Chemical characteristics of organic-based liquid inoculant of *Bacillus* spp.

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Abstract. *Bacillus* is an important soil rhizobacteria of many food crops and utilized as active substance in biofertilizer due to plant growth promoting activity. The objective of this research was to identify nutrient’s profiles and composition of primary and secondary metabolites in organic-based liquid inoculant of *Bacillus* spp. Proximate and molecular analysis had been carried out in laboratory by using standard method. The results verified that liquid inoculant contained organic carbon of 2.02%; small quantity of nitrogen, phosphor, potassium; and micronutrients iron and zinc. Heavy metals included lead, cadmium and chromium were detected in small amount, under the threshold level of heavy metal in organic fertilizer. The composition of primary metabolites in liquid inoculant was 0.31% of carbohydrate, 0.17% of protein, and 0.08% of lipid. The phytohormone of Indole Acetic Acid, gibberellin and cytokinin; as well as organic acids were detected in significant amount. This study suggests that consortia of *Bacillus* spp. enabled to synthesize phytohormone, organic acid, and exopolysaccharide which are important to promote plant growth.

1. Introduction

For environmental benefits, fertilizing soil with biofertilizer along with organic and inorganic fertilizers is recommended. Chemical fertilizers are still needed mainly because most of the agricultural soil in Indonesia is naturally low in nitrogen (N), phosphorus (P) and potassium (K); the three major essential plant nutrients. Inoculation of biofertilizer plays a significant role for substituting some inorganic fertilizers, especially N and P, and provides phytohormone that are not present in chemical fertilizers. To maintain the viability of microbial cells in liquid and solid biofertilizers, dormant cells are needed since they are resistant to harsh condition including high temperatures and low water availability. Biofertilizers are developed in many countries in the world to support sustainable agriculture systems. Nowadays, Indonesia gave an attention to biofertilizer development based on local microorganism strains.

The agricultural management strategy to reduce the use of inorganic fertilizers is biofertilizer development by using Plant Growth Promoting Rhizobacteria, PGPR [1]. The active ingredients of biofertilizers are beneficial soil microbes; including phosphate solubilizing microbes and phytohormone-producing bacteria [2] and exopolysaccharide-producing bacteria [3]. Rhizobacteria widely used as biological fertilizer are Gram-positive *Bacillus* which proliferate in the rhizosphere of plant roots [1].

*Bacillus* sp. are the prominent PGPR for the development of biofertilizers since they form spores [4] that withstand high temperatures in the tropics. The important *Bacillus* species for biofertilizer are *Bacillus megaterium*, *B. subtilis*, *B. cereus*, *Bacillus edaphicus* and *Bacillus mucilaginosus* [5]. The
production of phytohormone by Bacillus spp. has been extensively studied. Auxin produced by Bacillus sp. in bioassay increases the growth of potato plants (Solanum tuberosum) since auxin helps root development [6]. Bacillus megaterium Pl-04, and B. mucilaginosus B-1574 synthesize cytokinin (CK) and indole acetic acid (IAA); bacterial consortium increased CK and IAA levels by 35.6 and 21.3% and stimulating germination and growth speed of Cucumis sativus L. [7]. Some species of Bacillus are well known in solubilization of insoluble inorganic phosphorous in soil [8, 9]. The main mechanism for phosphate solubilization by Bacillus is excretion of organic acids. Bacillus megaterium, B. cereus and B. subtilis as well as their consortia produced at least two type of these organic acid: gluconic, lactic, acetic, succinic, and propionic acid[10]. Extracellular polymeric substance (EPS) plays a significant role to protect vegetative cell in harsh condition and to form biofilm in a surface [11]. Production of EPS by Bacillus species has been reported [12,13].

The initial stage of biofertilizers development is inoculant formulations. Liquid inoculant formulation is important to ensure bacterial viability as well as their metabolites production. Government Regulation no. 70 of 2011 about biofertilizer does not mention the content of primary and secondary metabolites even though the mechanism of Plant Growth Promoting Rhizobacteria is to increase the availability of nutrients and produce secondary metabolites. The objective of this research was to identify the composition of primary and secondary metabolites in organic-based liquid inoculant which contain 5.3 x 10^10 spore/mL of heat-resistance Bacillus spp.

