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Evaluation of anti-*Fusarium* and auxin production of *Trichoderma virens* InaCC F1030 isolated from rhizosphere of banana

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Abstract

Banana rhizosphere harbors a unique diversity of microbes including fungi that play critical roles in the growth of the plant host as well as might be important for biologically controlling the fungal soil-borne pathogens particularly *Fusarium oxysporum* f.sp. *cubense* (Foc), the causing agent of devastating Panama wilt. Among other fungi, we have succeeded to isolate a *Trichoderma* species from rhizosphere of healthy banana. Molecular identification revealed the isolate as *Trichoderma virens* InaCC F1030 (being collection of Indonesian Culture Collection or InaCC). Therefore, the aim of this study was to investigate the biological control of our isolate against Foc as well as plant growth promoting ability through its ability to produce auxin (indole-3-acetic acid/IAA). Two approaches were employed to evaluate the antagonism of our isolate against Foc, through direct confrontation test and volatile organic compounds (VOCs) producing. We found that our isolate was considered as antagonistic to the Foc, but not highly antagonistic according to direct confrontation assay. However, it was also revealed that our isolate produces the VOCs that inhibited around 50% of the mycelial growth of the test pathogen after six to seven days of exposure. Our isolate was able to produce the IAA in axenic submerged fermentation condition particularly in the presence of the precursor L-tryptophan. IAA production ability as well as the mycelial biomass of fungus were increased approximately 17% and 120% respectively as the effect of supplementation of 0.1% of L-tryptophan. These *in vitro* bioassays lead us to conclude that somehow our isolate *T. virens* InaCC F1030 has potency to be utilized as biocontrol and biofertilizer agent.

Keywords: banana, Foc, IAA, *Trichoderma virens*

Introduction

Banana is one of main important crops that has been used not only as food source but also for traditional customs and religious purposes. As a food source, the trending demand for banana fruit is increasing as a result of its expanding popularity worldwide (FAO 2018). Consequently, the effort to rise banana yield is inevitable, starting from the selection of desirable cultivar to improving the crop management practice including application of biofertilizers as growth promoter agents (Frison *et al*. 2004). However, for decades, various

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banana diseases, caused by plant pathogens from bacteria as well as fungi, have been devastated banana plantation, thus lowering or even threatening the sustainability of banana production. Therefore, two main focuses in banana productions, namely promotion of the growth and controlling the plant pathogens need to be continuously developed, but still environmentally friendly in nature as main consideration.

Species of the genus *Trichoderma* are widely distributed in natural environment and form mutualistic relationship with plants. The genus has been attracted numerous attentions due to its beneficial roles not only in agriculture field, particularly as biocontrol and plant promoting agent, but also for enzymes production in industrial usage (Błaszczyk *et al.* 2014). Our previous study showed that the ability of *Trichoderma harzianum* isolated from leaf litter and soil to inhibit the growth of *Fusarium oxysporum* f.sp. *cubense* (Foc), the causing agent of panama wilt that has been a major threat of banana cultivation worldwide for decades, was strain-dependent (Napitupulu *et al*. 2019a). Moreover, we also found that *T. harzianum* was able to promote plant growth through its capability to solubilize inorganic phosphate and produce growth hormone indole-3-acetic acid (IAA), but its action is modulated by various environmental factors and nutrition status (Napitupulu *et al*. 2019b, c).

Compared to bulk soil zone, rhizosphere of plant is well known to harbor a unique microbial diversity as the result of the interaction with the plant host. Some of these microbes have a mutualistic relationship with plant, while some have pathogenic in nature. In this study, we have succeeded in isolating a *Trichoderma virens* from rhizosphere of banana. The isolate is being collection of Indonesian Culture Collection (InaCC). Similar to *T. harzianum*, this fungus is associated as plant beneficial fungus that enhances biomass production and stimulates the growth of root (Contreras-Cornejo *et al*. 2009). However, beside the ubiquitously of *Trichoderma* spp., their high diversity, not only in taxonomic (Bissett *et al*. 2015) but also in metabolomic realm (Guo *et al*., 2019) has been noticed. Therefore, the aim of this study was to evaluate the biological control ability of our *T. virens* InaCC F1030 isolate against plant pathogen Foc and its potency to produce plant growth promoting hormone, IAA. Direct as well as indirect confrontation approach were employed in order to investigate the biological control ability of *T. virens* InaCC F1030 toward Foc.

