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

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Abstract

This research aimed to identify the indigenous MGPB bacteria that can increase the growth of *V. volvacea*. The research began by isolating MGPB indigenous bacteria 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 vegetative growth of mushrooms. The 23K bacterial isolate was the most significant increase in mycelium growth compared to other isolates and bacteria-induced controls. Identify of 23K bacterial isolate by gen analyze 16S rRNA used primer 518F (5'- CCA-GCA-GCC-GCG-GTA-ATA-CG -3') and 800R (5'- TAC-CAG-GGT-ATC-TAA-TCC -3') produce PCR ~1550 base pairs product. BLAST analysis results and phylogenetic tree adjustment has the closest diversity to *Bacillus thuringiensis* serovar *konkukian str*. 92-27 (equality value = 99%).

Keywords:

Bacillus thuringiensis, BLAST analysis, Indigenous bacteria, MGPB, *Volvariella volvacea*.

51 **1. Introduction**

52 *Volvariella volvacea* locally called straw mushrooms is one of the high nutritional food
53 mushrooms especially the protein content. Straw mushrooms also has some good mineral
54 content such as potassium and high phosphorus, coupled with the high enough riboflavin and
55 thiamine, make the mushrooms more desirable and needed by consumers today (Haq et al.,
56 2011). So, one of the efforts to find out the protein needs is by developing mushrooms
57 cultivation.

58 A mushrooms grows on a planting medium containing the source of cellulose and
59 hemicellulose as the main carbon source that can be used for the growth of mycelium straw
60 mushrooms (Sukendro et al., 2001). Usually, medium for growth mushrooms are the pulp of
61 oil palm, bagasse, waste cardboard, cotton waste, wood powder and so on. Even there have
62 been successful planting mushrooms using dried banana leaves (Balewu and Balewu, 2005).

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

70 The previous study reported that MGPB bacteria can induce the growth and productivity
71 of fungi. It is like research Young et al (2013) about bacteria contained in the growing
72 medium and mycelium Agaricus blazei that can induce the growth and productivity of the
73 fungus. It was reported that bacteria that can induce *A. blazei* growth is *Actinobacteria*
74 present in the planting medium. Research conducted by Familoni et al (2018), reported that
75 the bacteria are taken from the planting medium and oyster mushrooms fruit body (Pleurotus

ostreatus) obtained there is some isolate that can give effect to the growth of thick fungal colonies. Once identified using random amplified polymorphic DNA analysis (RAPD) with 10 primers the result was *Pseudomonas putida, Streptomyces spp., Trichoderma spp., Penicillium italicum*; and others.

Microbes become an important part of the growth of fungi. Research on the effect of bacteria on mushrooms productivity has been widely practiced. Zeranejad et al (2012) isolate and identifies bacteria that can induce *Agaricus bisporus* mushrooms production. In his research, he found two strains of bacteria that can induce mushrooms production. One of the molecularly identified strains is *Pseudomonas putida*. Research about the increasing production of straw mushrooms with the help of bacteria has been done by Payapanon et al (2011). It is known that bacteria that contribute to increasing nutrition in the composting phase are *Paenibacillus* dan *Bacillus sp*.

2. Materials and methods

2.1 Screening of selected bacteria

The process of obtaining indigenous bacteria that can induce the growth of mushrooms begins with a sampling of mushrooms substrate from 4 mushrooms producing regions, namely Karawang, Cikampek, Subang and Sukabumi. The substrate collection as a sample of harvesting and post-harvest is 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 are duplicated to be isolated by the four ways method. Then the pure isolate is separated into the NA medium inclined in the test tube (Cappuccino and Sherman, 2005). The pure isolates are tested for their ability by dual culture method. This method is done to selecting bacteria which can be increasing mycelia growth. This method used PDA in the Petri dish, and then bacteria cultured in the four sides of the

101 medium which is about 4 cm with pieces of mycelium in the middle. Then incubated in an
102 incubator with temperature 35°C. According to Chang & Miles (2004), the method of tissue
103 culture of straw mushrooms can be incubated at the temperature of 30-35°C. The selected
104 bacteria are the fastest bacteria reaching the edge of the Petri dish, so it can proceed to the
105 next step.

