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Evaluation of the Environmental Factors Modulating Indole-3-acetic Acid (IAA) Production by *Trichoderma harzianum* InaCC F88

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Abstract. Indole-3-acetic acid (IAA) is one of the most common plant hormones that regulate many aspects of growth and development of plants. *Trichoderma harzianum*, a plant growth promoting fungus, has the ability to produce IAA. However, various environmental factors influence the formation of the hormone. The objective of this study was to optimize the environmental condition for the production of IAA by *Trichoderma harzianum* InaCC F88 strain. The effects of L-tryptophan concentration, temperature, pH, salinity, and incubation time on IAA production and biomass were studied. The *in vitro* evaluations were carried out in the axenic condition of Luria-Bertani Medium. Supplementation of 1% L-tryptophan in the medium gives maximum IAA production. The most favourable initial pH and temperature for IAA production are 6.0 and 27°C, respectively. In the salinity test, the medium containing 1% NaCl yielded maximum IAA formation. After 4 days of incubation, the concentration of IAA reached equilibrium. Hence, optimum IAA production in liquid fermentation could be achieved by manipulating those factors.

1. Introduction

Some filamentous fungi, such as *Trichoderma harzianum* has been widely known as a biocontrol agent for various plant pathogens. This beneficial feature is complemented by its capability to promote plant growth through various characteristics, for example solubilizing insoluble phosphate, production plant hormone, and production of siderophore.

Indole-3-acetic acid (IAA), part of the auxin class, is one of the most common plant hormones that regulates many aspects of growth and development of the plants [1]. IAA has various roles in plants, such as the control of apical dominance [2], shoot elongation [3], root initiation [4], the arrangement of leaves on a plant stem [5], and tropism [6]. Many aspects of IAA, particularly its biosynthesis pathway, has been studied extensively. Plants as well as microbes, including bacteria and fungi, have been widely recognized to produce IAA. The search for biosynthesis pathways of IAA in both plants and microbes has been a subject to be investigated. In planta, two pathways have been commonly proposed, one of which is dependent on the amino acid precursor L-tryptophan and the other is independent of L-tryptophan, although the L-tryptophan-dependent pathway is likely to be dominant [7]. Microbes, including bacteria and fungi, have a similar pattern of the biosynthesis pathway [8] [9].

The production of IAA by microbes including fungi has been utilized to increase the growth of plants. Indonesian culture collections (InaCC) has many valuable resources of microbes particularly fungi. These fungi have the potential to be utilized for many purposes to control plant disease as well as

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promote plant growth. However, in the field application, many environmental physicochemical obstacles limit the optimization activity of the fungi. Due to the complexity variables in real condition, the *in vitro* evaluation approach is needed to simulate the field condition in order to study the influence of environmental stressors toward production of IAA. Therefore, the objective of this study was to evaluate the environmental factors modulating IAA production by *Trichoderma harzianum* InaCC F88 in axenic culture. Numerous environmental factors, including pH, salinity, temperature, as well as the presence of amino acid precursor L-tryptophan were *in vitro* evaluated.

2. Materials and Methods

2.1. Microorganisms

In the preliminary study, ten strains of *Trichoderma harzianum* of Indonesian Culture Collection (InaCC) were screened for the ability to produce IAA *in vitro* in Luria-Bertani (LB) broth supplemented with 0.1 % L-tryptophan (the data were not shown). One isolate that produces most IAA, *T. harzianum* InaCC F88, was chosen. This strain was used in this investigation. All fungi, including the chosen strain, were maintained on plates containing Potato Dextrose Agar (PDA) at 30ºC prior to use in the experimental procedures.

2.2. Fermentation condition for optimization of environmental factors modulating IAA production

2.2.1. Evaluation of L- tryptophan concentration on IAA production. The fermentation condition was done on 30 mL LB broth medium (pH 6.8) in 100-mL conical flasks. Different concentrations of L-tryptophan namely 0, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 % were selected for the production of IAA. The cultures were incubated at 30 ± 1°C for 7 days on a rotary shaker at 80 r/min. Uninoculated control was also maintained. The experiment was conducted with three repetitions for each concentration.

2.2.2. Evaluation of pH on IAA production. The fermentation condition was done on 30 mL LB broth medium in 100-mL conical flasks supplemented with L- tryptophan giving concentration of 0.75%. Different pH namely 5.0, 6.0, 7.0, and 8.0 were adjusted to the broth. The cultures were incubated at 30 ± 1°C for 7 days on a rotary shaker at 80 r/min. Uninoculated control was also maintained. The experiment was conducted with three repetitions for each concentration.