Materials and methods

Isolation and purification

The fungus was isolated from rhizosphere of banana planting in Experimental Greenhouse of Research Center for Biology-LIPI for 3 months. A total of 1 g of soil rizosphere was mixed with 9 mL of steriled water. The dilutions used were 10^{-1}, 10^{-3} and 10^{-5} dilutions. A total 25 µL of suspension was spread on Potato Dextrose Agar (PDA) with addition of 100 ppm of chloramphenicol with 2 replicates and incubated at 30 °C for 3 days in dark chamber. Daily observation was conducted to the plate until visible mycelium growth to keep away from contamination. Each macroscopically different colony of fungi were sub-cultured onto new PDA plates and incubated at 30 °C for 3 days. The fungal isolates were stored in −80 °C freezer for further use.

Genomic DNA extraction, PCR amplification, and DNA sequencing

DNA was extracted from fungal isolates. All fungal isolates were grown in the Potato Dextrose Broth (PDB) and incubated at 28 °C for 72 hours. Biomass fungal mycelia were used for DNA extraction. DNA was extracted using the Nucleon Phytopure Plant DNA Extraction Kit (Amersham Life Sciences, Buckinghamshire, UK). The ITS regions were amplified by polymerase chain reaction (PCR) using the pair of primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al*. 1990). Amplification reactions were performed with 12.5 µL of GoTaq Green

Master Mix (Promega, USA), 1 µL of ITS4 (10 µM), 1 µL of ITS5 (10 µM), 9.5 µL of nuclease-free water (NFW), and 1 µL of DNA. The amplification was run with the following program: 1 cycle of 3 minutes at 96 °C, 30 cycles of 30 s at 96 °C (denaturation), 30 s at 55 °C (annealing), 1 minute and 30 seconds at 72 °C (elongation), and 1 cycle of 10 minutes at 72 °C (elongation). The results of PCR amplification were revealed on a 1.2% agarose gel in TAE 1X solution at 100 V for 20 minutes with a 1 kb DNA ladder. The DNA bands were visualized using Gel DocTM EZ Imager (Bio-Rad Laboratories, Hercules, CA, USA). DNA sequencing was performed by the Macrogen sequencing service (Macrogen, Seoul, Korea). DNA was sequenced by the Sanger method (Sanger *et al*. 1977) using the 3370*xl* genetic analyzer (Applied Biosystem, USA). ITS4 and ITS5 primers were used for DNA sequencing.

Phylogenetic analysis

The new nucleotide sequence was assembled using SeqMan Pro version 7.1.0(44.1) in the DNASTAR lasergene core suite software (DNASTAR Inc., Madison, WI, USA). Analysis of sequences of *Trichoderma* fungi and closely related sequences obtained from the GenBank using BLAST (https://www.ncbi.nlm.nih.gov/BLAST/) was conducted using MUSCLE (Edgar 2004) in the MEGA 7 program (Kumar *et al*. 2016). The fungal taxa and their sequence accession number used in the phylogenetic analysis are shown in Figure 1. *Hypomyces subiculosus* (FN859452) was selected as an outgroup (Leylaie & Zafari 2018). The Neighbour-Joining (NJ) method (Saitou & Nei 1987) was obtained from the dataset by MEGA 7 using Jukes-Cantor + G model as the best evolutionary model for the dataset (Jukes & Cantor 1969). Clade robustness was assessed using bootstrap (BS) analysis with 1000 replicates (Felsenstein 1985).

Direct confrontation test

In order to assay the direct antagonism of *Trichoderma virens* InaCC F1030 against the Foc, one mycelium plug of our isolate and Foc (strain InaCC F822, from Indonesian Culture Collection) in Potato Dextrose Agar (PDA) medium (around 6 millimetres of diameter) was sourced from the margin of actively growing fungal colony and put on to a new nine-centimetre PDA plate, 4 centimetres aside of each other. The plates then were stored in an incubator (30 ºC) without light. Every day for 7 consecutive days, the occupation of our *Trichoderma* isolate against Foc was constantly monitored. According to the classification proposed by Bell (1982), the antagonism was judged at fifth day following these classification: grade 1— *T. virens* InaCC F1030 isolate entirely overgrew the Foc and occupied the whole PDA surface; grade 2— *T. virens* InaCC F1030 isolate overcome 75 % of the surface of PDA medium; grade 3— *T. virens* InaCC F1030 isolate occupies half of the PDA surface; grade 4— *T. virens* InaCC F1030 isolate occupies 25 % of the PDA medium surface; grade 5—the Foc completely overgrew the *T. virens* InaCC F1030 isolate (Bell, 1982). The experiment was conducted with three replicates.