106 2.2 Identification of selected bacteria

107 Identification using DNA sequence 16S rRNA method. DNA isolation in this research
108 was performed by obtaining one selected bacterial ose aged 24 hours into Eppendorf and
109 resuspended with 100 µl Deion. The sample was heated at 96°C for 1 minute then incubated
110 at -22°C for 3 minutes. The steps are repeated three times. Then the sample was centrifuged
111 at 14,000 rpm for 5 minutes. The resulting supernatant is used as a template to do PCR
112 (Baker et al, 2003; Araujo et al, 2001; Yuwono, 2006). The amplification step of encoding
113 gene 16S rRNA used PCR with composition dH_2O 16,9 μl, 10 mM dNTP (dNTP mix) 0.50
114 μl, 5x KAPA2G buffer DNA polymerase 5 μl, *forward primers* 518F (5'-
115 CCAGCAGCCGCGGTAATACG -3') and *reverse* *primers* 800R (5'-
116 TACCAGGGTATCTAATCC -3') forward, and then added KAPA2G robust (5U/ μl) 0,10
117 μl. The PCR condition used is pre-denaturation 95°C, 5 minutes; the denaturation step is
118 95°C, 15 seconds; the annealing step is 54°C, 90 seconds; and the elongation step is 72°C, 60
119 seconds with PCR process consist of 25 cycles. The next step is post PCR of 72°C in 7
120 seconds and the stop step PCR is 4°C. DNA template PCR result was performed
121 electrophoresis on 1% agarose gel and the formed band was seen using UV transilluminator
122 after immersion in ethidium bromide solution (Yuwono, 2006).

123 2.3 Sequence allignments

124 The sequence of nucleotide bases obtained from the sequencing results is then processed
125 using bioinformatics software BIOEDIT v.7.0.8.0. Further analyzed using 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 used MEGA 6.06 software.

3. Results and Discussion

3.1 The screening of selected bacteria for straw mushroom growth.

The isolation result was found 58 bacterial colonies ready for screening using dual culture method. **Figure 1** shows the difference in the length of mycelium between the four sides of bacteria. On the bacteria side of 1S35, the mushroom mycelium is not close to the bacteria, 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 2S35 and 3S35 bacteria, the mycelium length approaches 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. In bacteria 4S35 shows the existence of clear zone produced by bacteria so that the mycelium growth stops until the clear zone. Growth rate of bacteria 4S35 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 smoother mycelium growth with a growth rate of 6.5 mm / day.

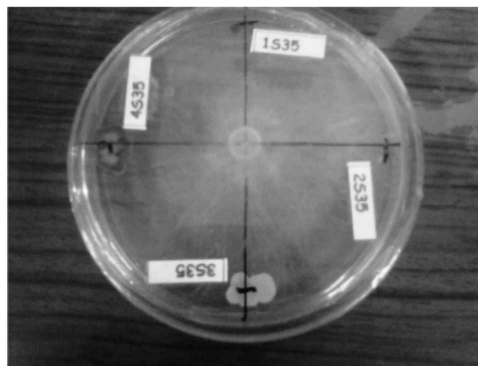
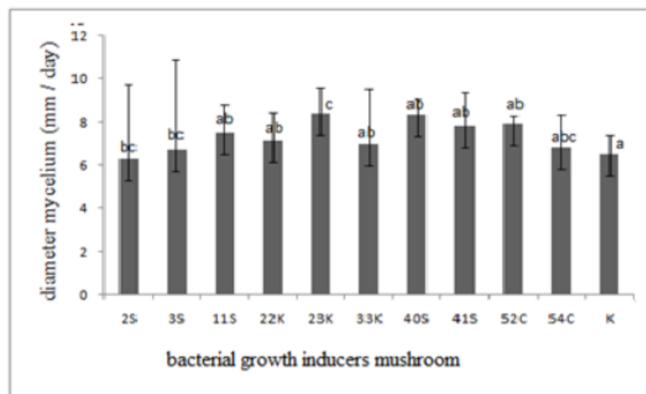


Figure 1. The screening of selected bacteria with *Dual Culture* method

145 The results of the screening showed two growth effects. The mycelium length and
146 mycelium growth are faster and longer than control. Both of these effects may be possible
147 due to the presence of bacteria on the PDA side of the medium. According to Pion et al (Pion
148 et al., 2013), the growth of fungi may be affected by the presence of inhibiting or antagonistic
149 bacteria or that induce growth according to the mechanism and potential of the bacteria.
150 Dual culture method that has been done to provide significant data on the difference of
151 mushrooms mycelium growth with given bacteria treatment. In **Figure 2**, of 10 bacteria that
152 can accelerate the growth of mycelium mushrooms, 23K bacteria have a faster rate of
153 mycelium growth than controls and other bacteria, which is about 8.4 mm / day.



