

PAPER • OPEN ACCESS

## Potency of nitrogen fixing bacteria isolated from POME disposal pond and their effect on the growth of *Caesalpinia pulcherrima* (L) Sw

To cite this article: S Widawati and Suliasih 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **308** 012043

View the [article online](#) for updates and enhancements.

# Potency of nitrogen fixing bacteria isolated from POME disposal pond and their effect on the growth of *Caesalpinia pulcherrima* (L) Sw

S Widawati\* and Suliasih

Research Centre for Biology- Indonesian Institute of Sciences, CSC-LIPI, Jl. Raya Jakarta-Bogor km 46, Cibinong 16911, West Java, Indonesia.

Email: widadomon@yahoo.com

**Abstract.** Nitrogen fixing bacteria (NFB) play an important role to promote plant growth through nitrogen fixation and IAA hormone production. The objective of this research was to obtain the functional bacteria (nitrogen fixing bacteria) isolated from POME disposal pond that show the ability in nitrogen fixation and the potential as plant growth promoting rhizobacteria (PGPR), as well as to investigate the potential of these bacteria through in vitro and in vivo growth of *Caesalpinia pulcherrima* (L) Sw, and its effect on bacterial populations in media contaminated by POME. In vitro plant growth promoting activity characterization included N-fixing activity, Indole Acetic Acid production, and *C. pulcherrima* germination (inoculant + POME). In vivo plant growth promoting activity used sterile sand inoculated with NFB and watered with POME, whereas the control was not inoculated and watered with aquadest. Five strains were found as potential PGPR, namely *Azotobacter* sp., *Azospirillum* sp1-3., and *Rhizobium* sp. In addition, *Azospirillum* sp.1 is the best PGPR that produced highest seed vigour index (SVI), seed germination in vitro, and seed germination in vivo respectively. *Azospirillum* sp.1 and *Rhizobium* sp. have the ability to promote growth of *C. pulcherrima* in vitro and in vivo with bacterial populations as much as  $10^7$ cfu mL<sup>-1</sup> in POME contaminated media. The bacteria and *C. pulcherrima* are potential for reclaiming infertile soil.

## 1. Introduction

Indonesia held the largest number of palm oil plantation in the world, for about 7.796 million hectares in 2010 [1]. Palm oil processing plants are spread out in several cities alongside various waste concerns. The waste impacts negatively on natural resources and the environment, for instance pollution and depletion of natural resources that degrade the quality of the environment. The resulting waste is classified into three types: solid waste, liquid waste and gas. The characteristics of palm oil plant liquid waste are brown, acidic, viscous, odorous, and non-toxic in nature. It is called POME or Palm Oil Mill Effluent [2].

Currently POME and sludge are used as fertilizer. Direct use can indeed increase phosphorus, nitrogen, calcium and magnesium in the soil [3], but blockages and puddles occur in the soil [4]. Whereas indirect use is by isolating bacteria from POME disposal pond. Thompson *et al.* [5] reported that correct and precise bacterial isolation determined the suitability of bacteria in the selected waste water remediation process. Therefore, the principle of the bacterial isolates selection provides the optimal reduction of pollutant level. Naturally, the preferred bacteria are available in small amounts



and unwanted bacteria are present in large numbers. Isolation process must be done in order to multiply the number of preferred bacteria [6].

Bacterial isolates obtained from palm oil waste usually have microscopic morphological characteristics of cells belonging to Gram-positive [7] and Gram-negative [8]. Gram-negative heterotrophic rods such as *Zooglea*, *Pseudomonas*, *Chromobacter*, *Achromobacter*, *Alcaligenes* and *Flavobacterium* [9]. Moreover, it is found the functional bacteria that can fix N, dissolve P, produce IAA, and effective as a bio-stimulant for the growth of chili plants [8].

The roles of bacteria in wastewater treatment and their ability in maintaining the ecological balance of aquatic environments have been extensively studied. However, the research on the isolation of microbes from POME storage pond was scarce. It is the bases to further study about functional bacteria, particularly the plant growth promoting rhizobacteria (PGPR) that has the effective role in the process of soil health rehabilitation, which will be able to optimize plant growth, especially in waste-contaminated soil.

Functional bacteria are bacteria that could work synergically with organic fertilizers. These bacteria possess the ability to stimulate plant growth (bio-stimulant). The procedure is by synthesizing and regulating the concentrations of various Plant Growth Regulators (PGRs) such as indole-3 acetic acid or IAA [10], producing ACC-deaminase, and providing nutrients as bio-fertilizers. The process was done by N<sub>2</sub> fixing from air through a symbiosis and dissolving P bound nutrients in soil by synthesizing PME-ase (Phosphomonoesterase). These bacteria should also be able to control the activity of pathogens from invasive organisms (bio-control), agrochemical compound modifier [11], and soil originated-pathogens (bio-protectant) by producing antipathogenic compounds or metabolites such as siderophore, chitinase, antibiotics, and cyanide [12].

Functional bacteria that have these abilities are usually classified as PGPR. When the bacteria are inoculated into seeds, sprouts, seedlings, or soil, they will directly colonize the root area of the plant (rhizosphere) aggressively and fertilize the soil, thus increase the supply of nutrients needed by the plant [13]. Some of the bacteria included in PGPR belong to the genus *Flavobacterium*, *Herbaspirillum*, *Acetobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Serratia*, *Enterobacter*, *Erwinia*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, and *Rhizobium* [14].

Some bacteria such as *Rhizobium*, *Azotobacter*, and *Azospirillum* are able to nitrogen fixation as well and produce plant growth hormones such as IAA, gibberellins, and cytokines [15]. Therefore, these genus are considered essential components of biological organic fertilizers [16; 17], especially as the fundamental materials of bioorganic fertilizers that are useful to stimulate plant growth in revegetating barren land due to pollution by POME waste.