Antagonism through volatile metabolites producing test

Separate nine-centimetre petri plates containing around 15 mL of PDA were inoculated in the centre with a mycelial disc (size around 6 millimetres) of Foc or the *T. virens* InaCC F1030. The cover was discharged and the bottom part of the plate containing Foc were placed on the top of the bottom part of plate inoculated with *T. virens* InaCC F1030. The plates were then sealed tightly. This arrangement was designed to allow the indirect confrontation between the two isolates, in which only gaseous matter can be exchangeable. The control plates, confronted *T. virens* InaCC F1030 and PDA without the Foc, were also maintained. Incubation of all the plates was conducted at 30 ºC under unlighted condition. After 7 days of incubation, the mycelial diameters of Foc with and without indirect

confrontation with *T. virens* InaCC F1030 were measured. The results were then used to assess the inhibition capacity of our isolate toward the Foc. The percentage reducing mycelial growth affected by VOCs produced by *T. virens* InaCC F1030 was calculated as following equation 1:

$$\% \text{ reducing mycelial growth} = \frac{D_{Foc\ Control} - D_{Foc-F1030}}{D_{Foc\ Control}} x100\% \quad (1)$$

where $D_{Foc\ Control}$ is diameter of mycelial Foc control (without conftrontation with *T. virens* F1030) and $D_{Foc\text{-}F1030}$ is diameter of mycelial Foc affected by *T. virens* InaCC F1030. This experiment was conducted with three repetitions.

Submerged fermentation for IAA production

The submerged fermentation in axenic condition was conducted on 30 mL of Czapek Dox Broth (sucrose 30.00 g/L, dipotassium phosphate 1.00 g/L, sodium nitrate 3.00 g/L, ferrous sulphate 0.01 g/L, potassium chloride 0.50 g/L, magnesium sulphate 0.50 g/L, pH 7.0) in 100-mL conical flasks with and without the precursor 0.1 % of L- tryptophan. The cultures were then incubated at room temperature for 7 days on a rotary shaker at 80 r/min. The prepared medium without fungal inoculation was also maintained as the uninoculated control. All experiments for each concentration were run in three repetitions.

Quantitative determination of IAA concentration

IAA concentration was estimated spectrophotometrically with some adjustments (Gordon & Weber 1951). As much 2 mL of Salkowski reagent (prepared by blended 1 mL $FeCl_3$ 0.5 M and 49 mL $HClO_4$ 35%) was mixed with 1 mL of culture supernatant. The mixture was then shaken vigorously for around 5 seconds and allowed to stand in dark chamber for 30 minutes at room temperature. IAA production, indicated by the pink colour developed, was measured with a spectrophotometer UV-Vis (JK-VS-721N, JKI, China) at wavelength of 530 nm. A standard curve of the indole-3-acetic acid standard solutions (Sigma-Aldrich) were prepared to calculate the IAA concentration in inoculated sample after correction with the absorbance of uninoculated control.

Dry biomass determination of the filamentous fungus

In order to know whether the addition of precursor influences the growth of fungus, we determined the biomass of fungi by collected the mycelial from liquid broth after quantitatively measured the IAA concentration. The fresh mycelial was put on pre-weigh paper and dried at 45^{o}C for 12 hours. The dried mycelial was then carefully weight.

Results

Our InaCC F1030 fungal isolate was obtained from banana rhizosphere. Besides it, we have succeded to isolate various members of class Ascomycota from Banana rhizosphere (data not shown). All fungal isolates are being collection of Indonesian Culture Collection or InaCC. The NJ tree of *Trichoderma* species showed that our InaCC F1030 isolate sequence nested in the same clade with *T. virens* G.J.S. 95-194 (AF328552) and *T. virens* CBS 249.59 (MH857855) (Figure 1). This result shows that InaCC F1030 sequence belongs to *T. virens*. Among other fungal isolates, we are interested in *T. virens* isolate as it belongs to *Trichoderma* genus that has been known to have potency as antagonist of various fungal plant pathogens and promoter of plant growth.

