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Qualitative *In Vitro* Evaluation of Plant Growth Promoting Activity of Selected Microbial Isolates Used for Biofertilizer Application

Marwanto[1*], Hendri Bustaman[2], Merakati Handajaningsih[1], Supanjani[1], Bambang Gonggo Murcitro[3]

[1]*Departement of Agronomy, Faculty of Agriculture, University of Bengkulu, W.R.Supratman, st. Kandang Limun, Bengkulu, 38371.*
[2]*Departement of Plant Prtection, Faculty of Agriculture, University of Bengkulu, W.R.Supratman, st. Kandang Limun, Bengkulu, 38371.*
[3] *Department of Soil Science, Faculty of Agriculture, University of Bengkulu, W.R.Supratman, st. Kandang Limun, Bengkulu, 38371.*
[*]*Corresponding author. Email:marwanto@unib.ac.id*

ABSTRACT

The use of microbial strains with plant growth promoting (PGP) properties is advisable as a sustainable alternative to chemical fertilizers to improve soil fertility and crop yields. The present study identify 12 specific microbial isolates (7 bacterial and 5 fungal isolates) to have PGP activity viz. atmospheric N fixation, phosphate and potash solubilization, indole acetic acid (IAA) production, and cellulase production. This research work was conducted at the Laboratory of Plant Protection Faculty of Agriculture University of Bengkulu in 2020. The results showed that N fixation ability was observed in *Bacillus megatrium*, *Bacillus* sp., *Azotobacter* sp., and *Pseudomomas fluorescent*. The highest phosphate solubilization index for bacterial isolate (1.95) was observed in *Bacillus* sp. and for fungal isolate (1.38) was in *Aspergillus niger*. The highest potash solubilization index for bacterial isolate (1.86) was observed in *P. fluorescent* andfor fungal isolate (2.06) was in *Paeceilomyces* sp. IAA production activity was observed in all isolates tested (except for *Paeceilomyces* sp. and *Trichoderma* sp.). Cellulase production trait was observed in all isolates tested. The highest cellulase production index was observed in *Azotobacter* sp. (1.71) for bacteria and *Saccharomyces* sp. (1.31) for fungi. Thus, the 12 isolates hold promise to be used as a biofertilizer through their potential PGP traits.

Keywords: *biocontrol cctivity; biofertilizer;indole acetic acid;nitrogen fixation; plant growth promoting trait*

1. INTRODUCTION

The low availability of soil nutrients required for plant growth especially nitrogen (N), phosphorus (P), and potassium (K) leads to low yields. The use of chemical fertilizers is one tool for raising plant yields and available plant nutrients. Chemical fertilizers may provide important macro nutrients, such as N, P, and K, and micro nutrients, such as copper (Cu) and iron (Fe). Chemical N fertilizers are essential to crop yields and are widely used in agricultural systems; however, their use also has some adverse environmental effects. They are vulnerable to leaching, denitrification and volatilization. All of the N fertilizers have the potential to be particularly toxic to humans[1].

Phosphorus can only be found in two types, H_2PO_4- and $HPO_4^{=}$. As phosphate fertilizer is applied to the soil, it can be quickly immobilized and made unavailable to plants. Most agricultural soils have large phosphorus reserves, many of which have accumulated as a result of repeated use of P fertilizers. However, a large amount of soluble inorganic phosphate applied to soil as chemical fertilizers is instantly immobilized after application and becomes inaccessible to plants as a result of phosphate fixation and precipitation by aluminum, calcium, iron, magnesium, soil type and soil pH. In acid soil, phosphorus is tightly fixed by aluminum and iron free oxides and hydroxides, resulting in a low efficiency of soluble P fertilizers such as super calcium phosphate [2].Potassium is the third most essential crop nutrient after nitrogen and phosphorus. It is important for agriculture to promote early growth, boost protein production, water retention, yield, nutritional value, color, texture, and disease resistance of food crops [3].

There are also advantages from the use of chemical fertilizers. They function instantly and provide all the necessary nutrients that are ready to be used. In addition, they are relatively inexpensive and easy to buy and use.

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However, in addition to the exponential growth of the world's population and the huge increase in the usage of chemical fertilizers, environmental issues such as leaching, runoff, deforestation and eutrophication of rivers have become critical due to the low absorption rate of chemical fertilizers by plants, which are only about 50%. Chemical fertilization can also build up toxic waste in soil and decrease the enzyme activity of soil microbes, soil pH and soil structure[4].With regards the price, chemical fertilizers are costly for many of the world's farmers who rely on organic matter as a nutrient source in subsistence agriculture. Problems related to NPK chemical fertilizers urge the need to develop cost - effective and environmentally safe fertilizers to substitute or partially replace chemical fertilizers.

