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Potential Mushroom Growth Promoting Bacteria (MGPB) in Optimizing Paddy Straw Mushroom Production

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Paddy straw mushroom (*Volvariella volvacea)* contains high protein content and delicious flavor, makes it highly demand each year. Production of *V. volvacea* does not merit the requirements due to its limited production. Therefore, approach in increasing production using Mushroom Growth Promoting Bacteria (MGPB) are needed. This study aims to obtain MGPB isolate as potential agent to increase *V. volvacea* strain WW-08 growth. This is experimental research in laboratory that consisted of indigenous bacteria isolation, MGPB screening with dual culture, MGPB inoculum optimization, molecular identification of selected MGPB using 16S rRNA, protein profiling with SDS-PAGE, and fruting body production. Indigenous bacteria obtained from growth medium were 58 isolate, and W34 bacteria at concentration of 10^6 cell mL^{-1} showed most significant result on micellium growth. Sequence of 16S rRNA region showed W34 bacteria is *Bacillus cereus.* Visualization of SDS-PAGE showed new protein in result of interaction between *B. cereus* and *V. volvacea* strain WW-08 with molecule weight of 17 kDa. Average of fruting body of *V. volvacea* strain WW-08 in treatment of *B. cereus* harvested for 7 days, was 240.19 g, whereas that without treatment of *B. cereus* was 82.15 g. These findings indicate treatement of *B. cereus* strain W34 increase *V. volvacea* WW-08 growth by 300%.

Key words: *Bacillus cereus*, Mushroom Growth Promoting Bacteria (MGPB), *Volvariella volvacea* WW-08

Jamur merang (*Volvariella volvacea)* mengandung protein tinggi dan rasa yang lezat, yang menyebabkan permintaan meningkat setiap tahunnya. Produksi *V. volvacea* ini sangat terbatas sehingga tidak mencukupi kebutuhan di masyarakat. Oleh karena itu salah satu cara yang dilakukan dalam meningkatkan produksi dengan menggunakan pendekatan *Mushroom Growth Promoting Bacteria* (MGPB). Penelitian ini bertujuan untuk mendapatkan isolat MGPB sebagai agen potensial untuk meningkatkan pertumbuhan *V. volvacea* strain WW-08. Penelitian ini merupakan penelitian eksperimental di laboratorium yang terdiri dari isolasi bakteri, penyaringan MGPB dengan metode dual culture, optimasi inokulum MGPB, identifikasi molekuler MGPB terpilih dengan menggunakan 16S rRNA, profil protein dengan menggunakan SDS-PAGE, dan produksi tubuh buah jamur. Bakteri yang diperoleh dari hasil isolasi media pertumbuhan sebanyak 58 isolat, dan bakteri W34 pada konsentrasi 10^6 sel mL^{-1} menunjukkan hasil yang paling signifikan pada pertumbuhan misellium. Urutan basa rRNA 16S menunjukkan bakteri W34 adalah *Bacillus cereus*. Visualisasi SDS-PAGE menunjukkan terdapatnya protein baru hasil interaksi antara *B. cereus* dan *V. volvacea* strain WW-08 dengan berat molekul 17 kDa. Rata-rata tubuh buah *V. volvacea* strain WW-08 dengan perlakuan *B. cereus* yang dipanen selama 7 hari, seberat 240,19 g, sedangkan tanpa perlakuan *B. Cereus* berat 82,15 g. Temuan ini menunjukkan perlakuan strain *B. cereus* W34 mampu meningkatkan pertumbuhan *V. volvacea* WW-08 sebesar 300%.

Kata kunci : *Bacillus cereus*, MGPB, *Volvariella volvacea* WW-08

Stable environment in Indonesia all year is good for mushroom growth (Aryantha 2005). Many mushrooms in Indonesia are often used as food, one of which is *Volvariella volvacea (V. volvacea)* (Chen and Buswell 2004). *V. volvacea* has distinct and delicious flavor, soft, elastic, makes it highly demand (Chen and Buswell 2004). Demand of *V. Volvacea* is high approximately 50-60% of total national production, with average of elevated demand was 20-25%/year (MAJI 2007). Production of *V. volvacea* peak at 70% that was from West Java, Middle Java, East Java, and Lampung (Tridjaja 2005; Sumiati and Djuariah 2007). In 2015, mushroom demands gradually increase that peaked 17500 ton/year, whereas only 13825 ton/year has been fullfiled (MAJI 2007). Obstacles commonly encountered during production of *V. volvacea* is decreased yields due to high contamination (Sinaga 2012). Approach increasing growth using Mushroom Growth Promoting Bacteria (MGPB) are therefore needed.