154

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

157 Bacteria 23K was selected to be selected bacteria against the growth of mushrooms. This
158 can be due to several factors, such as the hormone produced, the ability to degrade the
159 nutrients for the fungus and produce certain proteins required by the fungus. According to
160 Payapanon et al (2011) that the presence of bacteria can affect the growth of fungi that exist
161 around it by producing hormones and have the ability to dissolve phosphate for the
162 availability of bacterial nutrients. That bacteria can also affect the growth of fungi because it

163 acts as a mushrooms growth-promoting bacteria (MGPB) to stimulate the growth of fungi

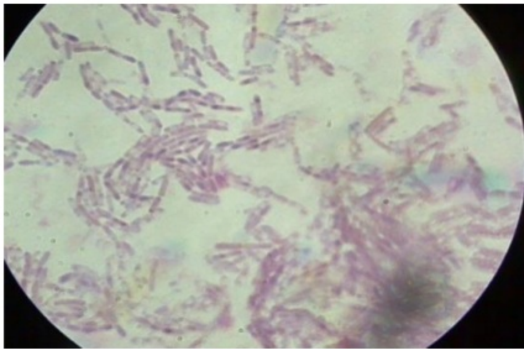
164 (Zeranejad et al., 2012; Familoni et al., 2018).

165 3.2 Identification of Bacteria 23K

166 Bacteria 23K have white colony color with the edge is rolled up. After four days, the

167 edge of the Bacteria 23K colony will be forming flagels, like moving in the medium (**Figure**

168 **3**).



169

170 **Figure 3**. Morphology of bacterial 23K with magnification 40x

171 According to Pakpahan et al (2013) the characteristic of *Bacillus* is motile with the

172 colonies' growth are spreading throughout the medium. The result of gram staining for

173 bacteria 23K is characterized by a gram-positive purple color of bacterial cells under

174 microscope observation and cell-shaped stem (bacillus).

175 Characterization is continued by identifying the bacteria molecularly that begun by

176 isolating the DNA of bacteria aged 24 hours. Samples were then performed by PCR to

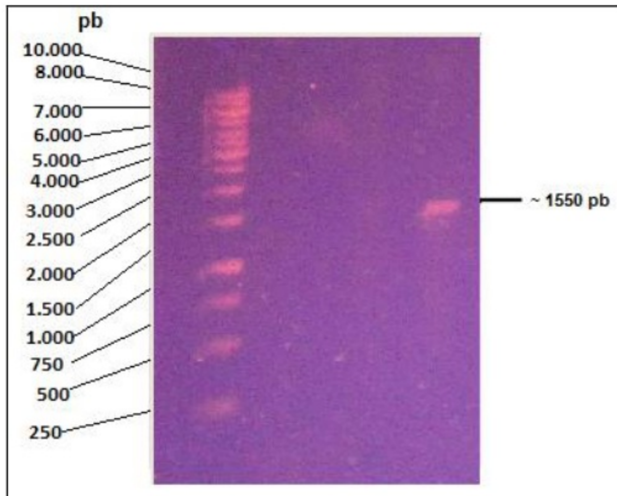
177 amplify DNA samples as DNA templates. Furthermore, electrophoresis was performed for 25

178 minutes with 1% gel to check the basepair of the bacterial genome which resulted in

179 visualization under UV light.