It has been shown that the use of beneficial living microorganisms as an alternative tool to enhance nutrient uptake, stimulate plant growth and increase yield has been beneficial. As a consequence of that, there is a growing incentive for the use of beneficial living microorganisms as biofertilizers [5].

Biofertilizers are, by definition, substances that contain living microorganisms that, when applied to seeds, plant surfaces or soils, colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant.The living microorganisms, including rhizospheric fungi, rhizospheric bacteria, symbiotic bacteria and non-symbiotic endophytic bacteria, have the capacity to stimulate plant growth by supporting plant nutrient status. Rhizospheric fungi, including arbuscular mycorrhiza and Penicillium bilaii are acknowledged to have growth-enhancing capabilities[5].

Nitrogen-fixing bacteria include symbiotic (*Rhizobium*) and free-living bacteria (*Pseudomonas* spp., *Bacillus* spp., *Azospirillum* spp., and *Burkholderia* spp.). These have also been known as plant growth enhancers [6]. Free-living, N-fixing bacteria are *Azospirulum*, *Azotobacter*, and *Pseudomonas*. These bacteria are able to fix atmospheric nitrogen without a specific host plant compared to *Rhizobium* legume.These free-living N-fixing bacteria replicate in plants and successfully settle in roots, stems and leaves as colony. During this association, the invasive bacteria rewarded the hosts acquired with substantial improvements in plant growth, vigor and yield [7]. *Azoarcus* sp., *Gluconacetobacter diazotrophicus*, *Herbaspirilium* sp.,*Azoarcus* sp., *Gluconacetobacter diazotrophicus*, *Herbaspirillium* sp., *Azotobacter* sp., *Acetobacter*, *Azospirillum*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Xanthobacter* are major N-binding bacteria [8] [9].The synthesis and secretion of plant growth regulators such as auxins, cytokines, gibberellins and nitric oxides are essential signals and components of plant growth promotion effects [10].

Pseudomonas, Bacillus, Rhizobium, Burkholderia, and *Erwinia* have been shown to have phosphate-solubilizing activity. These microorganisms are able to convert insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate, into usable plant forms [11]. The application of phosphate-solubilizing bacteria (PSB) provides an increase in maize yields and decreases the need for phosphate fertilizers [12].

Many fungal species can solubilize rock phosphate, aluminum phosphate and tricalcium phosphate, such as *Aspergillus niger*, *Aspergillus tubingensis*, *Aspergillus fumigatus*, *Aspergillus terreus, Aspergillus awamor*, *Penicillium italicum, Penicillium radicum, Penicillium rugulosum*, *Fusarium oxysporum, Curvularia lunata, Humicola* sp., *Sclerotium rolfsii, Pythium* sp., *Aerothecium* sp., *Phoma* sp., *Cladosporium* sp, *Rhizoctonia* sp., *Rhizoctonia solani*, *Cunninghamella* spp., *Rhodotorula* sp., *Candida* sp., *Schwanniomyces occidentalis*, *Oideodendron* sp., *Pseudonymnoascus* sp. [13] [14] [15].

Potassium-solubilizing bacteria (KSB) are capable of liquifying non-available potassium in accessible forms through organic acid secretion[5]. *Bacillus mucilaginosus* has been intensively studied as a biofertilizer among these bacteria. Other genera such as *Burkholderia* sp., *Paenibactillus* sp., and *Acidothiobacillus* sp. are also reported as KSB [16]. KSBs have the ability to solubilize plant non-accessible potassium into plant-available forms by organic acid release. KSB is a heterotrophic bacterium that can produce energy and cellular carbon from pre-existing organic materials. KSB also plays a vital role in maintaining the soil structure and helping to conserve soil water.

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a natural product of L-tryptophan metabolism generated by a variety of microorganisms, such as Plant Growth-Promoting Rhizobacteria (PGPR)[17].Bacteria that colonize the rhizosphere and plant roots and promote plant growth by any process is called PGPR. PGPR can demonstrate a variety of characteristics responsible for affecting plant growth. The ordinary traits encompass the development of plant growth regulators (such as auxin, gibberellin, ethylene), siderophores, HCN, and antibiotics [6].Bacteria produce auxins to disrupt host physiological processes for their own benefit. The microorganisms isolated from different crop regions of the rhizosphere have the potential to generate IAA as secondary metabolites due to the rich supply of substrates.IAA promotes longer root growth with an increased number of root hairs and root laterals responsible for the uptake of nutrients [18].IAA promotes cell elongation by altering certain factors such as increasing the osmotic content of the cell, increasing the permeability of water to the cell, decreasing the pressure of the wall, increasing the synthesis of the cell wall, and inducing particular RXA and protein synthesis.It encourages embryonic development, prevents or slows down the abscission of leaves, and stimulates flowering and fruiting[19].