2.2.3. Evaluation of temperature on IAA production. The fermentation condition was done on 30 mL LB broth medium (pH 6.8) in 100-mL conical flasks supplemented with L-tryptophan giving concentration of 0.75%. Different temperature namely 27ºC, 30ºC, 35ºC, and 40^{oC} were selected to incubate the broth. The cultures were shaken on a rotary shaker at 80 r/min. Uninoculated control was also maintained. The experiment was conducted with three repetitions for each concentration.

2.2.4. Evaluation of salinity on IAA production. The fermentation condition was done on 30 mL LB broth medium (pH 6.8) in 100-mL conical flasks with different concentration of NaCl namely 0, 1.0, 2.0, and 3.0 %. The pH of each broth was adjusted to 6.8. The cultures were incubated at 30 ± 1°C for 7 days on a rotary shaker at 80 r/min. Uninoculated control was also maintained. The experiment was conducted with three repetitions for each concentration.

2.2.5. Evaluation of incubation time on IAA production. The fermentation condition was done on 30 mL LB broth medium (pH 6.8) in 100-mL conical flasks. supplemented with L- tryptophan giving concentration of 0.75%. The cultures were incubated at 30 ± 1°C on a rotary shaker at 80 r/min. Sampling to measure the concentration of IAA was conducted every 24 hours for 7 days. Uninoculated control was also maintained. The experiment was conducted with three repetitions for each concentration.

2.3. Quantitative determination of IAA concentration
IAA concentration in the uninoculated control and culture was estimated by spectrophotometric method [10] with some modifications. An aliquot of 1 mL supernatant was mixed with 2 mL of Salkowski reagent (prepared by blended 1 mL $FeCl_3$ 0.5 M and 49 mL $HClO_4$ 35%). The mixture was vigorously shaken for 5 seconds and allowed to stand at room temperature for 30 minutes. The pink colour developed, indicating IAA production, was measured at 530 nm with a spectrophotometer UV-Vis (JK-VS-721N, JKI, China). Concentration of IAA was calculated using a standard curve prepared with standard IAA after correction with the absorbance of uninoculated control at 530 nm.

2.4. Determination of biomass of filamentous fungi
The biomass of fungi was determined by collected the mycelial from liquid broth. The mycelial was put on paper and dried at 45ºC for 12 hours. The dried mycelial was then separated from the paper and carefully weight.

3. Results and Discussion

3.1. Effect of L-Tryptophan concentration on IAA production and biomass of Trichoderma harzianum InaCC F88
Different concentrations of L- tryptophan between 0.0 to 2.0% were selected for the production of IAA. The spectrophotometric analysis showed that gradual increase in the IAA production with respective of L-tryptophan concentration. The production of IAA in LB medium without L-tryptophan is lower than in the medium supplemented with the precursor. The maximum IAA production was observed as 9.22 µg/ml when 1.0% L-tryptophan concentration was amended in the medium (Figure 1A). However, it is also showed that the presence of the precursor with concentration over 1% decreased the production of IAA. The biomass profile of *Trichoderma harzianum* in various L-tryptophan concentration is quite similar, in the range of 69.7 mg until 82.8 mg in such fermentation condition (Figure 1B). This showed that the production of IAA by *T. harzianum* is L-tryptophan dependent. Many existing reports revealed that some fungi are also showed similar pattern. The presence of 0.1% of L-tryptophan in Czapek-Dox broth increased the production of IAA by *T. harzianum* WKY1 around 5 times higher than the medium without the precursor [11], while in the same precursor concentration, our result showed increasing only 2.5 times higher. In other work, addition of exogenous L-tryptophan was not only increased the production of IAA by *Colletotrichum gloeosporioides* but also indole-3-acetamide (IAM), the intermediate during biosynthesis of IAA [12].

Among microorganisms, including fungi, the optimum of L-tryptophan concentration for the production of IAA is varied. Plant growth promoting Actinobacteria *Streptomyces* sp. isolated from rice rhizosphere showed maximum production of IAA namely 15.96 µg/ml in the presence of only 0.5% L-tryptophan, with concentration over 0.5% tended to decrease the production of IAA, in which this is showed similar pattern with our result [13]. A far higher concentration of IAA (362.53 µg/ml) was obtained in a condition of less amount of exogenous L-tryptophan concentration (0.1%) by white rot fungus *Pleurotus ostreatus* under fermentation condition of Jatropha seedcake in basal salt medium, but addition more L-tryptophan concentration to the medium did not increase the yield of IAA [14].

Although the production of IAA is dependent on L-tryptophan presence and concentration, the presence of L-tryptophan in the medium with concentration over optimum concentration decreases the IAA production. Our result shows that doubling the concentration of the precursor to 2% outcome decreasing the IAA production. At that condition, the IAA concentration was significantly dropped even in the same level of concentration in a condition without the presence of the precursor. Therefore, consideration of optimum concentration of L-tryptophan as the precursor for IAA biosynthesis is needed in order to enhance the production of IAA. For the field application, the presence of the precursor in medium is also need to be considered.

