 cycles. The next step is the post PCR step at 72°C in 7 seconds and the stop PCR step at 4°C. DNA template of PCR result has performed the electrophoresis on 1% agarose gel and the formed band was seen by UV transilluminator after immersion in ethidium bromide solution (Yuwono 2006).

Sequence alignments and construct the phylogenetic tree

Researchers were used the bioinformatics software BIOEDIT v.7.0.8.0 to proceed with the sequence of nucleotide bases. The researchers analyzed it with the BLASTN (*Basic Local Alignment Search Tool Nucleotide*) program on the NCBI website (<http://www.ncbi.nlm.nih.gov>). The determination of the phylogenetic tree and bootstrap value were used a MEGA 6.06 software. The bootstrap value was used to determine the degree of confidence in the construction of a phylogenetic tree. If the bootstrap value at a low level (less than 75), so the sequence has a low confidence level, while if the bootstrap value is high level, so the sequence confidence level is also high (more than 75) (Dharmayanti 2011).

RESULTS AND DISCUSSION

The screening of the selected bacteria for straw mushroom growth

The isolation results were found 58 bacterial colonies that can be seen in Table 1 and ready to be screened using the dual culture method (see Figure 1).

Table 1. The isolation result from screening using the dual culture method.

Bacteria code	Average growth rate (mm/day)	Mycelium thickness
Control	6.50 ± 0.90	Thin
1S	4.75 ± 3.61	Thin
2S	6.33 ± 3.40	Thick
3S	6.75 ± 4.13	Thick
4S	4.58 ± 2.65	Thin
5S	6.75 ± 0.66	Thin
6S	6.83 ± 1.15	Thin
7S	5.92 ± 0.58	Thin
8S	5.75 ± 2.38	Thin
9S	5.42 ± 1.53	Thin

Table 1. Contd.

Bacteria code	Average growth rate (mm/day)	Mycelium thickness
10S	5.25 ≤ 1.39	Thin
11S	7.50 ≤ 1.32	Thick
12S	4.92 ≤ 0.80	Thin
13S	5.25 ≤ 0.90	Thin
14S	5.25 ± 1.64	Thin
15S	5.5 ± 1.80	Thin
16S	5.08 ± 1.38	Thin
17S	5.08 ± 1.23	Thin
18K	5.58 ± 0.63	Thin
19K	7.33 ± 0.29	Thin
20K	5.50 ± 0.66	Thin
21K	6.33 ± 1.59	Thin
22K	7.17 ± 1.28	Thick
23K	8.42 ± 1.18	Thick
24K	5.50 ± 1.56	Thin
25K	6.25 ± 1.73	Thin
26K	6.00 ± 1.52	Thin
27K	6.00 ± 1.00	Thin
28SB	7.00 ± 1.09	Thin
29SB	6.67 ± 1.44	Thin
30SB	7.67 ± 0.14	Thin
31SB	6.00 ± 0.87	Thin
32SB	6.67 ± 1.44	Thin
33SB	7.00 ± 2.54	Thick
34SB	6.08 ± 1.66	Thin
35SB	6.83 ± 2.02	Thin
36SB	5.25 ± 0.25	Thin
37SB	7.83 ± 0.88	Thin
38SB	6.17 ± 1.15	Thin
39SB	6.67 ± 0.14	Thin
40S	8.33 ± 0.72	Thick
41S	7.83 ± 1.53	Thick
42S	6.42 ± 1.23	Thin
43S	6.00 ± 1.98	Thin
44S	6.42 ± 1.01	Thin
45S	8.33 ± 1.89	Thin
46S	5.92 ± 0.76	Thin

Table 1. Contd.