Isolation, characterization and identification of bacteria isolated from POME disposal pond is one approach to verify that nitrogen fixing bacteria (NFB) is beneficial in promoting soil fertility and plant growth in soil contaminated with POME waste. The effectiveness of NFB is proven by laboratory and field testing on *C. pulcherrima* (L) Sw plant was used for bacterial affectivity test. Consideration of the use of *C. pulcherrima* (L) Sw plant, because this plant has the potential for reboisation in urban areas or recreational forests and also for potentially improving soil fertility.

*C. pulcherrima* (L) Sw to grow in sandy soils, clay soils, acidic and alkaline soils, and produce bioactive compounds that are useful for health purposes such as anti-inflammatory agent [18] and antiviral producers that are modulated by certain flavonoid compounds recognized as quercetin [19].

The objectives of this research were to obtain the functional bacteria isolated from POME disposal pond that show the ability in nitrogen fixation and the potential as PGPR, to investigate the potential of these bacteria on the growth of *C. pulcherrima* (L) Sw in vitro and in vivo as well as its effect on bacterial populations in media contaminated with POME. So it is expected that in the future the use of POME as an organic fertilizer is not only inexpensive but also as an alternative for excessive chemical fertilizer applications [20].

## 2. Materials and Methods

### 2.1. Isolation and identification

Sampling was carried out randomly in the first reservoir of POME waste (soil pond) in PTP VIII palm oil plantation, Cikasungka, Bogor regency, West Java, Indonesia. The first pool is a direct shelter from factory waste, so that the waste conditions are warm. Samples were taken from POME disposal pond in the form of sediment or soft soil. The isolation of bacteria was carried out in a selective media such as yeast mannitol agar / YEMA [18], Caceres [21], and mannitol Ashby [18]. It was prepared by putting ten grams of sediment in an Erlenmeyer flask containing 90 mL of sterile distilled water. The flask was shaken on a rotary shaker (120 rpm in 30 minutes). Serial dilution of  $10^{-1}$  to  $10^{-7}$  was made from each sample. About 0.2 mL of soil extract was taken and put into sterile Petri dish, then was poured over by selective medium (temperature: 50°C): YEMA (10 g manitol; 0.5 g  $K_2HPO_4$ ; 0.2 g  $MgSO_4 \cdot 7H_2O$ ; 0.1 g NaCl; 1.0 g yeast; 20 g agar; 1 L aquadest + 2.5 mL  $L^{-1}$  Congo red solution) to obtain *Rhizobium*, mannitol Ashby (20 g manitol; 0.2 g  $K_2HPO_4$ ; 0.2 g  $MgSO_4 \cdot 7H_2O$ ; 0.1 g NaCl; 0.1 g  $K_2SO_4$ ; 5 g  $CaCO_3$ ; 20 g agar; 1 L aquadest) to obtain *Azotobacter*, and Caceres medium (0.5 g  $K_2HPO_4$ ; 0.2 g  $MgSO_4 \cdot 7H_2O$ ; 0.1 g NaCl; 0.5 g yeast extract; 0.02 g  $CaCl_2$ ; 0.01 g  $FeCl_3 \cdot 6H_2O$ ; 5.09 g DL mallic acid, 4.8 g KOH; 20 g Agar; 15 mL  $L^{-1}$  Congo red solution) to obtain *Azospirillum*. All media were made at pH 7.0 and incubated at room temperature for 7 days. The isolate obtained was purified and stored on a Luria-Bertani (LB) slanted media. After 3 days of incubation, the isolates were identified by observing the morphological characters such as cell shape (coccus, rod, short rod), gram positive / negative, cell motion (motile, spore formation, single, paired or chain), and cell movement (motile, spore formation, single, paired or chain). Biochemical properties of isolates were determined according to the Bergey's Systemic Bacteriology method [22-24].

### 2.2. Functional bacteria characterization as PGPR

**2.2.1 Nitrogen fixation.** The selection of nitrogen fixing bacteria was performed based on the method of Dobereiner [25]. The bacteria obtained from selective media were inoculated into a test tube containing Nitrogen Free Bromthymol blue (NFB) semi solid medium without N elements (0.5 % DL-mallic acid; 0.4 % KOH; 0.05 %  $K_2HPO_4$ ; 0.01 %  $MgSO_4 \cdot 7H_2O$ ; 0.005 %  $MnSO_4 \cdot H_2O$ ; 0.002 % NaCl; 0.001 %  $CaCl_2$ ; 0.005 %  $FeSO_4 \cdot 7H_2O$ ; 0.0002 %  $Na_2MoO_4 \cdot 2H_2O$ ; and 0.175 % bacto agar, and 2 mL 0.5 % bromthymol blue), then incubated at room temperature for 5-7 days. The bacterial activity of nitrogen fixing was indicated by the formation of a circular mist-like ring beneath the surface of the medium. Single bacterial cultures then were prepared on a Petri dish and on a test tube, both containing tilted Caceres media. It was incubated for 2 x 24 hours at 30°C for further growth observation. The growth was an indication that the bacteria were nitrogen fixing bacteria.

**2.2.2. The production of Indole-3 Acetic Acid.** The presence of Indole-3 acetic acid produced by bacteria is one of significant indication of PGPR group. Isolates were inoculated into a 50 mL flask containing King B broth with 200 ppm tryptophan as physiological precursors of auxin biosynthesis in the IAA analysis. After that, it was incubated at room temperature for 24, 48, and 72 hours. About 2 mL of culture suspension was taken from each incubation period and centrifuged for 5 min. The supernatant was transferred into a test tube, and then added with 4 mL of Salkowski reagent. The production of IAA was indicated by the pink colour on the bacterial extraction [26]. Quantitative analysis of IAA production was measured using spectrophotometer at  $\lambda$  540 nm with interpolation on IAA calibration curve, afterwards.