IAA is a metabolite produced from Trp by several Trp-dependent and Trp-dependent pathways in plants and bacteria. More than one pathway may be exist in the bacteria. Physiological evidence has been documented for various Trp-dependent synthesis pathways in *Azospirillum brasilense* [20].In the Trp-dependent pathway, tryptophan is transformed into indole-3-acetamide (IAM) by tryptophan-2-monooxigenase and IAM is assimilated to IAA by IAM-hydrolase. Horemans and Vlassak [21] have shown that *Azospirillum. brasilense* may have developed IAA in the void of tryptophan under aerobic growth. Some microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. The tryptophan increases the production of IAA in *Bacillus amyloliquefaciens* FZB42. Karnwal [22] tested *fluorescent Pseudomonas* isolates for their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan and found that for both strains, indole production enhanced with increases in tryptophan concentration.

Restu and Payangan [23] reported that five fungus genera (*Aspergillus, Trichoderma, Rhizopus, Penicillium*, and *Fusarium*) had ability to produce IAA. Among these fungus, *Fusarium* has the highest concentration, which was 38,611 ppm. *Fusarium* isolates have the potency to be developed as biological fertilizers.

It has shown that the highest levels of auxin were developed in the existence of NH4.It plays significant roles during embryogenesis when fine regulation of low levels of IAA is essential to polar development. Trp-independent pathways can make a major contribution to the newly synthesized IAA; however, comprehensive Trp-to-IAA conversion occurs in these preparations as well[6].

In addition to exhibiting PGP traits, certaian PGP microorganisms have the ability to produce hydrolytic enzymes suc as cellulase. These enzymes contribute to the destruction of pathogenic microbes cell wall and degradation of lignocellulotic waste. PGP micrdeveloped organisms producing this lytic enzme have been proven to demonstrate biocontrol properties against a wide range of pathogenic bacteria and fungi and ultimately increase crop yield [24].The degradation of lignocellulose is mainly accomplished by producing two types of extracellular enzyme systems: hydrolytic and oxidative catalytic systems. Microorganism responsible for the degradation of lignocellulose include species of bacteria (for example; *Clostridium*, *Cellulomonas, Bacillus, Pseudomonas, Fibribacter, Ruminococcus, Butyrivibrio*) and fungi (for example: *Aspergillus, Rhizopus, Trichoderma, Fusarium, Neurospora, Penicillium*). Of them, fungi are the principal agents involved in the degradation of lignocelluloses. Efficient cellulolytic fungi are represented by the species of *Aspergillus,Penicillium, Chaetomium, Trichoderma, Fusarium, Stachybotrys, Cladosporium, Alternaria, Acremonium, Ceratocystis, Myrothecium, Humicola*[25].

Finding cellulose degrading microbes is very important since they are highly needed in industrial and biotechnological sectors (Kasana *et al*., 2008). For these concern, the purpose of this study was to characterize the ability of selected microbial isolates to produce ammonia (NH_3) and plant hormone (IAA), solubilize phosphate (P) and potassium (K) and their biocontrol property checked by cellulase enzyme production ability. Theoretically, isolate having multiple traits have more chance to contribute plant growth promotion effects.

2. MATERIALS AND METHODS

2.1 Microbial Isolate Cultures

The 12 purified microbial isolates consisting of 7 bacterial and 5 fungal isolates were used in this study as shown in Table 1. The pure isolated colony of each microbial isolate was obtained from personal culture collection after sub-culturing several times on the nutrient agar plate. The 12 pure microbial isolates were screened in vitro for different PGP traits such as N fixation indicated by ammonia (NH_3) production, phosphate (P) and potash (K) solubilization, IAA production, and biocontrol property by checking cellulase enzyme production ability.