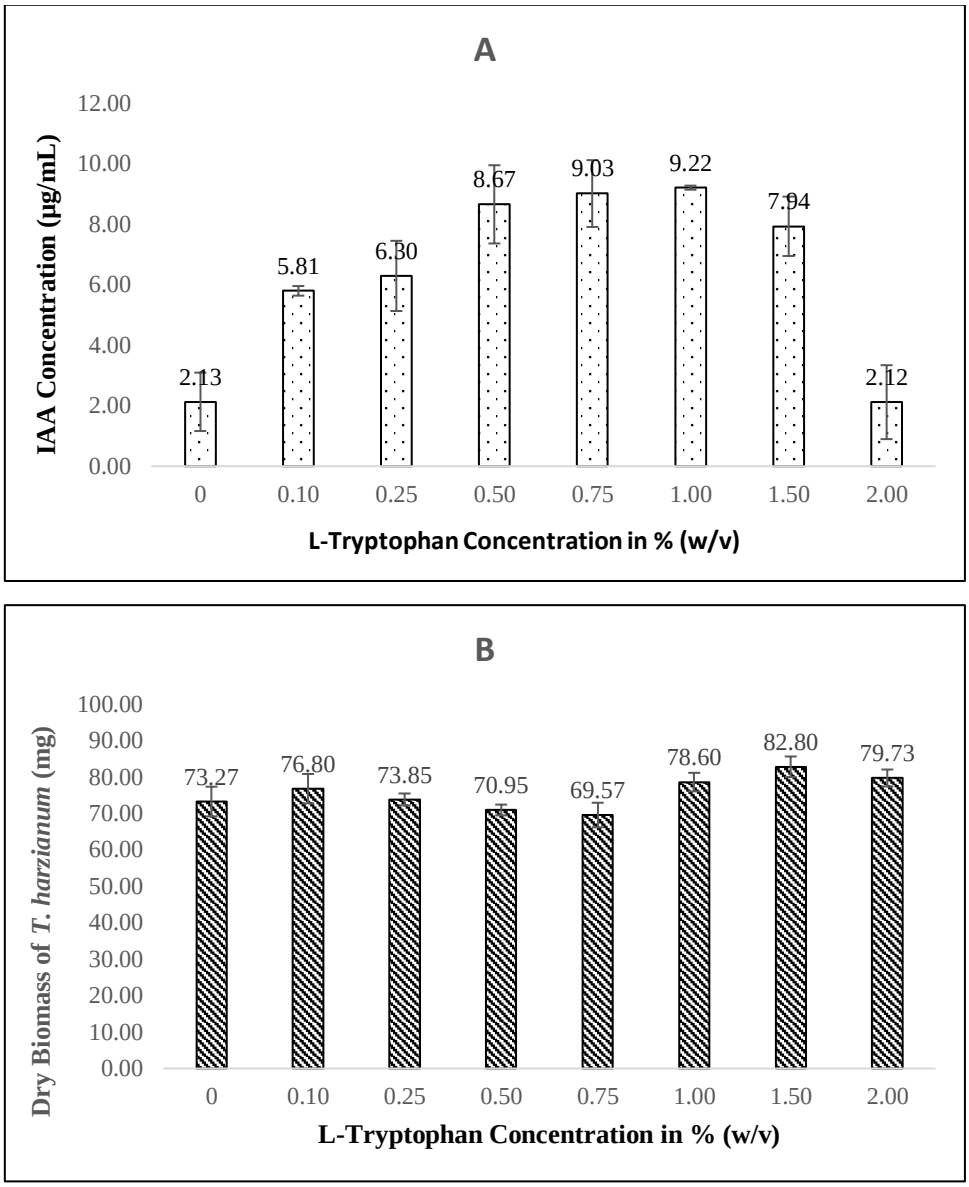

Figure 1. Effect of L-tryptophan concentration on IAA production (A) and biomass (B) of *Trichoderma harzianum* InaCC F88. The fermentation condition was done on LB broth medium (pH 6.8) at temperature of 30 ± 1°C on rotary shaker at 80 r/min for 7 days.

3.2. Effect of pH on IAA production and biomass of Trichoderma harzianum InaCC F88
The production of IAA is influence by pH (Figure 2A). The maximum IAA production of 8.41 µg/ml by *Trichoderma harzianum* InaCC F88 was observed at pH 6, increased from 6.32 µg/ml at pH 5. At more alkaline condition, at pH 7 and 8, the IAA production was reduced to the level of 5.8 µg/ml. The dry biomass profile of *T. harzianum* in fermentation condition with different level acidity (pH 5 to 8) is very similar, in the range 83.43.7 mg until 90.65 mg (Figure 2B).