Bacteria code	Average growth rate (mm/day)	Mycelium thickness
47K	8.00 ± 1.32	Thin
48K	6.00 ± 1.75	Thin
49S	3.17 ± 0.63	Thin
50K	6.75 ± 1.89	Thin
51C	6.92 ± 0.52	Thin
52C	7.92 ± 0.38	Thick
53C	5.50 ± 0.50	Thin
54C	6.83 ± 1.51	Thick
55C	5.25 ± 2.41	Thin
56C	4.42 ± 1.61	Thin
57S	5.17 ± 0.58	Thin
58K	4.33 ± 1.28	Thin

Figure 1 shows the difference in the length of mycelium between the four sides of bacteria. On the bacteria side of the 1S bacteria isolate, the mycelium does not move to the 1S bacteria isolate, the growth of the mycelium length is only about 20 mm and the growth rate of mycelium is about 4.75 mm/day. In the 2S and 3S bacteria isolates, the mycelium length moves to both sides of the bacterium, mycelium continues to grow until it passes through the bacteria-streaked side with a growth rate of about 6.75 mm/day. The 4S bacteria isolate shows the existence of a clear zone produced by bacteria so that the mycelium growth stopped until the clear zone. The growth rate of 4S bacteria isolate is slower than the other side, which is about 4.6 mm / day. Whereas in control, i.e., mycelium without bacterial cultures on the sides showed a widespread and thin mycelium growth with a growth rate of 6.5 mm / day.

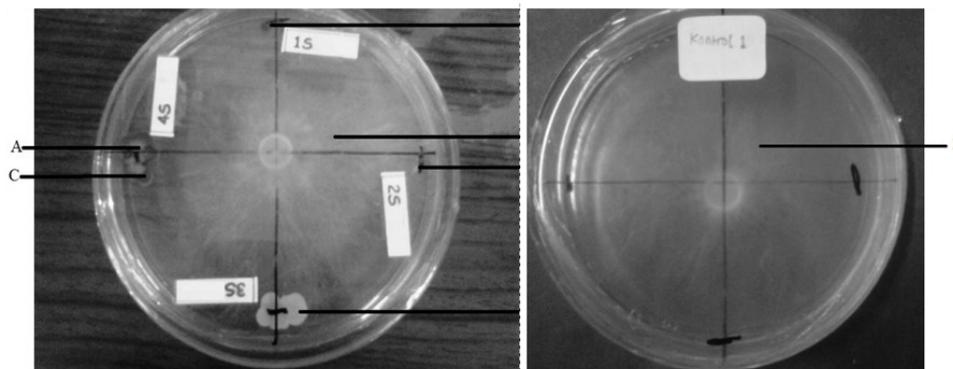


Figure 1. The screening of selected bacteria with *Dual Culture* method (left) and control (right) (A: Bacteria, B: Mycelium, C: Clear zone).

The results of the screening show two growth effects. The mycelium length and mycelium growth are faster and longer than control. Both of these effects may be possible due to the presence of bacteria on the PDA side of the medium. According to Pion et al. (2013), the growth of fungi may be affected by the presence of inhibiting or antagonistic bacteria or can induce

the growth of mycelium according to the mechanism and potential of the bacteria.