### 2.3. The potency of NFB as PGPR during the Germination of *C. pulcherrima* (in vitro)

**2.3.1. Bacterial augmentation on seed.** A total of 30 swollen seeds after soaking in sterile aquadest were taken with tweezers and placed into one of the nitrogen fixing bacteria cultures on LB liquid

medium. Seeds were incubated in bacterial culture for 1 hour. The same procedure was repeated for soaking seeds in other bacterial isolates. A total of 450 seeds were augmented with 5 different nitrogen-fixing bacteria and the rest (90 seeds) were not augmented (control). All treatments replicated 3 times.

**2.3.2. Germination.** Based on previous research (Unpublished), a total of 30 seeds, swollen by soaking in sterile aquadest, were collected and planted in Petri dish covered by filter paper moistened with 5 mL of inoculant and 5 mL of 10 mL POME in 100 mL sterile distilled water solution. In contrast, the controls were not mixed with bacteria and POME. The characteristics of seed germination observed were germination percentage and sprout length after 7-10 days incubation. Parameters in germination studied were percentage of germination and vigour index [% Germination  $\times$  (Average of shoot length + root length)]. The percentage of germination is the total number of germinated seeds divided by the total number of the seeds multiplied by 100 %. After that, the number of bacterial population that infected the sprouts root was calculated. One sprouts from each Petri dish (both control and treatment) was transferred into a test tube containing 9 mL of sterile aquadest (dilution to  $10^{-1}$ ), and mashed. The test tube was then pressed into a vortex for 2 minutes to mix the content. About 1 mL extract was diluted up to  $10^{-5}$  dilution series. As much as 0.2 mL of extract was taken from each dilution series  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-5}$  and placed in a sterile Petri dish. After that, selective media was poured over into the Petri dish to attain the *Azotobacter*, *Azospirillum*, and *Rhizobium*. The culture was homogenized and incubated for 3 days at room temperature. The number of growing colony was counted with a colony counter to obtain colony-forming units.

#### 2.4. The potency of BNF during the germination of *C. pulcherrima* (in vivo)

**2.4.1. Seedling planting.** Germination took place in Petri dish filled with sterile aquadest. After 3 days of incubation, the seeds that had germinated into sprouts were put into the Baker glass containing the liquid inoculants of the NFB (*Rhizobium*, *Azotobacter*, and *Azospirillum*). Two sprouts were planted on pots packed with sterile sand media (300 g). The sand media was dampened beforehand with a sterile distilled water (20 mL per pot) up to field capacity, then inoculated with 5 mL of liquid inoculant from each of the nitrogen-fixing bacterial isolates, and incubated for 5 days before planting. After planting, the moisture of the media and the maintenance of the potted plants were ensured by watering based on the treatment. POME solution with a ratio of 100 mL in 1000 mL was applied to potted plants containing *Rhizobium*, *Azotobacter*, and *Azotobacter* isolates 1 to 5, and for comparison watering with aquadest containing macro and micro elements or Müller's method were done as well. Control plants were watered with sterile aquadest. Three replicates were prepared for each treatment. In the age of five weeks, the plants were harvested. Some data were measured, such as plant height, root length, number of branches, wet weight, dry weight, presence of embryo, and bacterial population per pot of all treatments by Total Plate Count.

### 3. Results and Discussion

#### 3.1. Isolation and identification

Bacterial isolation from POME disposal pond on selective media (YEMA Congo red, Caceres, and Mannitol Ashby) resulted in 15 isolates and 5 isolates were identified manually. The identified isolates were *Rhizobium* sp. (1 isolate), *Azotobacter* sp. (1 isolate), and *Azotobacter* sp. (3 isolates).

#### 3.2. Functional bacteria characterization as PGPR

**3.2.1. Nitrogen fixation.** The growth of 5 strains inoculated in a test tube containing semi solid Nitrogen Free Bromthymol Blue (NFB) medium showed that all the strains could form a ring-like circular mist below the media surface after 3 days incubation at room temperature (Figure. 1).

The ring was formed due to the production of nitrogenase by nitrogen fixing bacteria. Comparable results were reported by Baldani *et al.* [27], Kusumawati *et al.* [28], which stated that the formation of nitrogenase by nitrogen fixing bacteria was indicated by the presence of a circular fog beneath the surface of a semi-solid NFB medium. The formation of nitrogenase signified that the nitrogen fixing bacteria were absolutely motile. Caceres [21] reported that the formation of ring-like white mist beneath the surface of a semi-solid NFB media implies that the bacteria, especially *Azospirillum* sp. are motile. Further results confirmed that the bacteria belong to the nitrogen fixing bacteria group with asymmetrical circle, pink, flat, glossy, and smooth colony with flat edges (Figure 2). The pink colour of the bacterial colony was affected by Congo red in Caceres media that was absorbed into the cells by simple diffusion and fused to the protein component of cell membrane [29; 30].