The level of acidity of soil is one of key factors to ensure the strong and healthy of plants. High acidity as well as high alkalinity of soil condition are able to limit the growth of both plants and microbes. Various of metabolic and physiological process of plants and microbes are also affected by the extreme level of acidity in soil. Although fungi have developed multiple mechanisms to adopt pH variations, very small group of fungi and yeasts such as *Penicillium* sp. and *Acontium* sp. have been reported to tolerate of extreme acidity up to pH of 1.0 [15]. However, in the alkaline range, hardly any

fungi are available to grow above pH 9.0 [16]. Therefore, the pH range for the experimentation of the effect pH toward IAA formation is designed in around of neutral pH, between 5 to 8.

The influence of level of acidity of medium in connection with IAA formation by microorganisms has been widely investigated. Most microorganisms optimally synthesized IAA in the range of neutral pH. *Aspergillus niger* produced IAA along with gibberellic acid at pH optimum of 6 and 5, respectively [17]. In the majority of yeast species, growth and IAA production are optimal in neutral and slightly acidic environment, but *Candida* sp. was reported to produce higher amount of IAA at more acidic condition, at pH 4 [18]. Similarly, the strains of Rhizobacteria *Klebsiella pneumoniae* were reported to yield IAA in a wide range of pH (6 - 9), but its maximum IAA production at pH 8 [19].