180

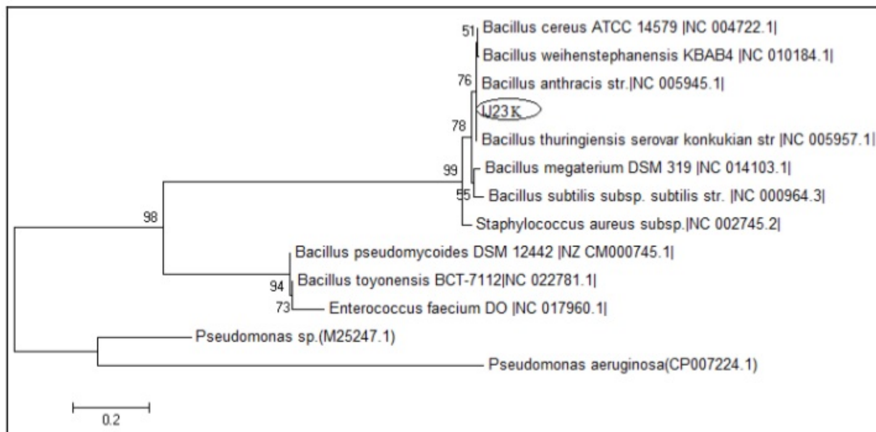
181



182 **Figure 4**. The result of amplification DNA 16sRNA for bacterial 23K

183 **Figure 4** shows that 23K bacterial DNA has an identical base length of ~ 1550 bp. The
184 DNA template is then entered into the stage of purification and sequencing. The sequencing
185 result is then processed using BIOEDIT software v.7.0.8.0. Furthermore, to find out the
186 identity of bacteria, the processed bases sequence is then analyzed using the BLASTN
187 program so it can be obtained its homolog. The last stage is to create a phylogeny tree to
188 determine the level of bacterial kinship using MEGA software version 6.06.

189



190 **Figure 5**. phylogenetic tree bacterial 23K

191 **Figure 5** shows that the construction of a 23K bacterial phylogenetic tree has the closest

192 kinship with some *Bacillus* such as *Bacillus thuringiensis serovar strucia str*. 97-27, *Bacillus*

193 *cereus* and *Bacillus anthracis* with a bootstrap value of 76.

194 *Bacillus thuringiensis* is grouped with *Bacillus cereus* and *Bacillus anthracis* whereby

195 all three have the ability to produce intracellular protoxin crystalline proteins (Roh et al.,

196 2007). It can also be seen that *Bacillus cereus* has a close relative to *Bacillus*

197 *weihenstephanensis*, but its bootstrap value is only 51. The bootstrap value is the value used

198 to determine the degree of confidence in the construction of a phylogenetic tree. If the

199 bootstrap value is low then the sequence has a low confidence level, whereas if the bootstrap

200 value is high, then the sequence confidence level is also high. In the phylogenetic tree, the

201 bootstrap value for each sequence is more than 75 so it can be categorized as having a high

202 trust value (Dharmayanti, 2011).

203 The phylogenetic tree shows that *Bacillus thuringiensis serovar konkukian str. 97-27*

204 is one of the bacteria closest to the Bacteria 23K. Classification of the NCBI as follows:

205 Kingdom : Bakteria

206 Division : Firmicutes

207 Clas : Bacilli

208 Ordo : Bacillales

209 Family : Bacillaceae

210 Genus : *Bacillus*

211 Species : *Bacillus thuringiensis serovar konkukian str. 97-27*

212 *Bacillus thuringiensis* is one of the millions of soil bacteria with pathogen characteristics

213 for insects (Hatmanti, 2000). Toxic compounds for insects from *Bacillus thuringiensis* are

214 specific to insect pests so they are harmless to other organisms and safe for humans (El-kersh

215 et al., 2012). The growth temperature for these bacteria is between 15°C - 40°C with an

216 optimum pH of 6.5 - 7.5 (Bernhard & Utz, 1993). *Bacillus thuringiensis* undergoes optimum
217 growth at 3-30 hours from the onset of inoculation and at the 30th hour undergoes a static
218 phase and decreases (Darwis et al., 2004).
219
220 **4. Conclusions**

221 Based on the results of this research, it can be concluded that Bacteria 23K is the most
222 significant bacteria increasing the growth of mushrooms than other isolates in the mycelium
223 phase. Based on the results of phylogenetic tree analysis, 23K bacterial isolates have the
224 closest to *Bacillus thuringiensis serovar konkukian str*. 97-27. It is necessary to test the
225 generative phase of mushrooms with bacteria 23K in the controlled clusters of temperature
226 and humidity.
227
228 **Acknowledgments**

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231 Pascasarjana Dalam Negeri).
232
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