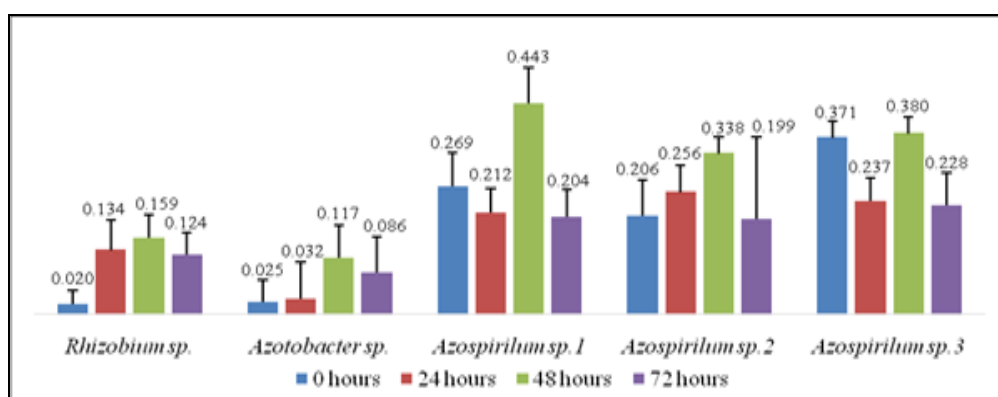
**Figure 1.** Nitrogenase production by *Azospirillum* sp. (1, 2, and 3), *Rhizobium* sp. (4), and *Azotobacter* sp. (5).



**Figure 2.** Single culture on Caceres media in Petri dish and test tube

**3.2.2. The production of Indole Acetic acid.** The ability of bacteria (*Azospirillum* sp.1-3, *Azotobacter* sp., and *Rhizobium* sp.) to produce of IAA growth hormone can be seen in Figure 3. The IAA test resulted in pink colonies of all 5 strains tested on NFB medium containing L-Tryptophan 200 ppm as precursor, after the addition of Salkowski reagent. Quantitatively, IAA test showed positive results as well, where the average IAA level reached the optimum level after 48 hours incubation. The IAA produced by bacteria was varied on the range from 0.117 – 0.443 $\mu\text{g mL}^{-1}$  and the highest yield of IAA were observed in *Azospirillum* sp.1 (0.443 ppm).

This occurred because the 48-hour incubation was a logarithmic phase in which the effect of the enzymes used in the bioconversion of tryptophan to IAA such as tryptophan monooxygenase, IAA hydrolase, indol-pyruvate decarboxylase and IAA dehydrogenase, was sufficient and parallel to the growth rate. Therefore, IAA production reached the optimum level. After that, IAA production decreased at 72 hours incubation phase since the bacteria passed into the phase of death, so that IAA production decreased. The highest level of IAA production in the 48-hour incubation phase was also obtained in Gusniar [31] and Kresnawaty [32] studies. According to Bhattacharyya and Dey [33], the decrease in IAA production at 72 hours was due to the release of IAA degrading enzymes such as oxidase and peroxidase.



**Figure 3.** The production of IAA by bacteria ( $\mu\text{g mL}^{-1}$ )

Shilev [33] reported that some bacteria from NFB group (symbiotic and non-symbiotic) are capable to generate growth hormone of IAA to stimulate lateral roots growth. The lateral roots can produce exudates and the nutrients that were absorbed by the roots thus increased bacterial population for enhanced inoculation effect [34].

### 3.3. The potency of NFB as PGPR during the germination of *C. pulcherrima* (in vitro)

The results of the potency of NFB as PGPR during the germination of *C. Pulcherrima* (in vitro), showed that the values of the radicle length, hypocotyls length, germination percentage, index vigour, and bacteria population in infected root are statistically significant if compared among control and bacterial plus POME treatments with bacterial plus aquadest treatments (Table 1). Shankar [35] reported that the value of vigour index on germination is important information to know the ability of growth of sprouts, normally under optimal and sub-optimal conditions. The vigour index is also an indication of seed strength for the seed growth and for dealing with environmental conditions that affect it [36]. The statement can strengthen the results of this study.

**Table 1.** The potency of nitrogen fixation bacteria during germination of *C. pulcherrima* (in vitro)

Treatment	Germination (%)	Radicle length (Cm)	Hypocotyl length (Cm)	Total length (Cm)	Index vigour	$\Sigma$ Bacteria ( $10^7\text{Cfu mL}^{-1}$ )
Control (uninoculated)	98±0.6 <sup>c</sup>	1.70±0.1 <sup>a</sup>	2.80±0.1 <sup>a</sup>	4.5±0.0 <sup>ab</sup>	441.00±2.6 <sup>b</sup>	0
<i>Azospirillum sp.1</i> + POME	76±0.9 <sup>b</sup>	2.54±0.0 <sup>b</sup>	3.00±0.6 <sup>a</sup>	5.54±0.6 <sup>b</sup>	421.04±50.2 <sup>b</sup>	2.83 <sup>b</sup>
<i>Azospirillum sp.2</i> + POME	70±1.7 <sup>a</sup>	1.52±0.3 <sup>a</sup>	2.40±0.3 <sup>a</sup>	3.92±0.3 <sup>a</sup>	274.47±22.5 <sup>a</sup>	2.33 <sup>a</sup>
<i>Azospirillum sp.3</i> + POME	70±0.6 <sup>a</sup>	1.50±0.3 <sup>a</sup>	2.41±0.3 <sup>a</sup>	3.92±0.3 <sup>a</sup>	274.23±24.1 <sup>a</sup>	2.37 <sup>ab</sup>
<i>Azotobacter sp.</i> + POME	70±1.2 <sup>a</sup>	1.52±0.0 <sup>a</sup>	2.19±0.2 <sup>a</sup>	3.80±0.1 <sup>a</sup>	265.69±4.9 <sup>a</sup>	2.47 <sup>ab</sup>
<i>Rhizobium sp.</i> + POME	75±0.6 <sup>b</sup>	2.52±0.6 <sup>b</sup>	3.04±0.2 <sup>a</sup>	5.56±0.4 <sup>b</sup>	417.42±31.0 <sup>b</sup>	2.6 <sup>ab</sup>
<i>Azospirillum sp.1</i> + aquades	98±0.0 <sup>e</sup>	4.14±0.0 <sup>c</sup>	5.51±0.1 <sup>b</sup>	9.65±0.0 <sup>c</sup>	945.70±3.4 <sup>e</sup>	4.6 <sup>d</sup>
<i>Azospirillum sp.2</i> + aquades	82±0.6 <sup>c</sup>	3.99±0.0 <sup>c</sup>	5.24±0.6 <sup>b</sup>	9.23±0.6 <sup>c</sup>	757.53±53.2 <sup>d</sup>	3.6 <sup>c</sup>
<i>Azospirillum sp.3</i> + aquades	80±0.0 <sup>c</sup>	3.98±0.0 <sup>c</sup>	5.13±0.1 <sup>b</sup>	9.11±0.1 <sup>c</sup>	728.8±6.9 <sup>cd</sup>	2.6 <sup>ab</sup>
<i>Azotobacter sp.</i> + aquades	80±0.6 <sup>c</sup>	4.00±0.3 <sup>c</sup>	5.33±0.6 <sup>b</sup>	9.33±0.9 <sup>c</sup>	745.40±63.9 <sup>d</sup>	3.3 <sup>c</sup>
<i>Rhizobium sp.</i> + aquades	86±1.2 <sup>d</sup>	4.02±0.0 <sup>c</sup>	5.22±0.0 <sup>b</sup>	9.39±0.1 <sup>c</sup>	807.39±5.4 <sup>d</sup>	3.5 <sup>c</sup>