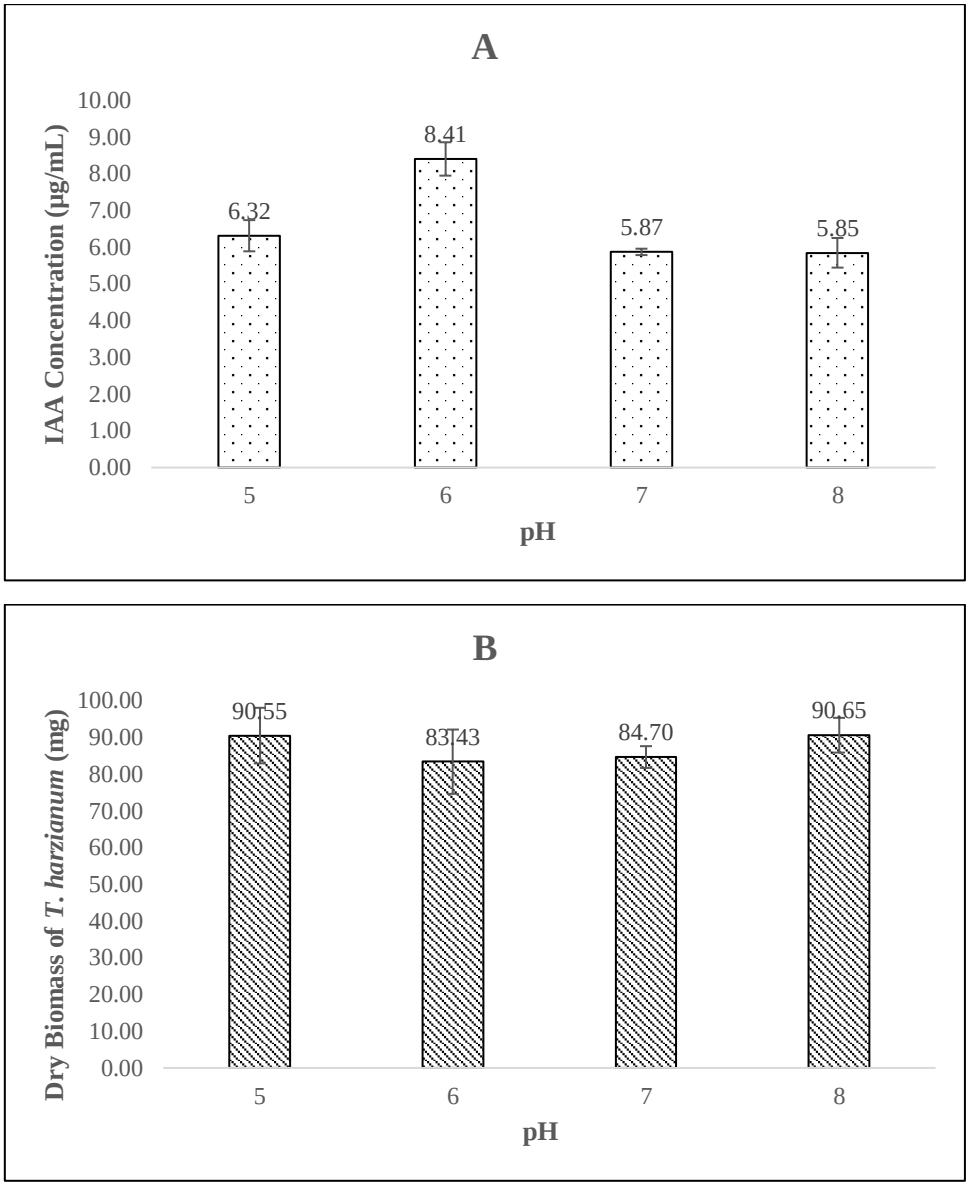

Figure 2. Effect of pH on IAA production (A) and biomass (B) of *Trichoderma harzianum* InaCC F88. The fermentation condition was done on LB broth medium supplemented with 0.75% L-tryptophan at temperature of 30 ± 1°C on rotary shaker at 80 r/min for 7 days.

Various external factors such as pH limit the physiological behaviour of the microorganisms by influencing the enzymes involved in the biosynthesis of IAA. Environmental pH could ionize the amino acids, the building blocks of the enzymes, that lead to the possibility of conformation alteration, affecting the enzyme activity. Moreover, accumulation of hydrogen ion as well as the presence of cationic metal may not only affect the shape of the enzymes involved in the complexity of metabolic pathway of IAA but they may also alter the shape or charge properties of the substrate so that either the substrate is unable to bind to the active site or it cannot undergo catalysis reaction. Hence, in general, enzymes have pH optimum, but the optimum value is varied between enzymes.

