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Protease and Phytohormone Production by Potassium solubilizers Isolated from Maize Rhizosphere

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ABSTRACT

Enzymes and phytohormones are essential substances required for plant development, especially at their vegetative stage. Microbial-plant-root interactions on rhizosphere region make possible for both nutrient mineralization and absorption. An in-vitro assessment was carried out to assess the protease, indole acetic and gibberellic acids production patterns by Lysinibacillus fusiformis and Providencia rettgeri. Both bacterial strains produced indole acetic and gibberellic acids; two essential phytohormones necessary for seed germination and fruit ripening. Time of incubation of both bacterial inoculants had significant effects ($p<0.05$) on protease activities. Lysinibacillus fusiformis produced more protease (0.6630±0.003 µg/mL) at 96 h after incubation, while the highest protease activity by Providencia rettgeri was observed at 72 h after incubation with a mean value of 0.4605±0.004 µg/mL. However, Lysinibacillus fusiformis produced more protease enzyme compared to Providencia rettgeri. The production trends of gibberellic and indole acetic acids were also study. The amount of gibberellic acid produced changed significantly ($p<0.05$) with time. The activity of gibberellic acid was at its peak 48 h after incubation for both Lysinibacillus fusiformis (76.25±0.21 µg/mL) and Providencia rettgeri (151.85±0.78 µg/mL), with Providencia rettgeri producing more gibberellic acid than Lysinibacillus fusiformis. The highest production of IAA was at its peak at 72 h after incubation for Lysinibacillus fusiformis (1.4530±0.001 µg/mL) but was not significantly ($p>0.05$) different with change in time. Providencia rettgeri produced more IAA at 72 h of incubation (1.1450±0.004 µg/mL) and was significantly ($p<0.05$) different with change in time.

Keywords: *Gibberellic acid, incubation time, indole acetic acid, protease, rhizobacteria*

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INTRODUCTION

Biochemical and physiological processes in plant are accelerated by enzymes and phytohormones activities. Potassium is needed in sufficient quantities for plant uptake (Hasanuzzaman *et al.,* 2018). Thomas *et al.* (2016) reported that K has the ability to activate sixty (60) enzymes, as well as enhance photosynthesis and regulate stomata opening. Enzymes are catalyst needed to facilitate chemical reactions in plants. Potassium serves as enzyme activator that regulates plant growth. Enzymes molecules are reshaped by the introduction of K. Potassium as a cation (K^+) neutralizes organic anions (negatively charged ions) within plants, which stabilizes pH and brings it to optimum for most enzymatic activities (or enzyme reactions). Potassium in plant cell determines the number of enzymes that can be activated and the rates of chemical reactions that can occur (Prajapati and Modi, 2012). Therefore, in K deficient plant, the amount of starch produced declines, while soluble carbohydrates and N compounds accumulate. Patil (2011) reported that in plant with high level of K,

starch produced moves from site of production to storage organs in the plants. Microorganisms especially bacteria are known to produce enzymes. These microbial enzymes act as catalysts that facilitates microbial activities, breakdown of protein into peptides and amino acids (Sumantha *et al*., 2004) and are also useful for sustaining microbial life (Johnson, 2005). Pant *et al*. (2015) reported that protease, one of the main enzymes produced for industrial use (Sharma *et al.,* 2017), is of the microbial origin. Most interestingly, these protease-producing bacteria are commonly found in the soil (Pant *et al*., 2015).

The function of protease enzyme is seen in the hydrolysis of protein (Schallmey *et al*., 2004). Protease-producing bacteria now draw attention in the fermentation industries (Rathakrishnan, 2012). Most *Bacillus* sp. has been reported to produce large amounts of proteases (Bhosale *et al*., 1995). Commercial proteases are mostly produced from bacteria species, and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources (Ferrero *et al*., 1996).In crops, protease functions are obvious in the cell division. Indole acetic acid, one of the common phytohormone in plants, is produced by beneficial microbes and acts a plant growth regulator (Yu *et al*., 2017). Gibberellic acid was report to trigger root shoot elongation, seed germination, flowering and fruiting in crops (Binenbaum *et al*., 2018).

Objective of the study

i. To determine the production of phtyohormones (indole acetic and gibberellic acids) by the potassium solubilizers in solution.
ii. To determine the ability of potassium-solubilizers to produce protease.