Young *et al.* (2013) found bacteria that produces metabolite in growth medium of *Agaricus blazei* in dissolving metal, calcium, and nitrogen fixation that

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take place in growth production. It has been reported that *Ochrobactrum pseudogrignonense* is able to induce and increase mushroom growth of *Pleurotus ostreatus* with significant increasing yield (Maryana and Aryantha 2013). Young *et al.* (2012) found six soil bacterial strain that increases *Agaricus blazei* growth. Studies regarding biogical factors such as induction by indigenous MGPB are rarely done. This study aims to determine effect of MGPB induction on mushroom growth. Results are expected to provide solution for Indonesia farmers in increasing *V. volvacea* growth that can merit market demand.

MATERIALS AND METHODS

Identification of *V. volvacea*. Mushroom confirmed with moleculer analysis using Internal Transcribed Spacer (ITS) marker, is *V. volvacea* strain WW-08 (Raju *et al.* 2014)

Isolation of Indigenous Bacteria. Indigenous bacteria was isolated from several growth medium of *V. volvacea* in West Java. Referring to Kumala and Siswanto (2010), isolation was performed with dilution method. Samples from growth medium of 1 g was diluted in eight dilution series. Samples was cultured in medium (NA OXOID CM3), incubated for 24 h. Bacteria colony was further subcultured to obtain single colony based on its morphology. Single colony obtained was cultured in stock medium (Jagessar *et al.* 2008)

Indigenous Bacteria Screening. Screening was performed on micellium growth of *V. volvacea* with dual culture using potato dextrose agar (PDA) medium (Aryantha and Maryana 2012). Increase in micellium growth was measured each day. Data was analyzed with one way (ANOVA) with SPSS. Indigenous bacteria that shows highest increasing was further subcultured in NA and number of colony was measured with total plate count (TPC) (Suarsa *et al.* 2011).

Identification Selected Indigenous Bacteria. Identification was conducted based on morphology characters (shape, color, and surface of colony) (Chen and Zhang 2009), and Gram staining. Moleculer identification was performed using 16s rRNA. DNA isolation was performed with boiling method. Sample in sterile deionized was boiled for 1 m at 96 °C and incubated for 3 min at -22°C in triplicate. Samples were centrifuged for 5 min at 14 000 rpm, and supernatant was taken to undergo polymerase chain reaction (PCR) (Langille *et al.* 2013). Primer used was universal primer of 16S rRNA: 63f (5'-CAGGCCTAACACATG CAAGTC-3') and 1387r (5'-GGGCGGWGTGTACA AGGC-3'). PCR protocol include following: pre-denaturation at 95 °C for 3 min, denaturation at 95 °C 30 s; annealing 45 °C 30 s, elongation 72 °C 2 min, post PCR 72 °C for 7 min and stop PCR 4 °C. PCR products were electrophoresed through 1% agarose. PCR products were sequenced and analyzed with basic local alignment search tool nucleotide (BLASTN) and at gene bank NCBI (http://www.ncbi.nlm.nih.gov). Sequences of 16S rRNA from databese was aligned Multiple Alignment Clustal W using Molecular Evolutionary Genetics Analysis (Mega 5.1). Phylogenetic tree was constructed with Distance Neighbor Joining (NJ Analysis) (Tamura *et al.* 2011).

Screening of Inoculum. Bacteria inoculum screening was performed with dilution. Bacteria inoculum at concentration of 10^{-8}, 10^{-9}, and 10^{-10} were cultured in PDA. Micellium of 3 mm was cultured grown in medium and incubated for 5 d, measurement of micellium length was conducted each day (Suarsa 2011). Data was analyzed with one-way ANOVA (Analysis of Variance) using software Statistical Package for the Social Sciences (SPSS) (Santoso 2012).