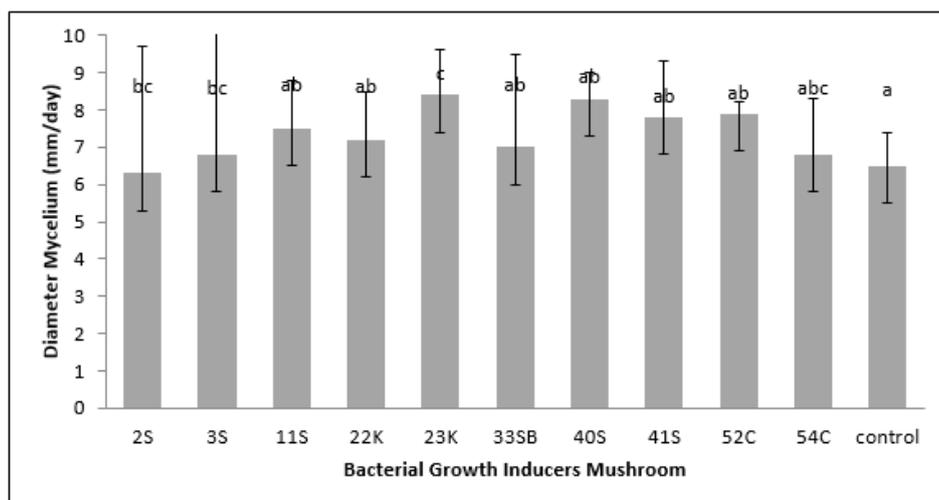


Figure 2. A Chart that diameter mushroom mycelium by indigenous bacteria (bar with the same letter are not significantly different at 5% Duncan test).

The dual culture method has been done to provide significant data on the difference in mushrooms mycelium growth by giving the bacteria treatment. From the 58 bacterial colonies, we selected the 10 highest growth rate average of mycelium and mycelium thickness (see Figure 2). The 10 bacteria isolate that can accelerate the growth of mycelium mushrooms, 23K bacteria has a faster rate of mycelium growth than controls and other bacteria isolates, which is about 8.4 mm / day. This finding is relevant to Payapanon et al. (2011) which found the presence of bacteria can affect the growth of fungi that exist around it by producing hormones and have the ability to dissolve phosphate for the availability of bacterial nutrients. The bacteria can also affect the growth of fungi because it acts as mushrooms growth-promoting bacteria (MGPB) to stimulate the growth of fungi (Zeranejad et al. 2012; Familoni et al. 2018).

Identification of Bacteria 23K

Bacteria 23K has the white colony color with the edge is rolled up. After four days, the edge of the Bacteria 23K colony will be formed the flagels (see Figure 3). These findings are relevant to Pakpahan et al. (2013) that found the characteristic of *Bacillus* is motile with the colonies' growth are spreading throughout the medium. The result of gram staining for bacteria 23K is characterized by a gram-positive purple color of bacterial cells under microscope observation and cell-shaped stem (bacillus).

Figure 4 shows that DNA of 23K bacteria isolate has an identical base length of ~ 1550 bp which be composed of both variable and conserved regions. The gene is large enough with sufficient interspecific polymorphisms of the 16S rRNA gene. Clarridge (2004) states that 500 and 1,500 bp are common lengths to be sequenced and compared. The sequences in databases have various lengths to provide valid measurements statistically.

Figure 5 shows that the construction of a 23K bacterial phylogenetic tree has the closest relationship with some *Bacillus* such as *Bacillus thuringiensis* serovar *strucia* str. 97-27, *Bacillus cereus*, and *Bacillus anthracis* with a bootstrap value of 76. *Bacillus thuringiensis* is one group with *Bacillus cereus* and *Bacillus anthracis* that have the ability to produce intracellular protoxin crystalline proteins (Roh et al. 2007). It can also be seen that *Bacillus cereus* has a close relationship to *Bacillus weihenstephanensis*, however the bootstrap value around

51. It means that the sequence has a low confidence level. Dharmayanti (2011) states that in the phylogenetic tree, the bootstrap value for each sequence is less than 75 so it can be categorized as having a low trust value.



Figure 3. Morphology of bacterial 23K with magnification 100x.

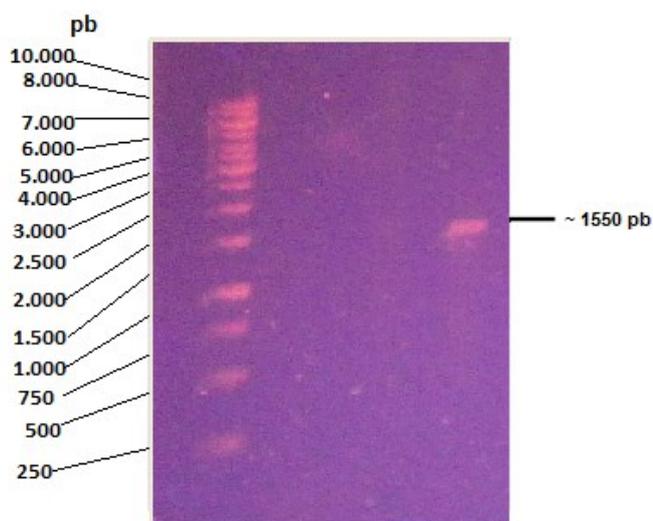


Figure 4. The result of amplification DNA 16sRNA for bacteria 23K.