2.2 In Vitro Characterization of Plant Growth-Promoting Traits of the Isolates

2.2.1 Test for Nitrogen Fixation Activity

Microbial ability in fixing atmospheric N asymbiotically.Ammonia production indicated by ammonia production of each isolate was tested and performed according to Dobereiner method (Dobereiner, 1995) using a semisolid medium of Nfb (nitrogen-free bromthymol blue) without N. The Nfb contained (g L^{-1}): malic acid, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$. $2H_2O$, 0.02; micronutrient solution ($CuSO_45H_2O$, 0.04; $ZnSO_4$ $7H_2O$, 0.12; H_3BO_3, 1.40; Na_2MoO_4 $2H_2O$, 1.0; $MnSO_4$ H_2O, 1.175); bromothymol blue, 2 mL (5 g L^{-1} in 0.2 N KOH) as an indicator; FeEDTA (solution 16.4 g L^{-1}), 4 mL; vitamin solution (biotin, 10 mg); pyridoxal-HCl, 20 mg; KOH, 4.5 g. A quantity of 1.6 to 1.80 g agar L^{-1} was added to prepare the semi-solid medium. Distilled water was added to bring the final volume to 1,000 mL and adjust pH to 6.5. Each isolate was inoculated and grown into test tubes containing the NFb and incubated at room temperature (30ºC) for 5–7 days. The strains that show a veil-like pellicle ring structure beneath the surface of the media were assumed as successful ammonia production or the microorganism had putative nitrogen-fixing activity. Observation of the initial pellicle formation was conducted 2–3 days after inoculation and growth was then observed every subsequent day as some bacteria and fungi could grow very quickly. Three replicate plates were used for each isolate.

2.2.2 Test for Phosphate Solubilization Activity

The ability of the 12 microbial isolates to solubilize insoluble P was identified using the plate method [26]

using Pikovskaya (PVK) agar medium containing $KH_3(PO4)_2$ as an insoluble inorganic form of P source. The appearances of the clear zone around bacterial or fungal growth (phosphate solubilization zone) were indicated a positive result for phosphate solubilization [27]. The Pikovskaya agar medium contained 10 g glucose; 5 g $[KH_3(PO_4)_2]$; 0.5 g $(NH_4)_2SO_4$; 0.2 g KCl; 0.1 $MgSO_4.7H_2O$; small amount of $MnSO_4$ and $FeSO_4$; 0.5 g of yeast extract, 15 g of bacto agar. Distilled water was added to bring the final volume to 1,000 mL and adjust pH to 6.5. Each isolate was spot inoculated by streaking in the solid PVK medium in a petri dish using ose needle and incubated at 28°C for seven days. Bacterial or fungal colonies surrounded by a clear halo zone, indicating phosphate removal, were visually observed and measured. The solubilization index was measured according to the ratio of total diameter (the sum of colony diameter and the halo zone diameter and the colony diameter [28] Three replicate plates were used for each isolate.

2.2.3 Test for Potash Solubilization Activity

A total of 12 microbial isolates were tested for potassium solubilizing activity on Aleksandrov agar medium having: 0.5% glucose, 0.05% magnesium sulfate heptahydrate, 0.0005% iron chloride, 0.01% calcium carbonate, 0.2% calcium phosphate, 0.2% feldspar (potassium aluminum silicate) and 1.8% agar; by spot plate method. Distilled water was added to bring the final volume to 1,000 mL and adjust pH to 6.5. Each isolate was spot inoculated by streaking in the solid Aleksandrov agar medium in a petri dish using ose needle and incubated at 28°C for seven days. Appearance of a clear halo zone developed around each colony after 5 days incubation indicated potash solubilization. The halo size produced by the respective strain was measured to calculate K solubilization index. The solubilization index was measured according to the ratio of total diameter (the sum of colony diameter and the halo zone diameter and the colony diameter [28]. All the observations were recorded in triplicate.

2.2.4 Test for IAA Production

IAA production was detected on Luria-Bertani agar medium (LB) containing the following (in grams per liter): Bacto-Tryptone (Difco), 10; yeast extract, 5; NaCl, 5; and Bacto-agar (Difco) [29]. The LB medium was added with 5 mM L-tryptophan the precursor of IAA. Distilled water was added to bring the final volume to 1,000 mL The pH was adjusted to 7.5 with 1 N NaOH before autoclaving. Each isolate was individually spot inoculated by streaking on the plates of LB medium in a petri dish using ose needle. Each inoculated plate was overlaid with an 82-mm-diameter disk of Whatman paper. All plates were incubated until colonies reached 0.5 to 2 mm in diameter. After an appropriate incubation period, the paper was removed from the plate and saturated with Salkowski reagent. The reagent was prepared by mixing 50 ml 35% of $HClO_4$ with 1ml 0.5 M $FeCl_3$ solution [30]. Membranes were saturated in a petri dish by soaking directly in 4.0 ml the Salkowski reagent and incubated for 0.5-2 hours for color development. The reaction was allowed to proceed until adequate color developed. All reagent incubations were conducted at room temperature. Bacteria or fungi producing IAA were characterized by the appearances of a specific red halo within the membrane immediately surrounding the colony, indicating a positive result for IAA production. All the observations were recorded in triplicate.