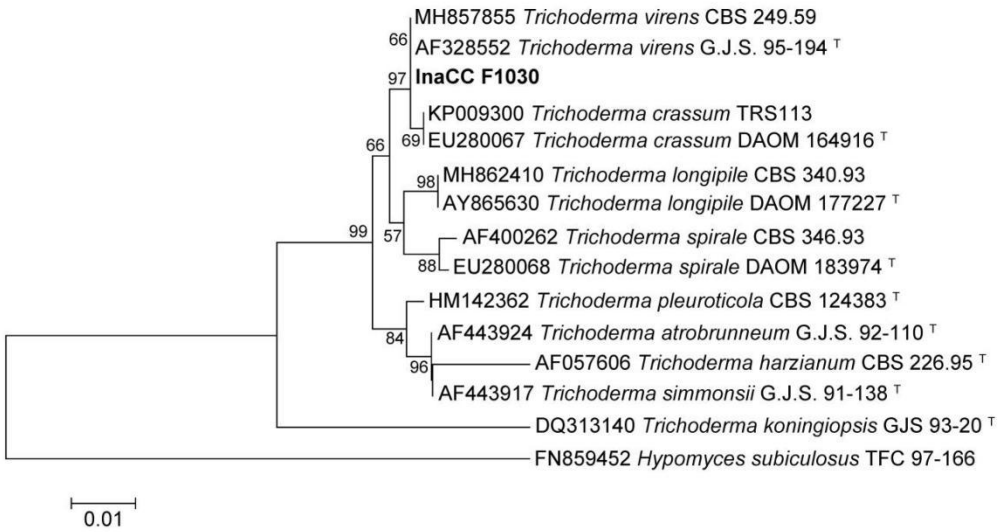

Figure 1. Neighbour-Joining (NJ) tree based on *Trichoderma* spp. ITS sequences. Bootstrap values>50% are indicated at the branch's node (1,000 replicates).

The in vitro antagonistic activities of *Trichoderma virens* InaCC F1030 against Foc were studied in direct confrontation to evaluate the direct contact between antagonist and pathogen. Every day, the growth profiles of *T. virens* in the presence of Foc were monitored and analysed using ImageJ software (Figure 2A) for 7 consecutive days (Figure 2B). Observations after 5 days, the antagonists averagely overgrew 48.2% of medium surface. According to Bell's classification, *T. virens* InaCC F1030 is belonged to grade 4, namely it occupies 25 – 50% of the PDA medium surface. The interpretation of this grade antagonism through dual culture test was also proposed by Bell (1982): a species of *Trichoderma* was regarded as antagonistic to the pathogens if the mean grade score for a given comparison was ≤2, but not highly antagonistic if the number was ≥3. Hence, in this direct confrontation assay, our isolate was considerably a weak antagonist against Foc.