Characterization of Protein Profil (SDS-PAGE). Both bacteria and mushroom were grown in PDB. Two days-aged sample were centrifuged at 13 000 rpm for 10 m at room temperature. Referring to Rajala (2013), supernatant was precitipated with addition of Sodium Deoxycholate (DOC) 25 µL, vortexed and left at 10 m, and incubated overnight. Samples were centrifuged at 13 000 rpm for 10 m at 4 °C. Pellet was taken and washed with 1 mL aceton, in duplicate. Buffer solutionof 6 × 50 mL of 20 mL (Tris-HCl pH 6.8 50mM 2,5 mL, sodium dodesil sulfat (SDS) 2% of 10% 2.5 mL stock, bromfenol blue 0.1 % 0.5 mL, glycerol 10% 5 mL, β-mercaptoethanol 100 mM 0.36 mL) was added, heated at 96 °C for 4 m. Running buffer used was 5 × 100 mL (tris base 1.51 gr, glycine 9.4 g, SDS 10% 5 g, added deionized water to reach final volume of 100 mL). Gel was composed of 30% acrylamid mix (acrylamid 2.92 g, metilen bis acrylamid 0.08 g, deionized water 10 mL), consisting of staking gel 4% (de Ion 1.67 mL, Tris pH 8.8 1.25 mL, SDS 10% 50 µL, acrylamid mix 2 mL, ammonium persulfate (APS) 25 µL, Tetra Etil Metil Ethilene Diamine (TEMED) 5 µL) and separating gel 12% (de Ion 3.05 mL, Tris pH 6.8 1.25 mL, SDS 10% 50 µL, acrylamid mix 0.65 mL, APS 25 µL, TEMED 5 µL). Gel was soaked with 200 mL staining solution (CBBR 0.5 g, metanol 100% 90 mL, pure acetetae acid 20 mL, deionized water 90 mL), and washed with destaining 400 mL (Metanol 100% 180 mL, deionized water 180

mL, pure acetic acid 40 mL). Result of SDS-PAGE was visualized by bands or press dry gel to obtain better result. Result of SDS-PAGE was analyzed with IMAGEJ to show detected protein peak (Susnea 2011).

Test of Selected Bacteria on *V. volvacea* Generative Phase. Selected bacteria grown in NA was subcultured in NB, and harvested after 24 h. Cultivation in mushroom house was modified using plastic pot of 30 cm diameter and height of 15 cm, referring to Aryantha and Maryana (2012). Each pot was filled with 5 logs composed of cotton as basic medium, bran of 2.5% and kapok of 1%, with log plastic of 2 kg. Treatment consisted of selected bacteria and watering, were sprayed to mushroom each day on growth medium surface. Treatments were conducted for 15 d. Humidity was maintaned with automatic water sprayer of 70-80%, and temperature was set to 26-28 °C. Initiation of fruting body emerged was measured at harvesting. Number and weight of fruitng body was measured at mature phase. Data was analyzed with ANOVA using software SPSS (Jackson *et al.* 2014).

RESULTS

Isolation of Indigenous Bacteria. Isolation of indigenous bacteria obtained 58 isolates (Table 1). Bacteria isolated from Subang were the highest among others, which was 25 originate from 3 mushroom house with different medium. Whereas 12 isolates was obtained from Karawang in 3 mushroom house, 12 isolates from Sukabumi in 2 houses, and 7 isolates from Cikampek in 2 houses (Table 1).

Screening of Indigenous Bacteria. Screening of 58 isolates was performed with dual culture. Results showed there were 2 bacteria that exhibited inhbition of mushroom, and bacteria that induced mushroom growth. There were 10 isolates obtained that possess ability to induce mushroom growth (Fig 1).

The highest micellium growth induced by bacteria, was exhibited by C bacteria, namely W34. W34 isolates was isolated from houses in Sukabumi, treated with sago medium and covering soil. Highest micellium growth rate influenced by W34, was about 13-15 mm each day, whereas lowest rate obtained at treatment of G bacteria B41, was about 5-7 mm. Growth rate in control without bacteria treatment, was about 3-5 mm each day. Statistical analysis showed results were significant among treatments, that suggested each bacteria exhibited significant effects on mushroom growth. W34 was able to highly enhance micellia growth of cold strain *V. volvaceae*, makes it chosen for further analysis. Other 48 isolates were considered to inhibit mushroom growth indicated by halo zone (Fig 2).