3.3. Effect of temperature on IAA production and biomass of Trichoderma harzianum InaCC F88

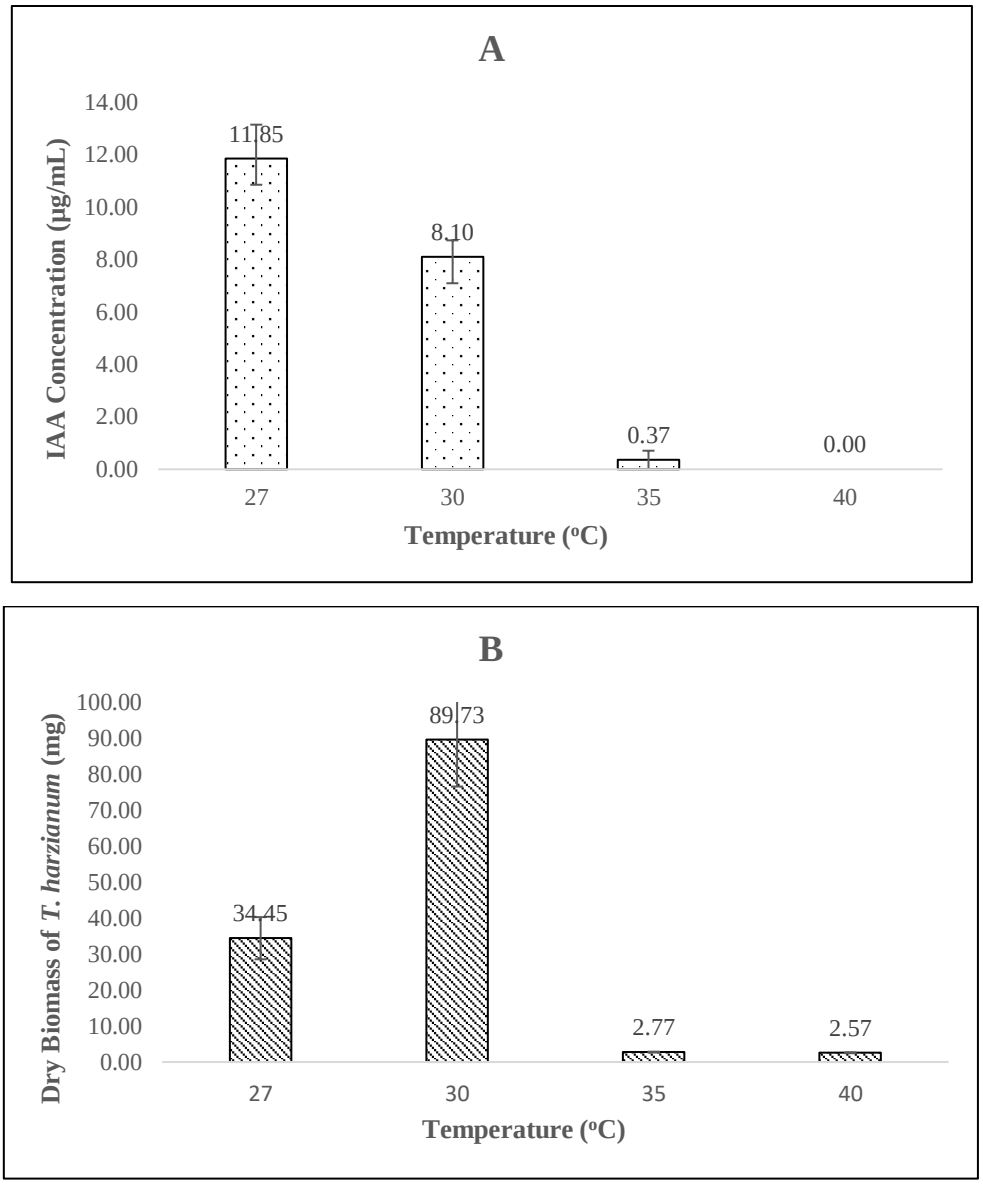

Figure 3. Effect of temperature on IAA production (A) and biomass (B) of *Trichoderma harzianum* InaCC F88. The fermentation condition was done on LB broth medium (pH 6.8) supplemented with 0.75% L-tryptophan on rotary shaker at 80 r/min for 7 days.

Figure 3A shows the effect of different temperature of fermentation condition toward the production of IAA. The highest IAA production, 11.85 µg/ml, was recorded at 27^{0}C then decreasing to 8.10

µg/ml at higher incubation temperature namely 30^0C. Increasing temperature to 35^0C was extremely knocked down the concentration of IAA to the level of 0.37 µg/ml while higher temperature of 40^0C was totally knocked out the production of IAA. Quite different, the biomass of *Trichoderma harzianum* at temperature of 27^0C was lower (34.45 mg) than higher temperature of 30^0C (89.73 mg), while almost no growth of fungus was observed in temperature of 35^0C and 40^0C (Figure 3B).

The temperature affects the growth of *Trichoderma harzianum* and its ability to produce IAA. According to some studies, the optimum temperature for the growth of *T. harzianum* is in the range of 25-30°C [20][21]. The sensitivity of *T. harzianum* toward temperature is clearly shown in our result. Increasing only 5^0C above 30^0C, the growth of the fungus in fermentation broth was stalled. Similar result showed that the growth of *Trichoderma* sp. on potato dextrose agar (PDA) at temperatures of 33^0C, 35^0C, and 37^0C decreased respectively by 1.5 times, 4.6 times and 10.5 times compared to considerably optimum temperature of 30^0C [22].

We found that production of IAA by *Trichoderma harzianum* at incubation temperature of 27^0C is higher 46% compared to 30^0C, even though the biomass was lesser approximately 3 times. As comparison, investigation on some sclerodermatoid fungi namely *Astraeus odoratus*, *Phlebopus portentosus*, *Pisolithus albus* and *Scleroderma sinnamariense* showed that optimum temperature for production of IAA is in the range of 20 - 35^0C in which 30^0C is the best temperature [23]. Moreover, the optimum temperature for IAA production and biomass formation by Actinomycetes *Streptomyces atrovirens* was observed at 30^0C [24]. Dissimilar, rhizobacteria *Bacillus licheniformis* showed better production of IAA at temperature of 35^0C, even still survive and have ability to produce IAA at temperature of 45^0C [25] while *T. harzianum* and other fungi are unable to grow at such extreme temperature.

Investigation on the influence of temperature on the formation of IAA in microorganisms are mostly focus on the search of the optimal temperature. It is obvious that IAA production has correlation with the growth ability of microorganism in certain temperature. An investigation on a Gall-inducing Fungus, *Ustilago esculenta*, revealed that IAA formation was significantly diminish when the fungus was grown in the condition of fluctuated temperatures, pointing out that a constant temperature has a profound effect on IAA formation [26]. Deeper explorations related to biosynthesis of IAA in world of fungi including enzyme studies is urgently needed to reveal more information about the influence of temperature on the IAA production.

3.4. Effect of salinity on IAA production and biomass of Trichoderma harzianum InaCC F86

Difference concentration of sodium chloride modify IAA formation by *Trichoderma harzianum* in Luria-Bertani (LB) fermentation broth (Figure 4A). The fungus was subjected to various salt concentrations namely 0%, 1%, 2%, and 3% to check its tolerance. The supplementation of 1% NaCl in the medium yielded maximum IAA namely 8.77 µg/mL, while addition 1% of NaCl to the medium decreased IAA formation to 7.41 µg/mL. More addition of 1% of salt in the medium causes even lower IAA production to reach the level of 5.62 µg/mL. The fungus was still produced IAA in medium without salt at 6.84 µg/mL. Figure 4B shows the fungal biomass at various sodium chloride concentration. The increase in sodium chloride concentration up to 2% slightly increased fungal biomass. However, the biomass was dropped about 43% compared at higher salinity condition of 3% NaCl.