Notes:  $\pm$  SD is value from 3 replications. The numbers followed by the same letter are not significantly different at ( $p < 0.05$ ) level of Duncan's test.

The results of germination in this study show that the germination of *C. pulcherrima* in *Azospirillum sp.1-3*, *Azotobacter sp.*, and *Rhizobium sp.* inoculation treatment watered with aquadest were better than in watered with POME and control treatment (germination without bacteria and POME); whereas the percentage of germination index vigour of *C. pulcherrima* in control were better than in *Azospirillum sp.1-3*, *Azotobacter sp.*, and *Rhizobium sp.* inoculation treatment watered

with POME, except parameters of radicles length, hypocotyl length, total length, and bacterial population. The highest percentage of germination (98 %) and vigour index (945.70) of *C. pulcherrima* was produced by seeds inoculated with *Azospirillum* sp.1 plus aquadest and the highest value of other germination is also found in the control (98%), but other values of parameters are very low. The values of the parameters with the lowest and highest effects of all treatment on the treated *C. pulcherrima* are shown in sequence i.e. *Azotobacter* sp. plus POME (percent of germination = 70%, radicle length = 1.52 cm; hypocotyl length = 2.19 cm; Total length = 3.8 cm, index vigour = 265.69; bacteria population =  $2.47 \times 10^7$  cfu mL<sup>-1</sup>); *Azospirillum* sp.1 plus aquadest (percent of germination = 98%, radicle length = 4.14 cm; hypocotyl length = 5.51 cm; Total length = 9.85 cm, index vigour = 945.7; bacteria population =  $4.60 \times 10^7$  cfu mL<sup>-1</sup>). This experiment showed the same results (low vigour index value), specially on the germination of *C. pulcherrima* in *Azospirillum* sp., *Azotobacter* sp., and *Rhizobium* sp. inoculation treatment watered with POME. This may be due to the inoculums concentration of *Azospirillum* sp., *Azotobacter* sp., and *Rhizobium* sp. plus POME that inhibits the imbibitions process, thus lowering the value of the vigour index and progress of germination process. This phenomenon occurred possibly due to the concentration of inoculants plus POME on the seeds that inhibited the process of seed imbibitions. The imbibitions stage is an early stage that determines the success of germination in which the fusion of fluid into the seeds is very important for the regulation and activation of growth hormone in the seeds.

Kuswanto [36], stated that the presence of inhibitors in seeds, seed exterior, solutions with high osmotic pressure, metabolic pathways inhibitors or respiratory rate inhibitors inhibits seed germination, for example by the competition of oxygen (O<sub>2</sub>) are an inhibitor of growth germination. These conditions restrain the germination or slow down the process, therefore germination will continue despite the suboptimal or stressed condition resulting in the low value of the vigour index.

According to Harper and Lynch [37] germination was inhibited due to the competition between *Azospirillum* sp., *Azotobacter* sp., and *Rhizobium* sp. all which are viable to the seeds of *C. pulcherrima* in the use of oxygen (O<sub>2</sub>) for respiration. Competition occurred due to the *Azospirillum* sp. and *Azotobacter* sp. are aerobic that requires oxygen (O<sub>2</sub>). These bacteria are also able to live independently in nature so that these bacteria will not be bound to the growth of sprouts from *C. pulcherrima* to survive. Sprouts at the beginning until the growth of first leaves are able to perform photosynthesis for the growth comes from the reaction of aerobic respiration that breaks down food stored on the seeds [38].

Thus presence of competition indicated by the vigour index was affected by the characteristic of each bacterial isolate in the competition of O<sub>2</sub> which was determined from the respiration rate of the bacteria and the ability to colonize during germination by sticking to the root surface and infecting the roots. Bacteria capable of colonizing during germination are bacteria that can be associated and symbiotic with *C. pulcherrima* (in vitro). Thus the result of the in vitro germination selection of bacterial potency showed that isolate *Azospirillum* sp.1 and *Rhizobium* sp. were effectively associated and symbiotic with *C. pulcherrima* plant.