Identification of Selected Bacteria. Identification was conducted based on morphology and gram staining of W34 (Fig 3). Colony appeared was milky white slightly cloudy, colony surface was glisten/oily, unshaped edge, and round shaped. This bacteria is ocassionally motile in certain environment that appears separated and form new colony. Bateria staining showed bacteria was gram positive, and bacil, categorized as bacillus. *Bacillus* sp. is commonly about 3-4 mm, dull, unshaped edge, flat, and glossy surface.

Molecular identification was performed using 16sRNA as house keeping gene. The PCR product showed the target size (Fig 4).

Optimization of Indigenous Bacteria Inoculum. The highest micellia growth was 1 mL addition of bacteria age of 24 h with density of 10^6 cell mL^{-1}

Table 1 Indegenous bacteria obtained from *Volvariella volvacea* growth medium

Source	Growth Medium	Number of isolates
Subang (1)	Paddy straw	7
Subang (2)	Soil	9
Subang (3)	Cotton waste	9
Karawang (1)	Cotton waste and Paddy straw	5
Karawang (2)	Cotton waste	5
Karawang (3)	Soil	4
Sukabumi (1)	Sago waste	3
Sukabumi (2)	Sago waste	9
Cikampek (1)	Paddy straw	3

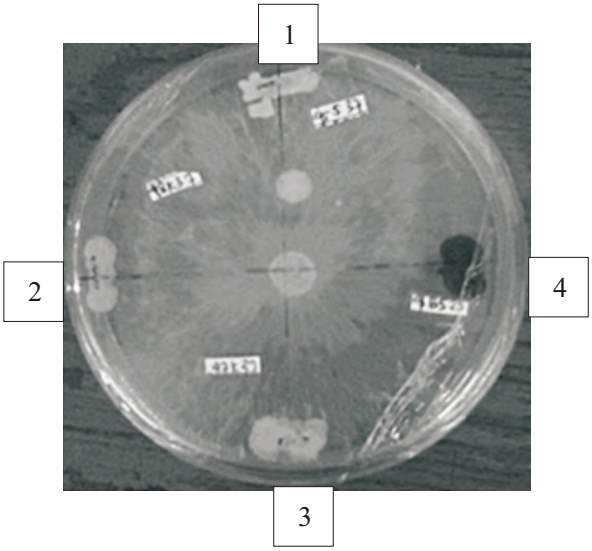


Fig 1 Screening of indigenous bacteria with dual culture. (1-2) Bacteria that induce mushroom growth, (3-4) Bacteria that inhibit mushroom growth.

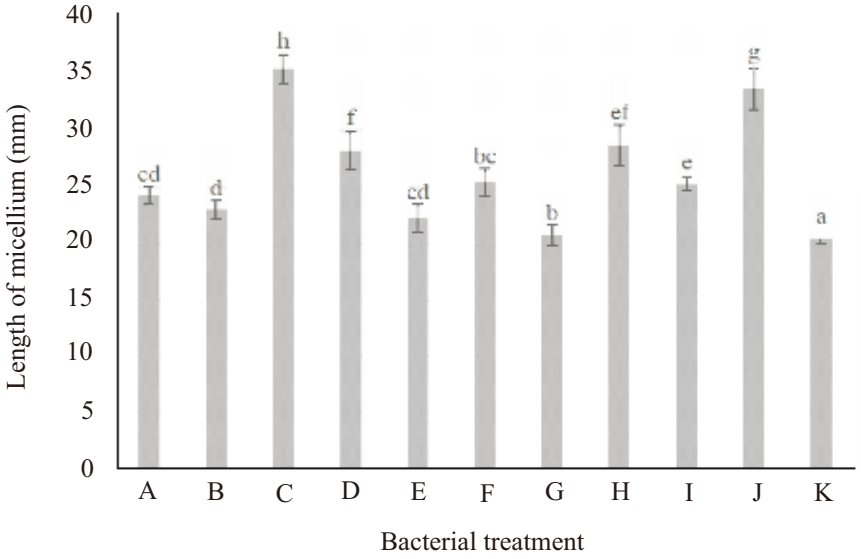
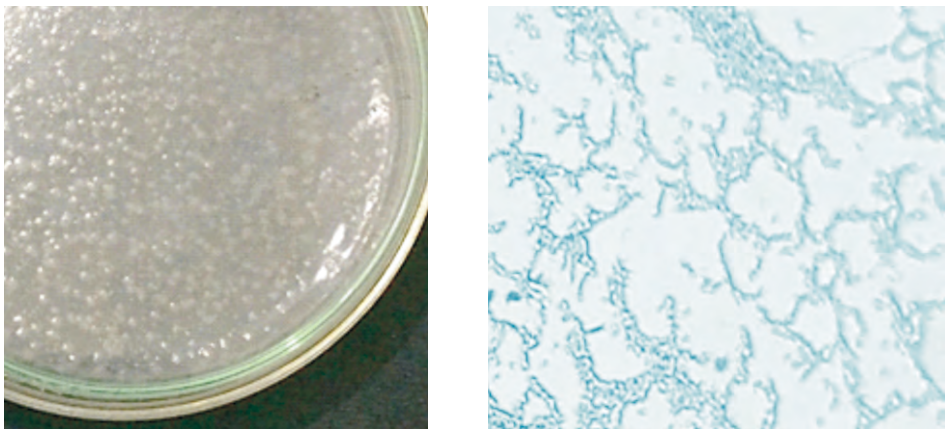