MATERIALS AND METHODS

Method of soil sample collection from maize rhizosphere

Soil sampleswere collected from the root zone of each maize plant. The maize plant was harvest and the soil collected from the root zone. Root samples of maize plants (at tasseling stage) were collected into a Ziploc for each location, and then taken to the laboratory for isolation of potassium solubizing bacteria. The rhizosphere soil samples were carried to the laboratory for physicochemical analysis. Samples from each location were properly labeled, air-dried and stored in a Ziploc.

Preparation of aleksandrov medium for KSR isolation

Aleksandrov medium was prepared using the following composition: Magnesium sulphate ($MgSO_4.7H_2O$) (0.50g), Calcium carbonate ($CaCO_3$) (0.10g), Potassium alumino silicate (3.00g), Glucose (5.00g), Ferric chloride (FeCl) (0.006g), Calcium phosphate ($Ca_3(PO_4)_2$) (2.00g), Agar (20.00g). One liter (1000 ml) of distilled water was added to the chemicals measured and the final pH will be adjusted to about 7.2±0.2 by adding 1N NaOH (25°C). The composition was heated to boiling to dissolve the mixture completely. The mixture was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. It was then allowed to cool at about 45°C, before dispensing into sterile Petri dishes containing the serially diluted samples.

Isolation of rhizobacteria from maize roots

Rhizobacteria were isolated from samples gotten from maize rhizosphere soils using Aleksandrov medium by serial dilution method (Hu *et al*., 2006).

Serial dilution method

Nine milliliters (9 ml) of water was transferred into different seven (7) test tubes and sterilized at 121°C for 15 minutes. The test tubes were placed in test tube rack and labeled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, $10^{-6}$and 10^{-7}. Ten (10) grams of the soil sample was weighed into 90 ml of sterile water and mixed thoroughly to get the stock solution. With a sterile syringe (or pipette tip), 1 ml of the stock solution was collected and serially diluted to 10^{-7}. This procedure was repeated for all the samples using different sterile syringes. One milliliter (1 ml) of the serially diluted (10^{-7}) sample was plated on the liquid Aleksandrov media using pour plate method. About 15 mL of the melted media is poured into each of labeled Petri dishes, covered with the lid and swirl gently so that the Aleksandrov medium covers the bottom of the plates and then allowed to solidify without disturbing. When the media solidifies, the Petri dishes were inverted and put in a biological oxygen demand incubator at 30±2°C for 48 hours to develop Colony Forming Units (CFU). After incubation, plates were observed for colonies of bacteria. Distinct colonies were sub-cultured repeatedly until pure cultures were obtained. The pure cultures were stored on agar slant for further studies.

Identification and characterization of KSR

The isolates that showed clear zones of K-solubilization were selected for further identification and characterization using morphological, biochemical characteristics and molecular methods. The identification of the selected KSR was carried out at Havenwaves laboratory, Ibadan, Oyo State.

Gibberellic Acid (GA) Production

The bacteria isolates were assessed for their ability to produce gibberelic and indole acetic acids, which are phytohormones and protease an enzyme. Production of gibberellic acid was detected by spectrophotometric method. The 48 h old growth of bacterial culture was centrifuged at 10,000 rpm for 15 – 20 minutes. The pH value of supernatant was adjusted to 2.5 using stock 3.75 N HCl. Supernatant was extracted using liquid liquid (ethyl acetate/$NaHCO_3$) extraction method. The amount of gibberellic acid in the ethyl acetate phase was measured by the UV spectrophotometer at 254 nm (Berryos*et al.*, 2004 and Umi*et al.*, 2014) at 24, 48, 72, 92 and 120 h.

Indole acetic acid (IAA) Production

All pure isolates obtained were determined for the abilities for Indole Acetic Acid production. The production of IAA was measured on TSB liquid media (20 ml) containing L Tryptophan (200 µg / ml). The culture was incubated in the dark on a shaker at room temperature, and harvested by centrifugation at 8000 rpm for 10 minutes at 24, 48, 72 96 and 120 h. One ml of supernatant was mixed with 4 ml of Salkowski reagent (dissolved in 2% of 0.5M $FeCl_3$ in 35% perchloric acid), then incubated at room temperature for 20 minutes. Indole production was indicated by color change into pink. IAA production was quantified from the supernatant absorbance using a spectrophotometer with a wavelength of 530 nm. It was then calculated using IAA standard curve. The control treatment was uninoculated TSB media with Salkowski's solution. Each treatment was repeated three times. A total of 10 ml of IAA standard 100 ppm was diluted into concentrations of 80 ppm, 40 ppm, 10 ppm, and 5 ppm in a test tube. As much as 2 ml of each concentration was placed in a test tube and added with 2 ml of Salkowski reagent (ratio 1:1). It was then incubated for 60 minutes at room temperature. After incubation, the solution turned pink. The IAA standard solution absorbance was measured at a wavelength of 530 nm and a standard curve was plotted to show the relationship between the concentration of the standard IAA solution (x) and absorbance (y) of IAA production. The production yield of IAA is presented in µg / ml (Patten and Glick, 2002; Gordon and Weber, 1951).