The results of bacterial population calculations showed that all isolates can be associated and symbiotic with *C. pulcherrima* plant. The highest bacterial population in root germination of *C. pulcherrima* was observed on *Azospirillum* sp.1 and *Rhizobium* sp. According to Dennis *et al.* [39], the number of bacterial populations associated with a plant is based on the bacteria characteristic and exudates preference and the characteristics of different plant exudates.

#### 3.4. The potency of functional bacteria during the germination of *C. pulcherrima* (in vivo)

Table 4 showed the potency of *Azospirillum* sp., *Azotobacter* sp., and *Rhizobium* sp. as PGPR in *C. Pulcherrima* in vivo seedling. It was shown the germination of *C. pulcherrima* in *Azospirillum* sp., *Azotobacter* sp., and *Rhizobium* sp. inoculation treatment watered with POME was better than in watered with aquadest and both treatment was better than control treatment (germination without bacteria and POME).

The treatment of bacteria inoculation as PGPR on *C. pulcherrima* had presented any difference in all parameters measured six weeks after planting, except for number of leaf branches. The best



bacteria as PGPR on the plant of *C. pulcherrima* watered with POME and aquades are belonging to *Azospirillum* sp.1 and *Rhizobium* sp. The highest value of parameter (shoot length, root length, fresh weight, dry weight, and bacteria population) generated by *Azospirillum* and *Rhizobium* bacteria in pot contaminated POME are respectively 10 and 9.9 cm, 6.63 and 6.60 cm, 0.57 and 0.57 g, 0.10 and 0.09 g,  $32.67 \times 10^7$  and  $14.67 \times 10^7$  cfu g<sup>-1</sup>. Thus POME watering has a better impact of *C. pulcherrima* growth and bacteria population than aquadest watering. The same results with the same treatment (POME watering) and the same test plant (*C. pulcherrima*) were obtained on the results of the study. This proves that the organic waste could be renewed into fertilizer, citric acid, bioethanol, biohydrogen, bioplastic, and low cost hydrolytic enzymes [40]. According to Basiron and Wang [41], it occurred due to the transformation of POME into fertilizer by functional bacteria. Other researchers also found functional bacteria isolated from POME. Alias and Tan [42] isolated bacteria from POME and identified it as *Burkholderia cepacia* that have the ability to produce poly- (3) - hydroxybutyrate. Whereas Alam *et al.* [43] obtained several species of microbes that were able to break down organic matter. These results supported the results of this study, because of the result of all studies on in vitro and in vivo germination selection of bacterial potency showed that *Azospirillum* sp.1 and *Rhizobium* sp. were effectively associate and symbiotic with *C. pulcherrima* plant in pot-infected waste of POME.

**Table 2.** Potency of PGPR during the germination of *C. pulcherrima* (in vivo) and effect of watering type on bacterial population

Treatments	Shoot length (Cm)	Root length (Cm)	Fresh weight (Gram)	Dry weight (Gram)	Number of leaf branches	Σ Bacteria (10 <sup>7</sup> Cfu g <sup>-1</sup> sand)
Control (uninoculated)	5.6±0.4 <sup>a</sup>	3.33±0.2 <sup>a</sup>	0.17±0.2 <sup>a</sup>	0.02±0.0 <sup>a</sup>	3±0.58 <sup>ab</sup>	0
<i>Azppirillum</i> sp.1+POME	10±0.2 <sup>f</sup>	6.63±0.4 <sup>e</sup>	0.57±0.1 <sup>c</sup>	0.10±0.0 <sup>f</sup>	4±0.58 <sup>ab</sup>	32.67 <sup>b</sup>
<i>Azppirillum</i> sp.2+POME	8.3±0.4 <sup>de</sup>	4.80±0.1 <sup>cd</sup>	0.40±0.1 <sup>bc</sup>	0.06±0.0 <sup>bcd</sup>	4±1.2 <sup>ab</sup>	3.67 <sup>a</sup>
<i>Azppirillum</i> sp.3+POME	7.2±0.6 <sup>c</sup>	5.10±0.1 <sup>d</sup>	0.39±0.1 <sup>bc</sup>	0.06±0.0 <sup>bcd</sup>	4±1.2 <sup>ab</sup>	2.40 <sup>a</sup>
<i>Azotobacter</i> sp.+POME	8.1±0.5 <sup>d</sup>	6.53±0.0 <sup>e</sup>	0.32±0.1 <sup>ab</sup>	0.05±0.0 <sup>bc</sup>	4±1.0 <sup>ab</sup>	5.80 <sup>a</sup>
<i>Rhizobium</i> sp.+POME	9.9±0.1 <sup>f</sup>	6.60±0.3 <sup>e</sup>	0.57±0.1 <sup>c</sup>	0.09±0.0 <sup>ef</sup>	4±1.2 <sup>ab</sup>	14.67 <sup>a</sup>
<i>Azppirillum</i> sp.1+aquades	9.2±0.1 <sup>f</sup>	5.0±0.0 <sup>d</sup>	0.35±0.0 <sup>abc</sup>	0.08±0.0 <sup>cdef</sup>	4±0.6 <sup>ab</sup>	2.00 <sup>a</sup>
<i>Azppirillum</i> sp.2+aquades	7.1 ±0.1 <sup>c</sup>	3.9±0.1 <sup>ab</sup>	0.23±0.1 <sup>ab</sup>	0.043±0.0 <sup>ab</sup>	3±0.6 <sup>ab</sup>	1.83 <sup>a</sup>
<i>Azppirillum</i> sp.3+aquades	6.2±0.1 <sup>ab</sup>	3.73±0.2 <sup>ab</sup>	0.23±0.0 <sup>ab</sup>	0.04±0.0 <sup>ab</sup>	3±1.0 <sup>ab</sup>	1.63 <sup>a</sup>
<i>Azotobacter</i> sp.+aquades	7.0±0.0 <sup>bc</sup>	4.2±0.2 <sup>bc</sup>	0.57±0.0 <sup>c</sup>	0.04±0.0 <sup>ab</sup>	3±0.6 <sup>ab</sup>	2.00 <sup>a</sup>
<i>Rhizobium</i> sp.+aquades	9.1±0.1 <sup>ef</sup>	6.3±0.6 <sup>e</sup>	0.37±0.1 <sup>abc</sup>	0.08±0.0 <sup>def</sup>	3±0.0 <sup>ab</sup>	1.21 <sup>a</sup>

Notes: ± SD is value from 3 replications. The numbers followed by the same letter are not significantly different at (p<0.05) level of Duncan's test.