2. Materials and methods
Metabolites identification was performed for organic based liquid inoculant containing four strain of Bacillus (Fig. 1) which contain 5.3 x 10^10 spore/mL All isolates were gram negative and motile. Laboratory analyses of the quality of liquid inoculant were conducted in May-June 2019 at the Soil Biology Laboratory of the Faculty of Agriculture, Universitas Padjadjaran, Jatinangor.

![Figure 1. Gram stain of four Bacillus isolates formulated in organic liquid culture](image)

Spores of Bacillus were counted after heating the fertilizer in a water bath at 80°C for 30 minutes in the mineral media by dilution plate method. The plat agars were then incubated for 24-48 hours at 30°C until free colonies was appeared. The chemical and biological properties of liquid biofertilizers to quantify organic carbon, N, P, K and micronutrients were performed by used of standardized methods (Table 1). Primary metabolites carbohydrate, protein and lipid were analysis proximately.

| Nutrients   | Analysis Method                                      |
|-------------|------------------------------------------------------|
| C-Organic   | Walkley and Black                                    |
| N-Total     | Kjeldahl                                             |
| P-Total     | HCl 25%                                              |
| K-Total     | HCl 25%                                              |
| Metal       | Atomic Absorption Spectrophotometer after Mixed acid extraction |
| Carbohydrate| Nelson-Samogyi                                       |
| Protein     | Kjeldahl                                             |
| Lipid       | Folch                                                |
Metabolite analysis was performed to quantify phytohormones, organic acid and exopolysaccharide. The content of Gibberellin (GA) and Cytokinin in liquid biofertilizer was carried out by HPLC after centrifugation at 10,000 rpm at 4°C for 15 minutes. The sample was evaporated and dissolved with acetonitrile for gibberellin and methanol for Cytokinin (Zeatin and Kinetin) prior to being injected into High Performance Liquid Chromatography (HPLC). Indole acetic acid analysis was carried out by using spectrophotometry with Salkowski’s solution. Organic acid levels were determined by HPLC.

Crude exopolysaccharide content was carried out by using the gravimetric method. Biofertilizer was centrifuged at 10,000 rpm at 4°C for 15 minutes. The supernatant was collected and 2 volumes of acetone was added before overnight at 4°C. The suspension was centrifuged again, the supernatant was removed and the EPS at the bottom of the tube was collected and transferred to Whatman filter paper no. 1, heated at 350°C for 30 minutes, cooled in a desiccator and weighed. Exopolysaccharide weight was the difference between the weight of filter paper containing EPS with the weight of empty filter paper after heating 350°C for 30 minutes.

3. Results and Discussion

Nutrients composition of liquid biofertilizer revealed that the concentration of major macronutrient N, P and K were low (Table 2), which explain that the biofertilizer was not the source of macronutrient for plant growth. Two metals, ferrous and zinc might contributed to plant development when biofertilizer is applied to the soil with low concentration of both nutrients.

Table 2. Nutrient composition of organic-based liquid biofertilizer

| Nutrients     | Amount  |
|---------------|---------|
| Organic-C     | 2.02 %  |
| Total-N       | 0.04 %  |
| P2O5          | 0.06 %  |
| K2O           | 0.02 %  |
| Ferrous       | 63.91 mg/L |
| Zink          | 12.02 mg/L |
| Carbohydrate  | 0.31 %  |
| Protein       | 0.17 %  |
| Fat           | 0.08 %  |

Aluminium was not detected, however biofertilizer contained some heavy metal with the concentration below the threshold of government regulation. Concentration of lead, cadmium and chromium were 0.77, 0.09 and 9.87 mg/L respectively. Very toxic heavy metal arsenic and mercury were not found. Since biofertilizer was formulated in organic-based liquid media; carbohydrate, protein and lipid were identified in low concentration (Table 2). The primary metabolite might be important for heterotrophic soil microbes which used organic material for energy, carbon and nitrogen source. Several phytohormone (IAA, GA₃ and Zeatin) which enable plants to growth better was found in liquid inoculants of Bacillus (Table 3). The research verified that liquid inoculant contained organic acids; demonstrated that Bacillus produced organic acid which was important for phosphate solubilisation.