Fig 2 Micellia growth induced by indingenous bacteria (A) 17, (B) 32, (C) 34, (D) 35, (E) 39, (F) 40, (G) 41, (H) 42, (I) 47, (J) 54, and (K) control.



A B

Fig 3 Morphology and Gram staining of bacteria. (A) colony, (B) Gram staining.

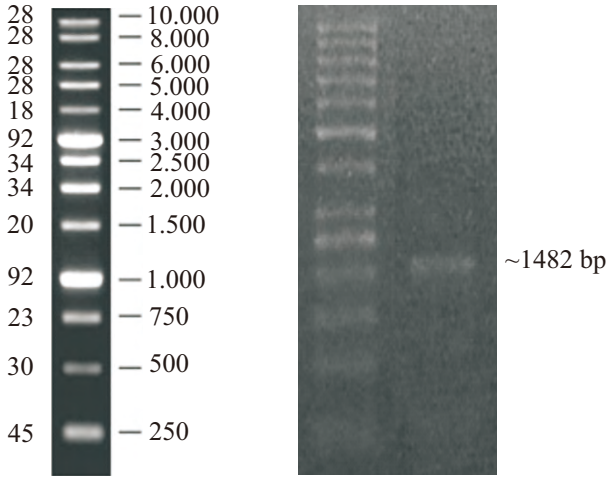


Fig 4 The PCR product of 16S rRNAgene of W34 isolate.

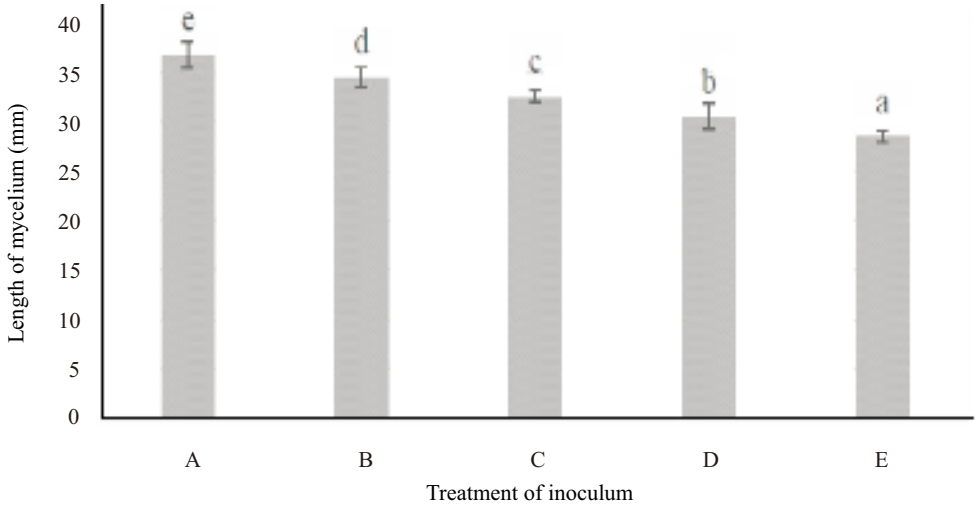


Fig 5 Optimized inoculum of selected bacteria A=10.6, B=10.5, C=10.4, D=10.3, E=10.10. Median of treatments followed by similar leter, is insignificant difference value (P>0.05) analyzed with Duncan post hoc test.

(Treatment A) (Fig 5).