#### 4. Conclusion

All the functional bacteria (NFB) isolated from POME disposal pond showed the potency to be plant growth promoting rhizobacteria (PGPR). *Azospirillum* sp.1 and *Rhizobium* sp. are the best PGPR (highest level of IAA production) and the ability to promote growth of *C. Pulcherrima* (L) Sw in in vitro and in vivo with as much as 10<sup>7</sup> bacterial populations in POME contaminated media.

#### 5. References

- [1] Gaol M R L L, Sitorus R, Yanthi S, Surya I, and Manurung R 2013 Pembuatan selulosa asetat dari α-selulosa tandan kosong kelapa sawit *J. Teknol. Kim. USU* **2** (3) 33-9
- [2] Kathiravale S and Ripin A 1997 Palm oil mill effluent treatment towards zero discharge apaper presented at *National Science and Technology Conference in Kuala Lumpur, Malaysia (July, 15<sup>th</sup>-16<sup>th</sup> 1997)* p 1-8
- [3] Alkarimiah R, Rahman R A 2014 Co-Composting of EFB and POME with the role of nitrogen-fixers bacteria as additives in composting process-a review. *Int. J. Eng. Sci. Innov. Technol. (IJESIT)* **3** (2) 132-45

- [4] Wood B J, Pillai K R, and Rajaratnam JA 1979 Palm oil mill effluent disposal on land *Agric. Wastes* **1** 103-27
- [5] Thompson I P, Gast C J van der, Ciric L and Singer A C 2005 Bioaugmentation for bioremediation: the challenge of strain selection *Environ. Microbiol.* **7**(7) 909–15
- [6] Barrow G I and Feltham R K A 2003 *Cowan and Steel's Manual for Identification of Medical Bacteria* 3<sup>rd</sup> edition (Cambridge University Press) pp1-331
- [7] Januar W, Khotimah S and Mulyadi A 2013 Kemampuan isolate bakteri pendegradasi lipid dari instalasi pengolahan limbah cair PPKS PTPN-XIII Ngabang Kabupaten Landak. *J. Protobiont* **2**(3): 137-40
- [8] Ningrum I Y 2018 *Isolasi dan Karakterisasi plant growth promoting bacteria dari limbah cair kelapa sawit untu knemacu pertumbuhan tanaman cabai (Capsicum annum L.)* Skripsi Departemen Biologi (Medan Universitas Sumatera Utara) p 42
- [9] Priadie B 2012 Teknik bioremidiasi sebagai alternative dalam upaya pengendalian pencemaran air *J. Ilmu Lingk.* **10**(1)38-48
- [10] Bottini R, Cassán F, and Piccoli P 2004 Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase *Appl. Microbiol. Biotechnol.* **65** 497-503
- [11] Gunalan 1996 Penggunaan mikroba bermanfaat pada bioteknologi tanah berwawasan lingkungan. *Majalah Sriwijaya* **32**(2)
- [12] Glick BR 1995 The enhancement of plant growth by free-living bacteria *Can. J. Microbiol.* **41** 109-17
- [13] Vessey J K 2003 PGPR as biofertilizer *Plant and soil* **255** 571-86
- [14] Sudhakar P, Chattopadhyay G N, Gangwar S K, and Ghosh J K 2000 Effect of foliar application of *Azotobacter*, *Azospirillum* and *Beijerinckia* on leaf yield and quality of mulberry (*Morus alba*) *J. Agric. Sci.* **134** 227–34
- [15] Bhattacharyya P N, Jha D K 2012 Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture *World J. Microbiol. Biotech.* **28** 1327-50
- [16] Sy A, Giraud E P, Jourand N, Garcia A, Willem P de Lajudie, Prin Y, Neyra M, Gillis M, Boivin-Masson C, Dreyfus B 2001 Methylophilic methylobacterium bacteria nodulate and fix nitrogen in symbiosis with legumes *J. Bacteriol.* **183** 214-20
- [17] Bhattacharjee R, Dey U 2014 Biofertilizer a way towards organic agriculture a review *Afr. J. Microbiol. Res.* **8**(24) 2332-42
- [18] Rao N S S 1994 *Soil Microorganisms and Plant Growth* 3<sup>th</sup> edition (New Hampshire: Science Publishers Inc) pp 1-352
- [19] Chiang L C, Chiang W, Liu M C, and Lin C C 2003 *In vitro* Antiviral activities of *Caesalpinia pulcherrima* and its related flavonoids *J. Antimicrob. Chemo.* **52** 194-8
- [20] Wu T Y, Mohammad A W, Jahim J M, and Anuar N 2009 A holistic approach to managing palm oil mill effluent (POME): Biotechnology advances in the sustainable reuse of POME. *Biotech. Adv.* **27** 40-52
- [21] Caceres, Enrique A, Rodriguez 1982 Improved medium for isolation of *Azospirillum* spp. *Appl. Environ. Microbiol.* **44** 990-1
- [22] Krieg N R and Doberiner J 1984 *The genus Azospirillum (Bergey's manual of systematic bacteriology vol 1)* eds NR Krieg and JG Holt (Baltimore: The Williams and Wilkins Co.) pp194-04
- [23] Brenner D J, Krieg N R, Staley J T 2005 *The Proteobacteria (Bergey's Manual of Systematic Bacteriology vol 2C 2<sup>nd</sup> edition)* ed GM Garrity (New York: Springer) p 1388
- [24] Brenner D J, Krieg N R, Staley J T 2005 *The Gammaproteobacteria (Bergey's Manual of Systematic Bacteriology vol 2B 2<sup>nd</sup> edition)* ed MG Garrity (New York: Springer) p 1108.
- [25] Doberiner J 1995 *Isolation and Identification of Aerobic Nitrogen Fixing Bacteria from Soil and Plant (Methods in Applied Soil Microbiology and Biochemistry)* Eds K Alef and P Nannipieri (London, UK: Academic Press) pp 134-41