2.2.5 Test for Cellulase Production

The 12 microbial isolates were confirmed for the ability to produce cellulose enzyme on a medium containing low viscosity carboxymethyl cellulose (CMC) agar as the sole carbon source. The composition of the CMC solid agar was as follows: $NaNO_3$ (3.0 $g\cdot L^{-1}$); K_2HPO_4 (1.0 $g\cdot L^{-1}$); $MgSO_4$ (0.5 $g\cdot L^{-1}$); KCl (0.5 $g\cdot L^{-1}$); $FeSO_4\cdot 7H_2O$ (0.01 $mg\cdot L^{-1}$); agar (20.0 $g\cdot L^{-1}$); CMC (15.0 $g\cdot L^{-1}$) [31] Distilled water was added to bring the final volume to 1,000 mL. The pH was adjusted to 6.5 prior to sterilization. Each isolate was individually spot inoculated by streaking on the plates of CMC agar in a petri dish using ose needle and incubated at 37 °C for 72 hours. After incubation, CMC agar plates were flooded with 0.1% (w/v) Congo red reagent and allowed to stand for 15 min. at room temperature, then washed with 1 M NaCl [32] (Ten *et al*., 2004). The formation of a clear pale halo zone with orange edge circling around each microbial colony indicated an area of cellulose hydrolysis by cellulase enzyme. This halo area was measured for subsequent calculation of the enzymatic index (EI) The EI was measured according to the ratio of total diameter (the sum of colony diameter and the halo zone diameter and the colony diameter [33]. The strains that showed an EI higher than 1.50 were considered to be potential producers of cellulases. For each strain the average EI of the three replicates was calculated.

2.3 Methods of Data Analysis

Data analysis was carried out using figure, table, and percentiles to evaluate PGP traits and biocontrol property of cultivable potential plant growth promoting microbial isolates.

Table 1 Multiple plant prowth promoting properties of selected microbial isolates.

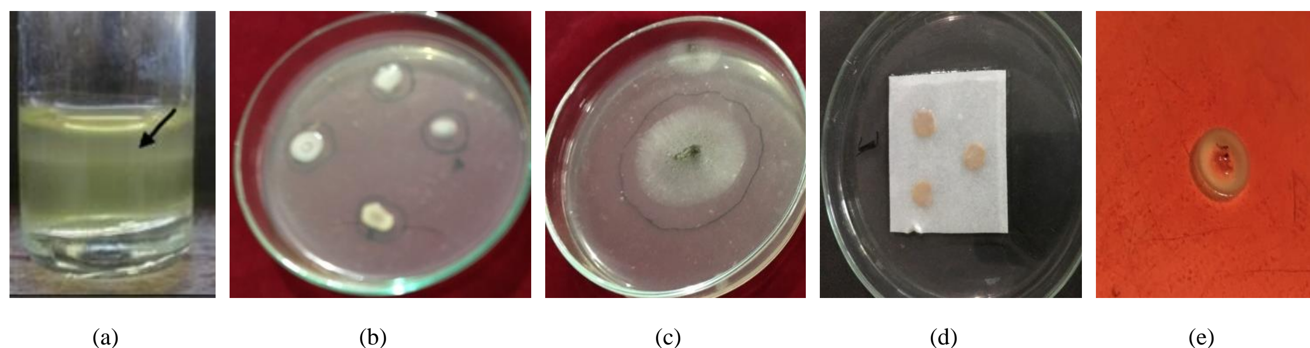
Isolate Code	N Fixation	P Solubilization	PSI	K Solubilization	KSI	IAA Production	Cellulase Production	EI
BI_1	+	+	1.46	+	1.52	+	+	1.35
BI_2	+	-	-	-	-	+	+	1.71
BI_3	+	+	1.95	+	1.63	+	+	1.63
BI_4	+	+	1.41	+	1.86	+	+	1.53
BI_5	+	+	1.30	-	-	+	+	1.18
BI_6	-	-	-	-	-	+	+	1.59
BI_7	-	-	-	-	-	+	+	1.34
FI_1	-	+	1.32	+	2.06		+	1.26
FI_2	-	+	1.38	+	1.28	+	+	1.08
FI_3	-	-	-	+	1.25	+	+	1.08
FI_4	-	-	-	-	-	+	+	1.31
FI_5	-	+	-	+	1.80	+	+	1.08