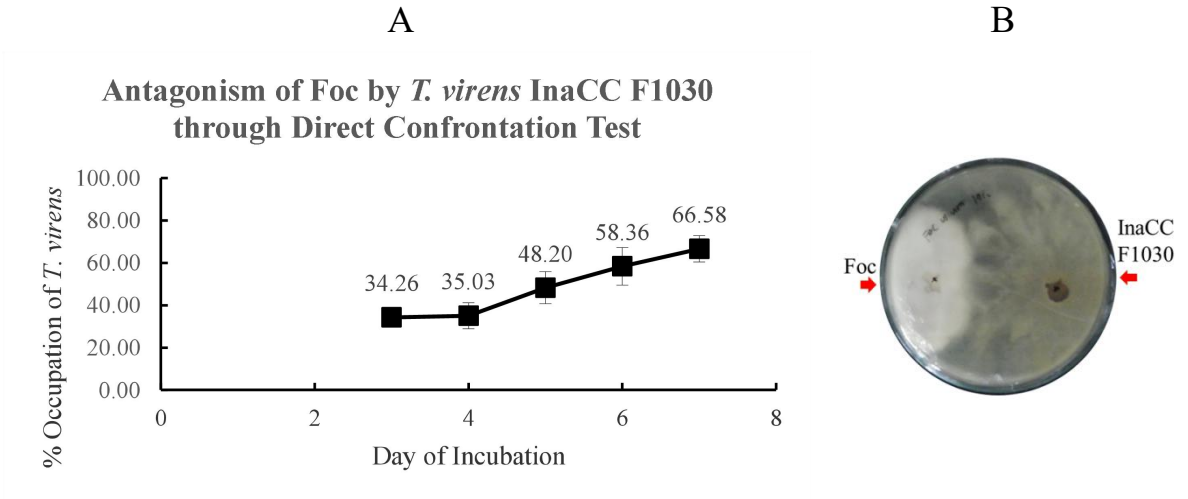

Figure 2. The direct confrontation profile of *Trichoderma virens* InaCC F1030 against Foc in PDA (A) and the appearance of confrontation between *T. virens* InaCC F1030 vs Foc after 7 days (B). The percentage of occupation in PDA medium was analysed daily using imageJ software. The experiment was conducted with three repetitions

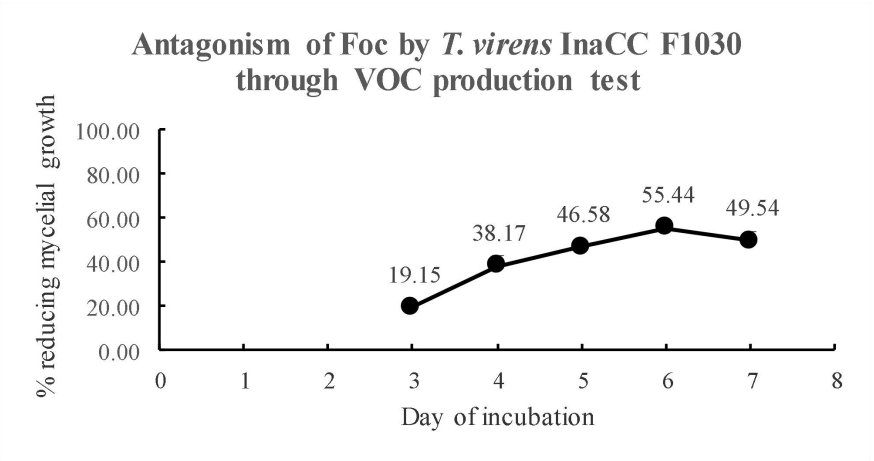

Figure 3. The percentage of reducing mycelial growth of Foc as affected by Volatile Organic Compounds of *Trichoderma virens* InaCC F1030. The diameter of Foc mycelial was measured daily for 7 days after inoculation. The experiment was performed in three repetitions.

Trichoderma virens InaCC F1030 tested in this study emitted volatile organic compounds (VOC) showed ability to influence the growth and development of the fungal plant pathogens (Figure 3). The VOCs emitted by *T. virens* InaCC F1030 inhibited the growth of Foc by impeding the area of mycelial surface compared to control (Figure 3). Our calculation showed that the growth mycelial of the Foc was inhibited around 50% after seven days of indirect exposure with *T. virens* InaCC F1030. As comparison, the inhibition of our isolates is higher than other results. An in vitro biocontrol study of *Trichoderma* species against *Fusarium oxysporum* associated chickpea wilt showed that the *Trichoderma virens* strains can inhibit 15 to 30 % of the pathogen mycelial growth (Dubey *et al*. 2007).

Figure 4 showed the production of IAA (A) and dry biomass (B) of *Trichoderma virens* InaCC F1030 in Czapek Dox Broth with and without the presence of the precursor L-tryptophan. Both IAA production ability and the biomass were affected by the presence of the precursor. The IAA formation and biomass were increased by approximately 17% and 120% respectively as the effect of supplementation of 0.1% of L-tryptophan.