Protease activity

Two culture media used for protease production and compared. One of them contained (% w/v): glucose-0.1, peptone-1, yeast extract-0.02, $MgSO_4$-0.01, $CaCl_2$-0.01, K_2HPO_4-0.05 (pH 7.0) (Qadar *et al.*, 2009). Other composed of (% w/v): glucose-1, peptone-0.5, yeast extract-0.3, $MgCl_2$-0.02, $CaCl_2$-0.04 (pH 7.0) (Sangeetha *et al.,* 2008). Glucose was sterilized separately and aseptically added to the flasks containing the liquid medium, after cooling. The pre-cultures were cultivated in Nutrient Broth medium (0.8% w/v) for 18 h. Then, overnight cultures with OD_{600}=0.3 were inoculated at 1% in enzyme production media (150 mL in 500 mL Erlenmeyer flasks) and incubated at 37 °C for 16, 24, 40, 48, 64 and 72 h in a shaking incubator (150 rpm). At the end of each period, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for determination of proteolytic activity. Bacterial biomass was determined by measuring optical density at 600 nm. Capabilities of protease production were examined and recorded.

RESULTS

Effect of incubation time on protease activity of two potassium-solubilizing rhizobacteria inoculants

The two potassium-solubilizing rhizobacteria were identified as protease-producing bacteria. Time of incubation of both bacterial inoculants had significant effects ($p<0.05$) on protease activities (Figures 2 and 3). For *Lysinibacillus fusiformis*, time of incubation from 0 h to 24 h significantly increased protease activity in solution. Invariably, as the time of incubation increased, protease activity also increased until it begins to decline after optimum production. This same trend was observed in *Providencia regtteri* inoculants.

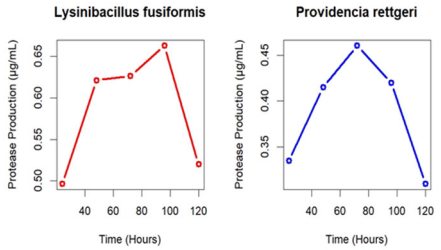

Figure 2: Effect of incubation time on protease activity.

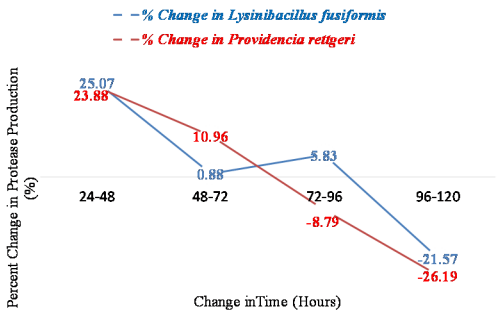

Figure 3: Percent (%) change in protease production in *Lysinibacillus fusformis* and *Providencia rettgeri*

At 24 h after incubation, *Lysinibacillus fusiformis* produced 0.4965±0.022 µg/mL of protease. There was an increase in protease production (0.6210±0.004 µg/mL) at 48 h after incubation, which was significantly higher ($p<0.05$) than that produced at 24 h after incubation. Production of protease stabilized at 72 h after incubation with a mean value of 0.6265±0.004 µg/mL. Further increase of protease activity (0.6630±0.003µg/mL) was observed after 96 h of incubation, followed by a sharp decline (0.5200±0.001 µg/mL) at 120 h after incubation.

A similar trend was observed in *Providencia rettgeri* strain (Figure 2). With *Providencia rettgeri*, protease activity was highest at 72 h after incubation (0.4605±0.004 µg/mL). The lowest production was observed at 120 h after incubation (0.3100±0.003 µg/mL). In this study, generally, it was revealed that *Lysinibacillus fusiformis* produced more protease enzyme than *Providencia rettgeri* and its highest protease activity was observed at 96 h after incubation (Figure 2). The lowest protease productions were observed in *Lysinibacillus fusiformis* at 120 h after incubation and at 24 h after incubation in *Providencia rettgeri*.