Remarks:

+ = activity detected
BI_1 = *Bacillus megatrium*
BI_2 = *Azotobacter* sp.
BI_3 = *Bacillus* sp.
BI_4 = *Pseudomonas flurescent*
BI_5 = *Chromobacterium* sp.
BI_6
BI_7

- = activity not detested
FI_1 = *Paeceilomyces* sp.
FI_2 = *Aspergillus niger*
FI_3 = *Trichoderma* sp.
FI_4 = *Saccarhomyces* sp.
BI_5 = *Aspergillus nodulan*

PSI = Phosphat Solubilization Index
KSI = Potash Solubiliation Index
EI = Enzymatic Index



(a) (b) (c) (d) (e)

Figure 1: (a) N fixation (pellicle formation indicated by an arrow); (b) phosphate (P) solubilization (indicated by a clear halo zone around isolate colony; (c) potash (K) solubilization (indicated by a clear halo zone); (d) IAA production (indicated by pink color formation); (e) cellulase enzyme production (indicated by clear halo zone around isolate colony).

3. RESULTS AND DISCUSSION

3.1 Nitrogen Fixation

The present research work used 12 different PGP isolates consisting of 7 bacterial strains and 5 fungal strains as shown in Table 1. Six out of seven bacteria isolates grew well on N-free agar media and produced ammonia after five days incubation at approximately 28°C as indicated by appearance of clear pellicle ring structure near the surface of the media (Fig. 1a). The bacteria were identified as*Bacillus megatrium*, *Bacillus* sp., *Azotobacter* sp., *Pseudomonasfluorescence*, B6, and B7. The formation of this structure confirms the potential for fixing atmospheric N. Similar structure was not found in all fungal isolates, meaning that all fungi considered negative for fixing atmospheric N. Reports on the bacterial ability to fix atmospheric N such as *Pantoea*, *Serratia*, *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Burkholderia* have been demonstrated by Laranjo *et al*.[34] ; Xu *et al*. [35]); Zaheer *et al*. [36].

3.2 Phosphate Solubilization

The microbes ability of phosphate solubilization are indicated by the formation of clear halo zone around their colonies when grown in PVK culture media ammended with $KH_3(PO4)_2$ as an insoluble inorganic form of P source. The results of this study showed that 4 out of 7 bacterial isolates and 3 out of 5 of fungal isolates generated clear halo zone around the colonies after 5 to 7 days of incubation, demonstrating the ability of phophorus solubilization (Fig. 1b). Thus, they are potentialphosphorus solubilizers. The putative bacteria was *Bacillus megatrium*, *Bacillus* sp., *Pseudomonasfluorescence,* and *Chromobacterium* sp. The putative fungi was *Paeceilomyces* sp., *Aspergillus niger*, *Trichoderma* sp., and *Aspergillus nodulans*. According to Fankem *et al*. [37], the halo zone is produced due to solubilization of insoluble phosphates mediated through the secretion of organic acid by the isolates in the surrounding medium combined with a reduced pH medium. Phosphate solubilizing bacteria release organic acids which dissolve phosphate mineral through anion exchange or chelation of Fe and Al ions associated with phosphate [38].

The ability of miroorganism to solubilize insoluble phosphate was measured by the solubilization index: the ratio of the total diameter (colony + halo zone) and the colony diameter, after 5 days of inoculation as shown in Fig. 1b[28]. Phosphate solubilization index (PSI) values of the isolates varied from 1.30 to 1.95 for bacteria and from 1.25 to 1.38 for fungi. The PSI average of all isolates was 1.44 (Table 1). The highest PSI (1.95) were found in *Bacillus* sp. and the lowest PSI (1.30) in *Chromobacterium* sp. for bacteria. The highest PSI (1.38) was observed in *Aspergillus niger and the lowet PSI (1.25) in Aspergillus nodulan* for fungi (Table 1). Based on colony diameter and halo zone formation, *Bacillus* sp. was the most efficient rock phosphate solubilizer on PVK agar plates among rock phosphate-solubilizing bacteria, and *Aspergillus niger* for fungi. The PSI of the most efficient bacteria was greater than that of the fungi although fungi produce large halo zone compared to bacteria.