Some microorganisms have ability to cope salt stress. A study of fungal diversity in a saline soil in a dryland-agricultural region reported that *Penicillium* was the dominant species [27]. Gram-positive bacteria were abundance in saline habitats, with *Bacillus* and *Micrococcus* were the dominant genera [28]. For Trichoderma, the salinity tolerance ability was species and strain dependent [29]. Moreover, a screening of IAA production by some salt tolerance *Trichoderma* species in Czapek-dox broth supplemented with 0,02 % of L-tryptophan also showed variability within species and strains, with the

result range from 5.5 to 36.4 µg/mL [30]. However, little information is available regarding to the formation of IAA by Trichoderma species under salt stress. According to our result, *T. harzianum* did produce IAA in the presence of 1 % NaCl, but the quantity of IAA production was decreased with the salt stress increased 3% of sodium chloride concentration. It also has been widely observed that the increasing salinity and then the osmotic pressure of medium decreased the biomass of fungi [31].

Salinity is one of the important abiotic stresses that limit plant growth and crop yield. The negative effects of high salinity condition towards plants including dehydration, reduction of metabolic and photosynthetic activity, and dysfunction of membrane [32][33]. Many strategies have been proposed and experimented to cope this obstacle. A recent study reported that *Trichoderma harzianum* effectively promoted wheat growth and enhanced plant tolerance to NaCl stress through the increased of ACC-deaminase activity and IAA production [34].