Characterization of Protein (SDS-PAGE). Protein obtained from isolate was performed with SDS-PAGE. Sodium dodesil sulfat (SDS) is ionic detergent that dissolves hidrophobic resulting negative muatan in overall protein structure and further visualized with imageJ. Visualization of SDS page with ImageJ is shown in Fig 6.

Treatment of Selected Bacteria in Generative Phase of *V. volvacea*. Weight and number of fruting body were measured for 7 harvesting days. Fig 7 showed fruiting body and mycelium with and without treatment. Highest result treated with bacteria, was at 3rd day or 7th day after mushroom seeds cultivated, that resulted 681.77 g of 29 fruitng body. Weight harvested at 5th day was 406.65 g of 21 fruiting body, 308.43 g of 16 fruiting body at day 4,246.84 g of 18 fruiting body at day 2, 217.75 g of 15 fruiting body at day 6, and 66.06 g of 13 fruiting body at last day.

Non-treated mushroom was sprayed with water once a day to maintain its water supply. Highest result was obtained in day 3 that produced 188 g of 10 fruiting body, followed by 101.04 g of 9 fruiting body at day 5, 83.48 gram of 6 fruiting body at day 4, 54.87 g of 7 fruiting body at day 2, 55.54, g of 5 fruiting body, 10.02 g of 4 fruiting body. These results indicate treatment of bacteria enhance mushroom production of 200% compared to non-treated mushroom.

DISCUSSION

Isolation was performed to obtain promising bacteria. Indigenous bacteria obtainedwas different each area. These results might be due to various houses and growth medium used. Study conducted by Nannipieri *et al.* (2003) shows that soil is complex and dynamic system biology. Bacteria diversity is present due to nutrient content contained in soil such as C, N,

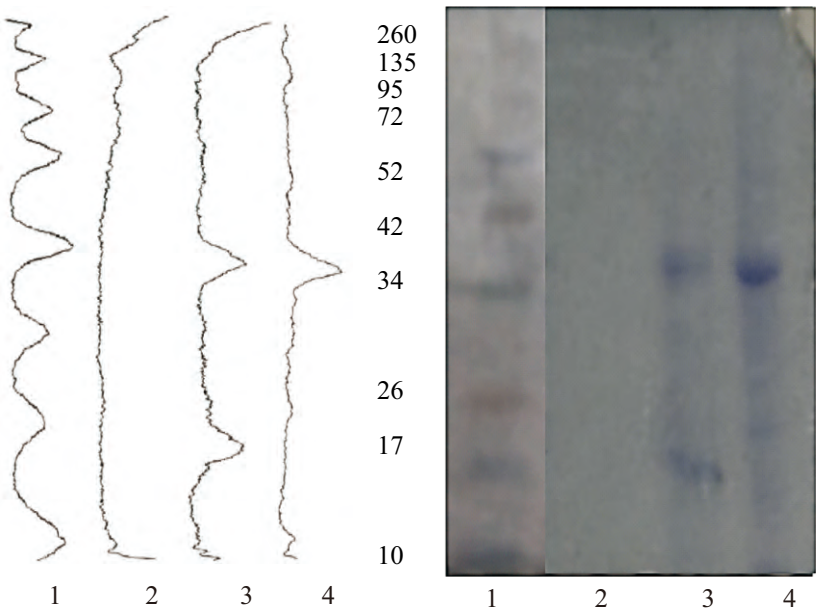


Fig 6 SDS-PAGE (A) visualization of protein peak (B) protein bands. (Line 1) Ladder (spectra broad range pre-stained), (line 2) JVC mushroom (line 3) JVVC + W34, and (line 4)W34 bacteria.