The different ability of phosphate solubilization based on SI in the present study occurs due to the varying type, amount, and diffusion rates of diverse organic acids produced by fungal isolates as previously reported by Yadav *et al*. [39]. Iman [40] reported that the SI of the test phosphate solubilizing fungal strains (*Penicillium italicum* and *Aspergillus niger*) were 2.42 and 3.15, respectively. On the contrary, Mahamuni *et al*. [41] reported that SI for different fungal strains isolated from sugarcane and sugar beet varied from 1.13 to 1.59. Alam *et al*. [39] also reported that the fungal cultures isolated from maize rhizosphere had SI ranging from 1.53 to 1.80.

No halo zone formation was detected around the bacterial and fungal isolate colonies during the rock phosphate-solubilization tests performed on PVK solid medium. This can, perhaps, be explained by the low diffusion of the organic acids produced by these microorganisms during their growth or the use of a solubilization process without organics acids.

This qualitative test offered feasibility as it can be used for an initial selection of strains, tests in addition to being simple, rapid and well adapted for screening of a large number of samples. However, further test such as ARA (acetylene reductase assay) is needed for confirmation.

3.3 Potash Solubilization

The microbes ability of potash (K) solubilization are indicated by the formation of clear halo zone around their colonies when grown in Aleksandrov culture media ammended with feldspar as an insoluble inorganic source of K. The results of this study showed that 3 out of 7 bacterial isolates and 4 out of 5 of fungal isolates generated clear halo zone around the colonies after 5 to 7 days of incubation, demonstrating the ability of K solubilization (Fig. 1c.). Thus, they are potential K solubilizers. According to Premono*et al*. [28], the halo zone is produced due to solubilization of insoluble potash mediated through the secretion of organic acid by the isolates in the surrounding medium. These acids convert insoluble K such as feldspar to the soluble form of K (soil solution form) with the availability of major plant nutrients [44] (Bahadur *et al*. 2014). The putative bacteria was *Bacillus megatrium*, *Bacillus* sp., *Pseudomonasfluorescence* andthe putative fungi was *Paeceilomyces* sp., *Aspergillus niger*, *Trichoderma* sp., and *Aspergillus nodulans*. Previous works reported that microbes from the genus *Aspergillus and Bacillus* are efficient K-solubilizers [45].

The ability of the isolate to solubilize insoluble feldspar was measured by the solubilization index: the

ratio of the total diameter (colony + halo zone) and the colony diameter after 5 days of inoculation, as shown in Fig. 1c. Potash solubilization index (KSI) values varied from 1.52 to 1.83 for bacteria and varied from 1.25 to 2.06 for fungi. The KSI average of all isolates was 1.63 (Table 1). The highest PSI (2.06) were found in *Paeceilomyces* sp. and the lowest (1.25) in *Trichoderma* sp. for fungi. KSI value of 1.86 was observed in *Pseudomonas fluorescent.*

Based on colony diameter and halo zone formation, *Paeceilomyces* sp. was the most efficient K solubilizer on Aleksandrov agar plates for fungi, and *Pseudomonas fluorescent.*for bacteria. The KSI of the most efficient fungi was greater than that of the bacteria. The previous research results also evidenced that K solubilization is done by a wide range of saprophytic bacteria and fungal strains [46], [47].

This test in plates presented feasibility as it can be employed for an initial selection of strains, tests in addition to being simple, rapid and well adapted for screening of a large number of samples.

3.4 Indole Acetic Acid (IAA) Production

The presence of bacteria and fungi in rhizosphere contributes to positive impact on plant growth indirectly by playing a role as a biocontrol agent or directly by secreting plant hormone and by assissting resource acquisition (mostly N, P, and K). A lot of attention have been paid on the auxin indole3-acetic acid (IAA) recently. For this concern, 12 isolates were tested for IAA production on the solid Luria-Bertani medium supplemented with tryptophan. When the isolates were inoculated on a nitrocellulose membrane, bacteria or fungi capable of synthesizing IAA exhibits red color on the membrane. The results showed that a concentrated pink color on the membrnae was observed in each bacterial isolate and only 3 out of 5 for fungal isolates tested, indicating that they have the ability to produce IAA (Fig. 1d). The result of this study confirms the results of other scholar. Patten and Glick [48] reported that Tryptophan dependent IAA synthesis had been also determined in several bacteria. Swain *et al.* [49] have reported IAA producing *Bacillus subtilis* spp. Auxin production by *Bacillus subtilis* spp increased when culture medium supplemented with an IAA precursor (tryptophan), which confirm the results of other scholar [50]. In *Pseudomonas syringae,* IAA biosynthesis occurs mostly from tryptophan via indole-3 acitamide [51] and in *Pseudomonas fluorescens,* tryptophan bypassing the indole 3-actaldehyde, which is further converted into IAA [52].