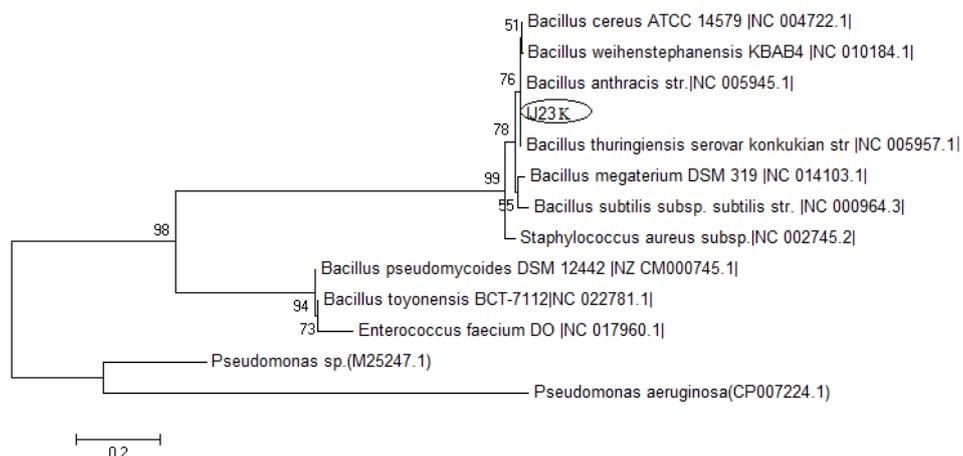


Figure 5. Phylogenetic tree bacteria 23K based on the 16S rRNA gene analysis using Maximum Likelihood method.

The phylogenetic tree shows that *Bacillus thuringiensis* serovar *konkukian* str. 97-27 is one of the bacteria that closest to the Bacteria 23K. The classification of *Bacillus thuringiensis* in the NCBI as follows:

Kingdom : Bacteria
Division : Firmicutes
Class : Bacilli
Ordo : Bacillales
Family : Bacillaceae
Genus : *Bacillus*
Species : *Bacillus thuringiensis* serovar *konkukian* str. 97-27

Bacillus thuringiensis is one of the millions of soil bacteria with pathogen characteristics for insects ([Hatmanti 2000](#)). Toxic compounds for insects from *Bacillus thuringiensis* are specific to insect pests so they are harmless to other organisms and safe for humans ([El-kersh et al. 2012](#)). The growth temperature for these bacteria is between 15°C - 40°C with an optimum pH of 6.5 - 7.5 ([Bernhard & Utz 1993](#)). *Bacillus thuringiensis* undergoes is optimum growth at 3-30 hours from the onset of inoculation and at the 30th hour undergoes a static phase and decreases ([Darwis et al. 2004](#)).

CONCLUSION

Based on the results and discussion, it can be concluded that Bacteria 23K is the most significant bacteria that can increase the growth of mushrooms than other isolates in the mycelium phase in vitro. Based on the results of phylogenetic tree analysis, the 23K bacterial isolates have the closest to *Bacillus thuringiensis* serovar *konkukian* str. 97-27. It is necessary to test the generative phase of mushrooms with bacteria 23K in the controlled clusters of temperature and humidity.

AUTHORS CONTRIBUTION

I.J.S and I.N.P.A designed the research and supervised all the process, I.J.S and I.N.P.A collected and analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

The authors don't have a conflict of interest.

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