The rate of change in protease activity was also studied, and observations made indicated that the rate of increase was more in *Lysinibacillus fusiformis* than in *Providencia rettgeri*. For instance, the percent increase in *Lysinibacillus fusiformis* between 24 h to 48 h after incubation is 25.07 %, between 48 to 72 h after incubation the percent increase was 0.88% and later increased by 5.83 % from 72 to 96 h after incubation. However, there was a sharp decrease of 21.57 % observed between 96 to 120 h after incubation (Figure 3). In *Providencia rettgeri* the percent increase between 24 to 48 h was 23.88 %. While from 48 to 72 h after incubation, the percent increase was 10.96 % (Figure 3). At 72 to 96 h after incubation the change in protease activity declined by 8.79 %, which further decreased sharply by 26.19 %, from 96 to 120 h after incubation. Generally, based on the study, the rate of change in protease activity is more between 24 and 48 h after incubation, while a decline in the rate of activity reflected at 96 to 120 h after incubation. Protease enzyme activity was optimum at 96 h of incubation for *Lysinibacillus fusiformis* and 72 h of incubation for *Providencia rettgeri.*

Effect of incubation time on gibberellic acid (GA) production of two potassium-solubilizing rhizobacteria inoculants

The effects of incubation time on gibberellic acid (GA) production of the two potassium-solubilizing rhizobacteria inoculants were measured and the results presented in (Figure 4). It was observed that the inoculants produced gibberellic acid (GA) and the amount of gibberellic acid produced changed significantly ($p<0.05$) with time. *Lysinibacillus fusiformis* inoculants for instance produced 37.85±0.78 µg/mL at 24 h after incubation which further increased significantly ($p<0.05$) to a peak of 76.25±0.21 µg/mL at 48 h after incubation. At 72 h after incubation, the GA produced dropped significantly ($p<0.05$) to 34.00±0.28 µg/mL. The decrease in GA production between 72 to 120 h after incubation were significantly the same ($p>0.05$) (Figures 4 and 5). *Providencia rettgeri* inoculants also produced GA which showed significant

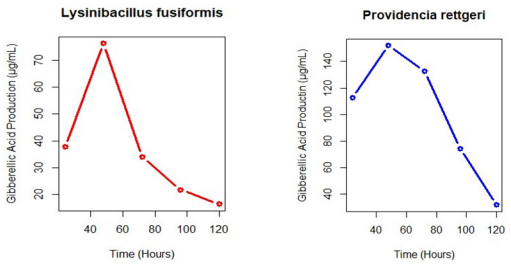

Figure 4: Effect of incubation time on gibberellic acid production.

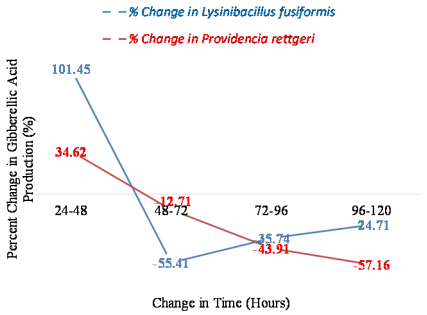

Figure 5: Percent (%) change in gibberellic acid production in *Lysinibacillus fusiformis* and *Providencia rettgeri*

differences ($p<0.05$) with time of incubation. The highest GA production was observed at 48 h after incubation (151.85±0.78 µg/mL). At 24 and 72 h after incubation the GA productions were significantly the same ($p>0.05$) giving mean values of 112.80±0.85 µg/mL and 132.55±0.78 µg/mL respectively. However, the least production was observed at 120 h after incubation (31.85±0.64 µg/mL).The percent change in GA production by *Lysinibacillus fusiformis* followed a similar trend with that of *Providencia rettgeri*. The GA production of *Lysinibacillus fusiformis* increased by 101.45 % between 24 to 48 h after incubation, however the production rate dropped by 55.41 % at 72 h after incubation. The decline in production continued till 120 h after incubation (Figure 5). *Providencia rettgeri* production rate of GA increased by 34.62 % at 48 h after incubation and later declined by 12.71 % at 72 h after incubation. It is observed that gibberellic acid production

is more in *Providencia rettgeri* bacteria strain than in *Lysinibacillus fusiformis* bacteria strain. For instance, at 48 h after incubation, the production of gibberellic acid in *Lysinibacillus fusiformis*(76.25 µg/mL) is about double the production in *providencia rettgeri* bacteria strain (151.85 µg/mL).