The two fungal isolates viz. *Paeceilomyces* sp. and *Trichoderma* sp. did not develop a pink color formation on the membrane after saturated with Salkwoski reagent, indicating negative IAA formation. These fungus do not have the ability to covert tryptophan into IAA. On the contrary, the other fungus viz. *A. niger, A. nodulan*, and *Saccarhomyces* sp. were considered as IAA producer, which partly confirm the results reported by Restu and Payangan [23].They reported that fungus genera *Aspergillus, Trichoderma, Rhizopus, Penicillium*, and *Fusarium* exhibited IAA production ability.

This test in plates offered feasibility as it can be utilized for an early selection of strains, tests in addition to being simple, rapid and well adapted for screening of a large number of samples.

3.5 Cellulase Production Activity

This test is based on the observation of the halo zone produced by hydrolysis of cellulose (CMC) by cellulase secreted by bacteria or fungi strains examined. The halo zone produced by hydrolysis of cellulose is directly related to the region of action of the cellulolytic enzymes, since the dye only remains attached to regions where there are β1,4-D-glucanohydrolase bonds. The results of the present study showed that the pale halo zone around the colonies (Fig. 1e), which corresponds to the zone of CMC degradation, was observed in all 12 isolates (equivalent to 100% of the strains evaluated). Clear halo zones surrounding microbial growing colonies after incubating them for a suitable period confirm their ability for cellulases production [53]. These enzymes are produced by various fungus such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma* species [54]. Azzaz *et al*. [54] fyrther stated that *Aspergillus niger* produces highly active cellulase when grown in liquid media by both surface and submerged culture methods and recently by solid state fermentation. Production of cellulase also reported by using substrates like cellulose, xylose and lactose using *T. reesei*[55]. Saravanan *et al*. [56] studied the production of cellulose using *Trichoderma reesei* in solid state fermentation. Some species of *Penicillium* i.e. *Penicillium iriensis* and *P. citriviride* produce significant quantities of cellulase, when grown under different conditions. Their enzyme activity was confirmed by Congo Red plate screening assay. It was found that *Aspergillus niger* showed the highest cellulase activity when compared with *Trichoderma viride* and *Bacillus subtilis*.

The ability of the isolate to degrade cellulose was measured by the solubilization index: the ratio of the total diameter (colony + halo zone) and the colony diameter after 5 days of inoculation, as shown in Fig.1e. The results showed that he enzyme index (EI) values varied from 1.18 to 1.71 for bacteria and varied from 1.08 to 1.31 for fungi. The EI average of all isolates was 1.353 (Table 1). The highest PSI (1.71) was observed in *Azotobacter* sp. for bacteria and the lowest (1.08) in *A. niger, A. nodulan*, and *Trichoderma* sp. for fungi. Based on the colony diameter and halo zone formation, *Azotobacter* sp.. was the most efficient amorphous cellulose (CMC) degrader through Congo red tes for bacteria, and *Saccarhomyces* sp. for fungi. The EI of the most efficient bacteria was greater than that of the fungi. The previous research results also evidenced that cellulase is produced by a wide range of microorganism such as fungi and bacteria [54], [57]. Cellulase is the common enzyme degrading cellulose. Endo-bglucanase, Exo-b-glucanase and b-glucosidase are

three main components comprising in cellulase enzyme which have been shown to act synergistically in the hydrolysis of cellulose to glucose [58].

According to Ten *et al*. [59] the diameter of the halo zone is useful for selection of strains that can efficiently degrade polysaccharides such as cellulose. Moreover, the enzymatic index can be used as a simple and rapid methodology to select strains within the same genus that have potential for the production of [60]. Table 1 shows the EI results obtained for cultivation of the fungi in synthetic medium containing CMC as sole carbon source, after 4 days of incubation at 30◦C. This test in plates presented feasibility as it can be employed for an initial selection of strains, tests in addition to being simple, rapid and well adapted for screening of a large number of samples.

4. CONCLUSION

The qualitative assessment by testing in plates with different medium and assay procedures showed that these applied tests were feasible for confirming the multiple ability of the 12 isolates to have PGP properties viz. atmospheric N fixation, phosphate and potash solubilization, indole acetic acid (IAA) production, and cellulase production.These methods in plates offered feasibility as they can be utilized for an initial selection of strains in addition to being simple, rapid and well adapted for identifying of a large number of samples. The 12 isolates tested exhibit multiple plant growth promoting properties. Hence, these strains can be further formulated into biofertilizer through co-culturing and then applicated for greenhouse and field experiments.

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