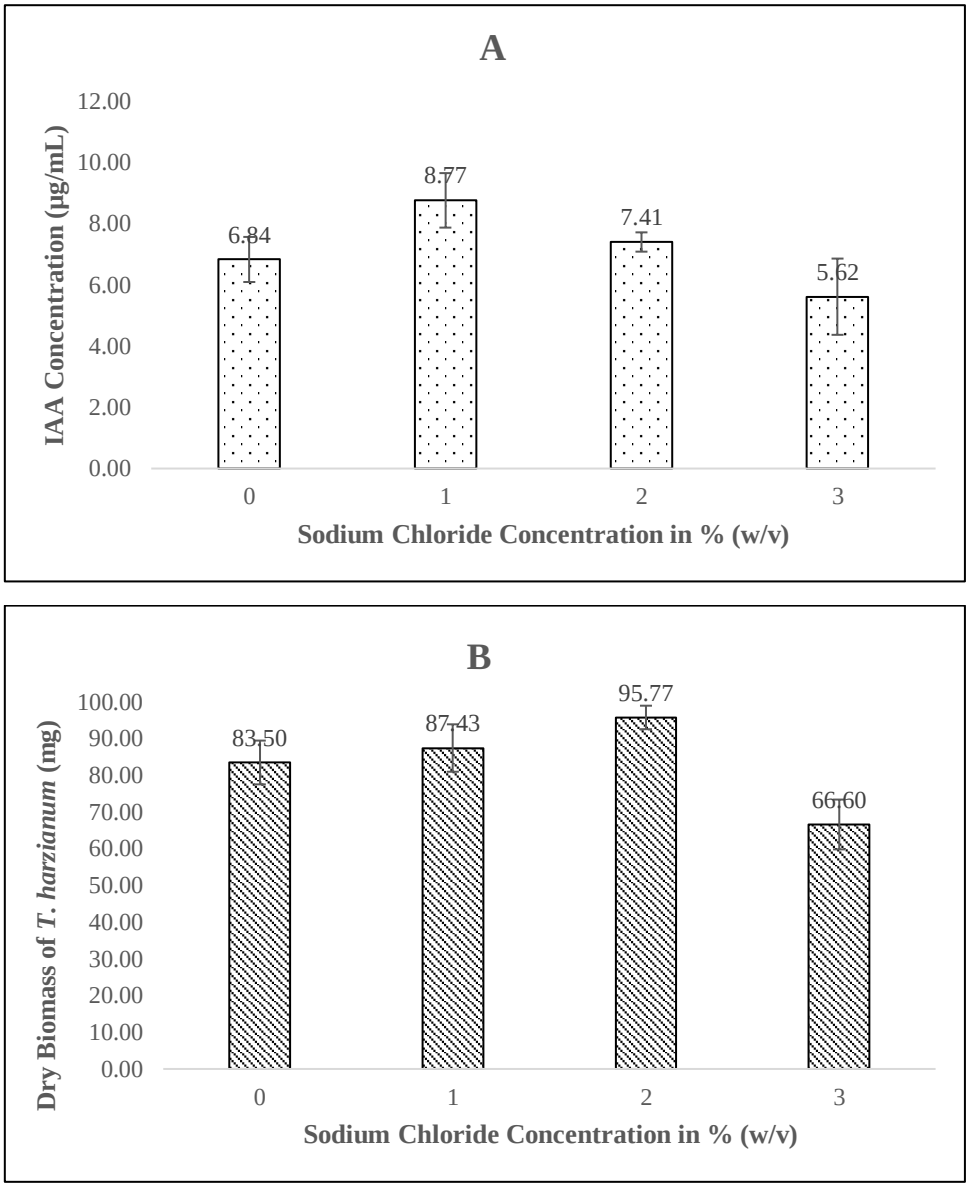

Figure 4. Effect of salinity on IAA production (A) and biomass (B) of *Trichoderma harzianum* InaCC F88. The fermentation condition was done on LB broth medium (pH 6.8) supplemented with 0.75% L-tryptophan at temperature of 30 ± 1°C on rotary shaker at 80 r/min for 7 days.

3.5. Effect of incubation time on IAA production of Trichoderma harzianum InaCC F88

The effect of IAA production was estimated up to 7 days. *Trichoderma* is considerably a fast-growing fungus that presence in wide range of ecosystems and produce a great number of metabolites for versatile purposes. Among genera of *Trichoderma*, the growth profile of *Trichoderma harzianum* is faster than *Trichoderma viride* and *Trichoderma longibrachiatum* in various nutritional sources as well as physical conditions [35]. In correlation with IAA biosynthesis, our result shows that the maximum IAA production was observed in fourth day of incubation (9.34 µg/ml), and after that the concentration of IAA was seemingly stagnant (Figure 5). Other filamentous fungi, *Mortierella* sp., showed longer time of incubation to reach maximum IAA formation up to 9 days when fermented in Czapek–Dox modified [36]. Moreover, observation in axenic culture of *Tricholoma vaccinum*, a spruce ectomycorrhizal fungus, showed that the IAA production is still increased up to 28 days [37]. However, maximum IAA production of plant growth promoting rhizobacteria *Arthrobacter agilis* was obtained in the stationary growth phase at 24 hours [38], a significantly shorter time than *Trichoderma*. Along with other beneficial physiological characters, *T. harzianum* is a good candidate for the inexpensive production of IAA in such short period. Further investigation regarding scale up of IAA production is commenced.

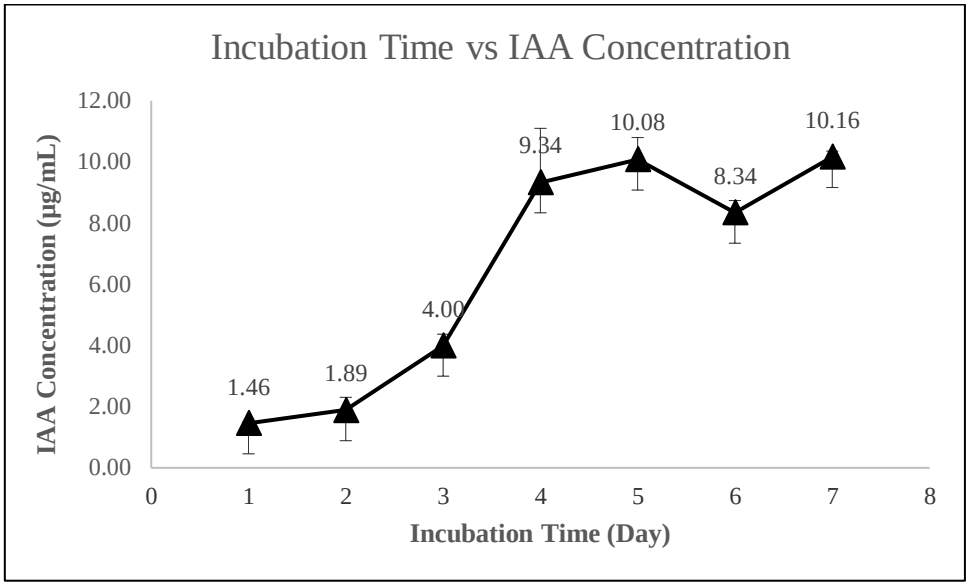

Figure 5. Effect of incubation time on IAA production of *Trichoderma harzianum* InaCC F88. The fermentation condition was done on LB broth medium (pH 6.8) supplemented with 0.75% L-tryptophan at temperature of 30 ± 1°C on rotary shaker at 80 r/min.

4. Conclusions

Environmental psychochemical variables influence the biosynthesis of metabolites such as indole acetic acid (IAA) by *Trichoderma harzianum* InaCC F86 strain in axenic culture. The IAA production is affected by the presence of L-tryptophan and its concentration, level of acidity, temperature, salinity, and incubation time. Hence optimum IAA production in liquid fermentation could be achieved by manipulating those factors. This work can also be extended to give initial information regarding the action of *T. harzianum* as IAA producer in field condition.

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