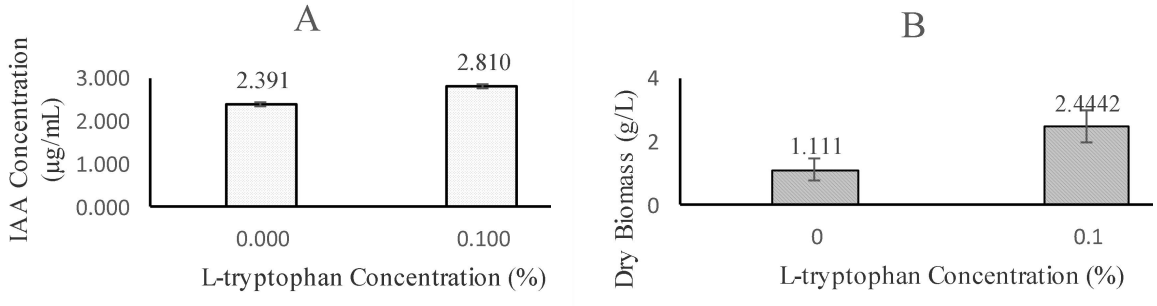

Figure 4. IAA production (A) and biomass (B) of *Trichoderma virens* InaCC F1030 in the condition of without and with supplementation of exogenous L-tryptophan. The condition for submerged axenic fermentation was on Czapex Dox Broth on rotary shaker at 80 r/min for 7 days at room temperature. The experiment was conducted with three repetitions for each concentration.

Discussion

Direct confrontation test modelled the common proposed of mode of actions of *Trichoderma* species against fungal pathogen, namely mycoparasitism (Benítez *et al*. 2004; López-Mondéjar *et al*. 2011). Direct contact between *Trichoderma* as antagonist and pathogen opens the interaction trough the production of cell-wall degrading enzymes (Steyaert *et al*. 2003). Our result showed that *T. virens* InaCC F1030 was not strongly occupied both medium and the Foc. In comparison, a study conducted by Qualhato *et al*. (2013); evaluation of antagonism of four *Trichoderma* species, namely *T. harzianum*, *T. ghanense*, *T. asperellum*, and *T. tomentosum*, showed efficient to highly efficient antagonism against *Fusarium solani* according to Bell's classification. Obviously, the antagonism activity of *Trichoderma* species against Foc via dual culture test shows variability among species. Moreover, for *T. virens* InaCC F1030, a report showed that the antagonism ability was strain-dependent (Taghdi *et al*. 2015).

Volatile organic metabolites produced by microbes including filamentous fungi have been reported to have biocontrol ability against various plant pathogens (Campos *et al*. 2010) as well as plant growth stimulating effects (Hung *et al*. 2013). Our results corroborate previous reports that showed *Trichoderma* species produce VOC that significantly inhibit the mycelial growth of certain plant pathogens (Meena *et al*. 2017). *In vitro* comparative study between *Trichoderma* species demonstrated that the VOC producing ability by *Trichoderma* to inhibit the growth of plant pathogen was species and strain dependent (Moya *et al*. 2018; Napitupulu 2019a).

Although *T. virens* formatted the phytohormone IAA on L-tryptophan free medium, an indication of dependency on L-tryptophan presence was noticed. Similarly, a study of production of IAA by other *Trichoderma* species reported a dependency on the precursor presence and concentration (Saber *et al*. 2017). However, comparison with other plant growth promoting microorganisms, in a condition without L-tryptophan, it seems that our isolate, *T. virens*, produced less amount of IAA. For example, *Streptomyces* isolates after incubation for 5 days in axenic condition of YM broth produced approximately 20 µg/mL of IAA (De Fretes *et al*. 2016). Our result showed that in condition without the precursor, the isolate was only produced 2.391 µg/mL. We also noticed that the presence of L-tryptophan increases the biomass of isolate in this minimum nutrient broth medium. The effect of amino acids did influence not only biomass but also the physiological the fungi, with maximum growth obtained by the addition of L-tryptophan (Gunasekaran & Weber 2010).

Conclusion

We found that our isolate, *Trichoderma virens* InaCC F1030, was considered as antagonistic to the Foc, but not highly antagonistic according to direct confrontation assay. However, it was also revealed that our isolate produces the VOCs that inhibited up to 50% of the mycelial growth of the test pathogen compared without the presence of *T. virens* InaCC F1030. Our isolate was able to produce the IAA in submerged fermentation condition particularly in the presence of the precursor L-tryptophan. Our isolate has potency to be utilized as biocontrol and biofertilizer agent.

Conflict of interest

The authors declared that no possible conflict of interest from this manuscript.

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study.

Author contributions

All authors have reviewed the final version of the manuscript and approved it for publication. TPN, IR, AK, IMS designed the study; TPN and IR performed research and collected the data; TPN, and IR analysed the data; TPN, IR, AK, IMS wrote and reviewed the paper. TPN is the main contributor of this manuscript.

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