Effect of incubation time on indole acetic acid (IAA) production by two potassium-solubilizing rhizobacteria inoculants

Indole acetic acid (IAA) productions by the two KSR

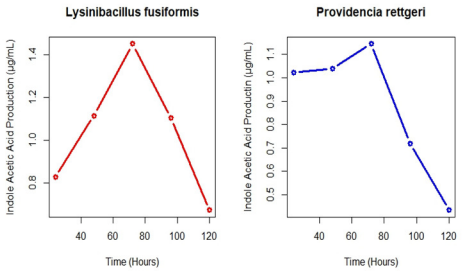

Figure 6: Effect of incubation time on indole acetic acid production.

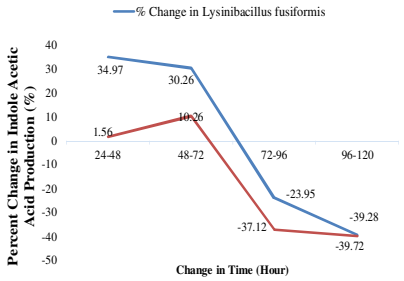

Figure 7: Percent (%) change in indole acetic acid production in *Lysinibacillus fusiformis* and *Providencia rettgeri*

inoculants were determined and the results presented in (Figure 6). Incubation time did not significantly affect ($p>0.05$) IAA production by *Lysinibacillus fusiformis*, although at 72 h after incubation, *Lysinibacillus fusiformis* produced 1.443±0.001 µg/mL IAA, while the least IAA of 0.671±0.004 µg/mL, was produced at 120 h. after inoculation but was not significantly different ($p>0.05$) from other time variations. *Providencia rettgeri* produced the highest IAA of 1.1450±0.004 µg/mL at 72 h after incubation which was significantly different ($p<0.05$) from those produced at different times of incubation (Figure 7). At 24 and 48 h after incubation the IAA production were significantly the same with mean values of 1.0225±0.002 and 1.0385±0.004 µg/mL respectively. The least IAA production (0.4340±0.001 µg/mL) was observed at 120 h after incubation. Generally, the highest production of IAA was at its peak at 72 h after incubation for both *Lysinibacillus fusiformis* (1.4530±0.001 µg/mL) and *Providencia rettgeri* (1.1450±0.004 µg/mL).

In observing the rate of change of IAA production, between 24 – 48 h., the percent (%) increase of IAA produced by *Lysinibacillus fusiformis* was 34.97 % which later declined to 30.26 % between 48 – 72 h of incubation. The production of IAA by *Providencia rettgeri* indicated that the percent increase between 24 – 48 hafter incubation was 1.56 % which later rose to 10.26 % between 48 – 72 h. after incubation. Beyond this peak, there was sharp decrease of 23.95 % between 72 – 96 h. for *Lysinibacillus fusiformis* and 37.12 % for *Providencia rettgeri*. Similarly, the activities of indole acetic acid later declined by 39.28 % and 39.72 % between 96 – 120 h. of incubation for *Lysinibacillus fusiformis* and *Providencia rettgeri* respectively (Figure 7).

DISCUSSION

Proteolytic enzymes (proteases) are almost everywhere in nature in all living organisms, and they play a unique role in growth and differentiation of living cells (Tiwari *et al*., 2015). Chu (2007) documented that proteases are one of the three major enzymes used in industries. Tiwari*etal*. (2015), in their study, observed that protease production by different bacterial isolated, reached their maximum values at 48 h after incubation. Previous studies also reveal similar trend (Olajuiyigbe and Ajele, 2005). In a study that involved a protease-producing fungi, *Aspergillus flavus* and *Aspergillus terreus*, the production pattern gave a maximum enzyme production at 72 h after incubation, and gradually declined with increase in time (Chellapandi, 2009). The study by Mushtaq *et al*. (2023) also indicated that maximum production of protease was observed at 72 h after incubation.