- [26] Mehboob A, Stal L J, and Hasnain S 2010 Production of indole-3-acetic acid by the *cyanobacterium arthrospira platensis* strain MMG-9 *J. Microbiol. Biotechnol.* **20**(9) 1259-65
- [27] Baldani V L D and Dobereiner J 1980 Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* **12** 433-39.
- [28] Kusumawati D I, Widawati S, Lisdiyanti P, and Sudiana IM 2017 Isolation and Screening for IAA production, Nitrogen Fixation, P-Solubilization and Cellulolytic Activity of Plant Growth-Promoting Rhizobacteria from *Imperata cylindrical* Grasslands. *Proceedings The 1<sup>st</sup> SATREPS Conference. "The Project for Producing Biomass Energy and Material Through Revegetation of Alang-alang (Imperata cylindrical) Fields"*. **1** pp 125-33.
- [29] Hammar M, Arnqvist A, Bian Z, Olsen A, and Normark S 1995 Expression of two cs goperons is required for production of fibronectin and congo red binding curli polymers in *Escherichia coli* K-12. *Mol. Microbiol.* **18** 661-70
- [30] Bastarrachea F, Zamudio M, and Rivas R 1988 Non encapsulated mutants of *Azospirillum brasilense* and *Azospirillum lipoferum* *Can. J. Microbiol.* **34** 24-9.
- [31] Gusnaniar 2007 Produksi IAA oleh *Rhizobium* spp, *Pseudomonas* spp, dan *Azotobacter* spp Dalam media sintetik dan serum lateks *Hevea brasiliensis* Muel. Arg dengan suplementasi tryptophan Skripsi Fakultas Biologi (Yogyakarta: Universitas Gajah Mada)
- [32] Kresnawaty I, Andanawarih S, Suharyanto dan Tri-Panji 2008 Opmimisasi dan pemurnian IAA yang dihasilkan *Rhizobium* sp. dalam media serum lateks dengan suplementasi tryptophan dari pupuk kandang *Menara Perkebunan* **76**(2) 74-82.
- [33] Shilev S 2013 *Soil Rhizobacteria Regulating the Uptake of Nutrients and Undesirable Elements by Plants (Plant Microbe Symbiosis Fundamentals and Advances)* ed NK Arora (India: Springer) pp147-50
- [34] Spaepen S, Vanderleyden J and Remans R 2007 Indole-3-acetic acid in microbial and microorganism-plant Signaling *FEMS Microbiol. Rev.* **31** 425–48.
- [35] Shankar U 2006 Seed size as a predictor of germination success and early seedling growth in 'hollong' (*Dipterocarpus macrocarpus* Vesque) *New Forests* **31** 305–20. DOI 10.1007/s11056-005-8198-6
- [36] Kuswanto H 1996 *Dasar-dasar Teknologi Produksi dan Sertifikasi Benih* (Yogyakarta: Penerbit Andi offset) pp 1-192
- [37] Harper S H T and Lynch J M 1979 Effects of *Azotobacter chroococcum* on barley seed germination and seedling development. *J. Gen. Microbiol.* **112** 45-51
- [38] Raven P H, Evert R F, and Eichhorn S E 2005 *Biology of Plants* 7<sup>th</sup> Edition (New York: WH Freeman and Company Publishers) p 114
- [39] Dennis P G, Miller A J, and Hirsch P R 2010 Are root exudates more important than other sources of rhizo deposits in structuring rhizosphere bacterial communities *FEMS Microbiol. Ecol.* **72** 313-27
- [40] Salihu A and Alam M Z 2012 Palm oil mill effluent: a waste or a raw material *J. Appl. Sci. Res.* **8**(1) 466-73
- [41] Basiron Y and Weng C K 2004 The oil palm and its sustainability *J. Oil Palm Res.* **16**(1)1-10
- [42] Alias Z and Tan I K P 2005 Isolation of palm oil-utilising polyhydroxy alkanoate (PHA)-producing bacteria by an enrichment technique *Bioresour. Technol.* **96** 1229-34
- [43] Alam M Z, Jamal P and Nadzir M M 2008 Bioconversion of palm oil mill effluent for citric acidproduction: statistical optimization of fermentation media and time by central composite design *World J. Microbiol. Biotechnol.* **24** 1177-85

### Acknowledgments

This research was financially supported by a grant-in-Aid for Scientific Research from the TEMATIK DIPA (Daftar Isian Pelaksanaan Anggaran) programe, Research Center for Biology, Indonesian Institute of Sciences. The authors also thank to Ety Suryati, Prasetya Adi, Lucky Rahma Fadilah, and Hasna Hafida for technical help for data collection.