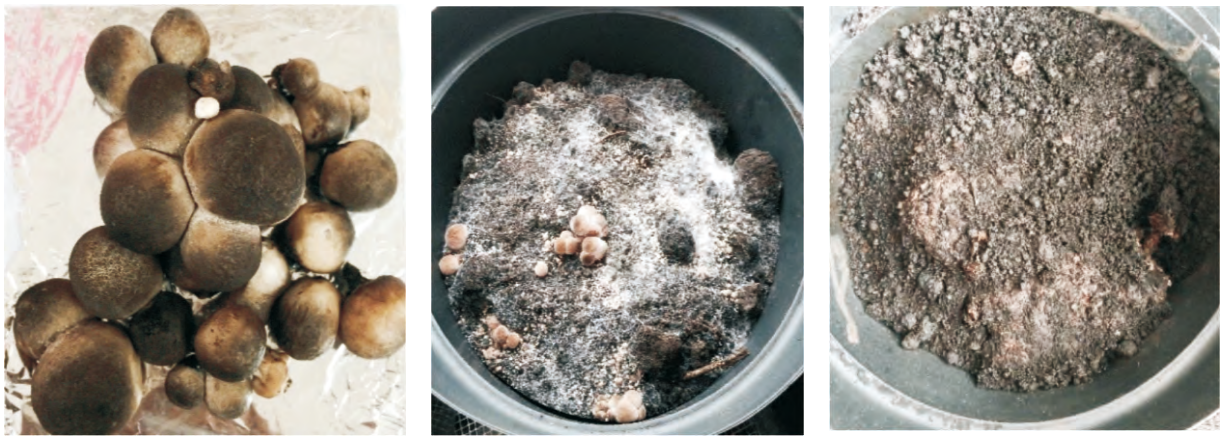


Fig 7 *Volvariella volvacea*. (A) fruiting body, (B) mycellium +34, (C) mycellium -34.

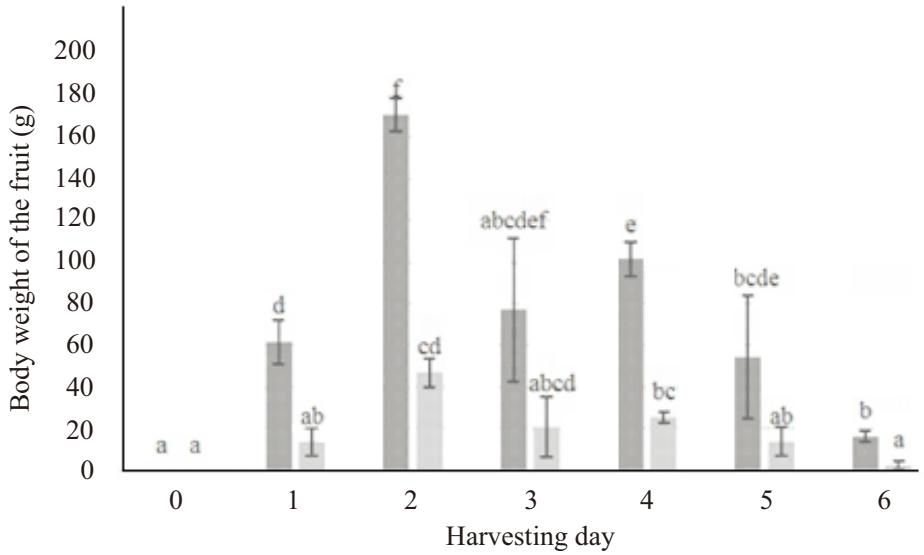


Fig 8 Production of *Volvariella volvacea* fruiting body. : bacteria, : without bacteria.

P, S, and other compounds. Composition of substrate as growth medium also affect bacteria diversity (Fig 1). Referring to Poyedinok *et al.* (2008) dan Sales-campos *et al.* (2011), bacteria growth is strongly influenced by environmental factors such as temperature, pH, oxygen supply, as well as internal factors such as nutrient content and other compounds to feed microorganism.

Bacteria also has antagonist effect in inhibiting mushroom growth or other microorganism. As shown in Fig 2, selected bacteria enhanced micellium growth indicated by growth to the edge. Enhancement of mushroom bacteria is commonly associated with ability of bacteria to produce secondary metabolite (Kim*et al.* 2008). There were 10 bacteria chosen based on its ability to enhance highest micellia growth.

Bacteria has different response to micellia growth, either synergistic or antagonistic (Frey-klett *et al.* 2011). Micellia growth relies upon secondary metabolite secreted by bacteria, either growth factor or antibiotic (antifungi) (Berendsen*et al.* 2012). Several interactions occurred between mushrooms and bacteria is physical association consisting of Planktonic, mixed biofilm, and intrahphal colonization. Molecullar communication such as antibiotis, metabolite alteration, chemotaxis signaling, metabolite convertion, phsiochemical changes, adhesion, protein production, and genetical alteration. Such interactions is commonly employed by both bacteria and mushroom to induce growth, development, and reproduction, transportion, gen aquisition, survival, and mutualistic and pathogenic simbiosis (Frey-Klett *et al.* 2011). Bacteria induce growth of *V. volvacea* belongs to Gram negative and Gram positive. According to Allen (1995), both Gram-positive and Gram-negative bacteria possess ability to intake metal cation present in soil. Gram-positive bacteria such as *Bacillus* sp., *Thiobacillus* sp. and Gram-negative such as *Pseudomonas* sp., commonly found in nature, are also able to dissolve phospate and utilized by other organism growth (Sharma *et al.* 2013).