Table 3. Metabolite in liquid inoculant composed of some isolates of Bacillus sp.

| Metabolites          | Unit | Amount | Metabolites | Unit | Amount |
|----------------------|------|--------|-------------|------|--------|
| Indole Acetic Acid   | mg/L | 8.47   | Oxalic acid | mg/L | 367.1  |
| Gibberellin (GA₃)    | mg/L | 5.31   | Maleic cid  | mg/L | 0.7    |
| Cytokinin (Zeatin)   | mg/L | 0.04   | Lactic acid | mg/L | 6.40   |
| Exopolysaccharide    | g/L  | 4.00   | Tartaric acid | mg/L | 200.1  |
| Phosphatase          | mg/L | 0.04   | Acetic acid  | mg/L | 305.8  |
Indole Acetic Acid determined in liquid culture was higher than the ability of Bacillus to produce auxin in other experiment: 60-80 µg/mL at 48 hours of incubation [6]. Bacillus subtilis LK14 isolated from Moringa peregrine plant produced IAA to improve the growth of Solanum lycopersicum [14]. The ability of Bacillus to produce Gibberellin and Cytokinin was not a novel knowledge. Some Bacillus strains isolated from rhizosphere of Dipterocarpus sp. produce gibberellin up to 0.221-0.897 mg/mL [15]. Identification of auxin and gibberellins from ethyl acetic fractions using HPLC revealed that Bacillus subtilis LKM-BK produced 1-naphthalene acetic acid (NAA), tryptamine, 3-indole propionic acid (IPA), indole-3-butyric acid (IBA), 3-indole acetic acid (IAA), gibberellic acid, and trans-zeatin [16]. Zeatin Riboside was the main cytokinin in Bacillus subtilis liquid culture [17].

Organic acid is is a typical production in phosphate-solubilizing Bacillus to solubilize inorganic phosphorous to phosphate ion that available for plant uptake. The mechanisms of PGPR to solubilize the unavailable form of inorganic phosphorus (Ca-phosphate; Al-phosphate; K-phosphate) to available ions of HPO₄²⁻ and H₂PO₄⁻ [18]. This was an important bacterial metabolisms that gave the benefit the plants. However, the solubilization of inorganic P is a complex process which also depend on soil properties. Phosphorous was found in soil and also in organic phosphate form. Phosphatase in liquid culture (Table 3) was an enzyme which catalyse organic phosphate degradation to release available inorganic phosphate. Bacillus mycoides and B. laterosporus isolated from forest trees enabled to mineralize through phosphatase activity [8].

Exopolysaccharide was found in the significant amount in liquid culture of Bacillus consortium (Table 3). Production of EPS by five Bacillus species had been demonstrated in vitro; they excreted 3-35 µg EPS/10⁶ cell depended on the species, media composition and glucose concentration [12]. Medium with cane molasses enriched with rice bran, salt and organic acid increased EPS production of B. subtilis up to 4.86 g/L [13]. In agricultural land, EPS contribute to improve soil aggregation which induce better nutrient uptake [3].

Metabolite production by Bacillus spp. in liquid culture were valuable initial information to ensure that the bacterium are benefits plant and might be formulated as biofertilizer. However, deep and intensive research concerning the influence of media composition; as well as organic and inorganic substance composition are necessary prior to biofertilizer formulation.

4. Conclusion
Bacillus consortium liquid culture with the bacterial density of 3.4 x 10¹⁰ cfu/mL contained small amount of macronutrient N, P and K but significant amount of micronutrient of Fe and Zn. The phytohormones, various types of organic acids as well as phosphatase and exopolysaccharide in the liquid inoculant showed that Bacillus spp might play a significant role to enhance plant growth. This finding suggested that Bacillus consortium demonstrated the abilities to produce multiples metabolites which can help plant to better growth and yield. The whole analyses of this liquid inoculant explained that the formulation are necessary to improve the quality of liquid inoculant prior to biofertilizer development.

Acknowledgement
The research was funded by Directorate Generale of Higher Eduction Kemenristek-Dikti, Republik Indonesia year 2019.

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