One of the complex organic molecules acting as a phytohormone is the gibberellic acids having a molecular formula, $C_{19}H_{22}O_6$ (Slipa *et al*., 2018). A study conducted by Slipa *et al*. (2018) revealed that at 48 h after incubation, *Bacillus lichenformis* DS3, isolated from banana field soils, recorded a maximum production of gibberellic acid, 83.7 µg/ml at a pH of 7.0. In another study, *Bacillus subtilis* strain from paddy rhizosphere recorded a maximum level of gibberellic acid production, 4.8 µg/ml (Sivasakthi *et al*., 2013). There seem to be variations in the levels of gibberellic acid production by different strains of bacterial isolates. It is also possible that the variations observed may be due to environmental differences that control microbial activities. Apastambh *et al*. (2016) also reported another *Bacillus* species which produced highest level of gibberellic acid, 52 µg/ml. Similar result obtained in this study also indicated that after 48 hof incubation, gibberellic acid production declined (Slipa *et al*., 2018). One factor that might be

responsible for the level of gibberellic acid is pH. Several authors have reported maximum production of gibberellic acid at near neutral or neutral pH of 7.0 (Slipa *et al*., 2018; Sagar Desai, 2017; Karakoc and Aksoz, 2006).

Indole Acetic Acid (IAA) and Gibberellic Acid (GA) are major phytohormones produced by certain rhizobacteria (Kejela, 2024). Plant growth and development is significantly influenced by indole-3-acetic acid as reported by (Kejela, 2024). Niranjan *et al.* (2024) reported that, naturally, phytohormones are produced in low concentrations.

Boiero *et al*. (2007) reported that certain bacterial species can produce phytohormones, and their ability to produce phytohormones is strain specific. Indole acetic acid (IAA) is functional in root enhancement, and bacteria-producing phytohormones create a link for interactions with plants (Kejela, 2024).

Biosynthesis of phytohormones produced by bacteria species show great potentials in agriculture and biotechnology (Stirk and van Staden, 2020). The advantage of using microorganisms to enhance crop production is in its ability to sustain and stabilize both plant and human ecology. It goes as far as initiating nutrient cycling as well as accelerating the process of biodegradation.

Indole acetic acid is a common product produced by microorganisms, it is also one of the most physiologically active auxins (Hariharan *et al*., 2014; Karnwal, 2017). Previous studies showed that some gram positive strains of the *Bacillus* family are known to produce indole acetic acid (Wahyudi *et al*., 2011). In another study by Islam *et al*. (2015), isolates from cucumber rhizosphere were able to produce indole acetic acid with or without L-tryptophan. They also reported that with 50 gmL^{-1} of L-tryptophan, their isolates produced higher concentrations of indole acetic acid ranging from 1.4 – 2.6 gmL^{-1}.

In a study carried out in East Java, Indonesia, Ramadhani *et al*. (2020) documented that the optimum time for bacterial isolates to produce indole acetic acid hormone was at 48 h after incubation. They also discovered that indole acetic acid production between 0 – 24 h increased, and entered it peak at 48 h after incubation. A drop in IAA production was observed at 96 h after incubation.

Suliasch and Widawati (2020), reported similar trend when they studied the effect of incubation time on IAA production by *Bacillus siamensis*. The observed an increase in IAA production from 24 h after incubation, which reached a maximum production 96 h after incubation. Their study indicated an obvious decrease in IAA production from 120 h after incubation which continued to 144 h after incubation. Similar result was also reported by Sonali*et al*. (2018), where maximum production of IAA was observed at 96 h after incubation.

In a study conducted by Suliasch and Widawati (2020), they obtained a range of between 2.88 – 5.14 μgmL^{-1} productions of IAA from 19 bacterial isolates. In another study, *Bacillus*sp produced IAA ranging from 0.75 – 21.3 μgmL^{-1} (Araujo *et al*., 2012).

Generally, the production of IAA by bacterial species has been observed to decline after an optimum production time. These findings is similar in several other studies (Dipanwita*et al*., 2015; Nita *et al*., 2011; Apine *et al*., 2011; Dhara, 2009; Hariharam *et al.,* 2014). However, there could be variation in time of optimum production of different bacterial species which may be due to some abiotic factors such as temperature and pH (Baggam *et al*., 2017).

Conclusion

Lysinibacillus fusiformis exhibited peak protease activity at 96 h after incubation, while *P. rettgeri* showed highest protease production at 72 h after incubation. Both K-solubilizers demonstrated highest gibberellic acid production at 48 h after incubation, and highest indole-3-acetic acid (IAA) production at 72 hours of incubation. These findings suggest the potential use of *Lysinibacillus fusiformis* as a bio-inoculants to stimulate seed germination and growth, highlighting its potential application in agricultural practices.

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