Phylogenetic tree shows relationship among species with similarity value shown in bootstrap. Results showed W34 has close relationship to *B. cereus* (Dharmayanti 2011). Outgroup used was *Paenibacillus brasiliensis*. W34 is located near *B. cereus*, SBTBC-001 strain, JN66, dan etc. These results are in accordance with BLAST that indictae selected indigenous bacteria belongs to *B. cereus*. *B. cereus*is Gram-positive bacteria, bacil, aerobic, and facultative anaerobic, motile, ocassionally produce spora. This bacteria is abundantly found in nature, and soil (Huang and Tang 2007). Several *B. cereus* strain potetially act as probiotic, mesophilic, optimal at 20 °C - 40 °C, and adapt to various environments (Vilain *et al.* 2006).

Rajkovic (2006) reported highest cell obtained from *B. cereus* is at 24 h, in which approximately 59 × 10^6 CFU mL^{-1}, indicated as stationary phase. Further treatment with bacteria of 10^5 cell CFU^{-1} resulted length of 34.5 mm. Treatment of 10^4 cell CFU^{-1} resulted length of 32.5 mm. Lowest decreased mycellium was 30.5 mm obtained in treatment of bacteria (F) with inoculum of 10^3 cell CFU^{-1}. Mycellium of 28.5 mm was obtained for 5 d of growth with cells of 10^{10} CFU mL^{-1}. Based on measurement, induction by *B. cereus* toward cold *V. volvacea* strain was significantly different among treatments.

The new protein are formed by interaction between mushroom and bacteria weighs around 17-26 kDa. In this interaction between mushroom and bacteria also obtained a new extracellular proteins which previously were not present at each isolates, with molecular weight about 17 kDa. Research Hao-chi*et al.* (1997) showed that a successful immunomodulatory protein purified from *V. volvacea*, resulted in protein of about 17-18 kDa. Protein molecule is not activated when the fungus stands alone, but further activated upon interaction with bacteria. Protein *B. cereus* obtained on the value 34-42 kDa. Referring to Berber (2004), protein with a molecular weight of approximately 29-45 kDa protein is believed to originate from *B. cereus*.

Based on the data in Fig 7 can be seen that the treatment of bacteria produce fruiting bodies are heavier in higher mass. Suharnowo and Isnawati (2012) states that the high growth of fruiting bodies is due to the secondary metabolites produced by bacteria that this compound could help increase the formation of fruiting bodies. Bacteria generally produce metabolites that can be utilized by fungi to protect themselves from environmental stress, heavy metal stress and are also useful in inducing the growth of fungus. Production of biofertilizer bacteria can form compounds that can increase the solubility of minerals and nitrogen fixation. Research conducted by Zarenejad *et al.* (2012) showed that MGPB is able to dissolve phosphate to be easily digested by fungus as a source of energy (ATP) and growth. Another role as biostimulan is production of phytohormones such as auxin, cytokinin and ethylene that enhance the growth of fungi (Payapanon *et al.* 2011; Ahemad *et al.* 2015). Harni *et al.* (2015) reported that *B. cereus* isolated from the growth media patchouli is able to enhance plant growth by producing indole acetic acid (IAA).

Watering also should not be done too often in which excess water on the media causes mycelium subemerged and rotting. According to Li *et al.* (2014), excess water in media causesaltered colors, resulting in death. Research conducted by Mishra (2011), reported that the fungus will flourish on the materials that have been weathered or decomposed. Organic materials containing cellulose and lignin in large numbers supports the growth of mycelium and fruiting body development related to enzymes produced by the fungus. Temperature also influences the growth of fungal mycelium especially for *V. volvacea* cold strain. The temperature of the growth of *V. volvacea* or button mushrooms ranged between 24-30 °C, and optimum growth at 27°C (Thakur and Singh 2014).

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