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ASSESSMENT OF PHOSPHATE SOLUBILIZATION AND INDOLE ACETIC ACID PRODUCTION IN PLANT GROWTH PROMOTING BACTERIA ISOLATED FROM GREEN HOUSE SOILS OF GONJU-GUN, SOUTH KOREA.

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ABSTRACT

Phosphate solubilizing and indole acetic acid (IAA) producing bacteria are considered to be beneficial microorganisms as they have a profound effect on plant growth. A total of 35 phosphate solubilizing bacterial strains were isolated and screened for the production of IAA. Two best performing *Enterobacter* species were selected and employed in elucidating their phosphate solubilizing potential and IAA production under different conditions. Both strains were found to increase available phosphorus content in the medium profusely (640 µg/ml in *E. ludwigii* and 621 µg/ml in *E. hormaechei*). They recorded the maximum IAA production (240 and 332 µg/ml respectively in *E. ludwigii* and *E. hormaechei*), in NB medium supplemented with tryptophan (0.8% and 0.6% respectively in *E. ludwigii* and *E. hormaechei*) at 30°C. It is evident from results that both strains possess great potential to be developed as bio-fertilizers which could enhance soil fertility and plant growth through phosphate solubilization and IAA production.

Key words**:** *E*nterobacter *hormaechei, Enterobacter ludwigii,* IAA production, phosphate solubilization

INTRODUCTION

It is well known that a considerable number of bacterial species, which can be found in the plant rhizosphere, at root surfaces and in association with roots are able to improve the quality of plant growth directly and or indirectly. They are called as "plant growth promoting rhizobacteria" (PGPR).

Phosphorus is a key nutrient required for plant growth and it is applied as chemical fertilizers. Though, a large portion of the applied soluble forms of P is initially available for plant uptake, they rapidly react with the soil and becomes progressively less available (Mundra *et al.,* 2011), due to immobilization into insoluble forms particularly, $CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$. PGPR possess the ability to promote plant growth under different conditions through dissolving insoluble P enabling it available for plant uptake. They are called as phosphate-solubilizing microorganisms (PSMs).

The phytohormones produce by PGPR may exert pronounced effects on plant growth and establishment (Spaepen *et al.,* 2007). Indole-3 -acetic acid (IAA), the most abundant member of the auxins family of phytohormones has many roles in important physiological processes including root initiation, cell enlargement and division, tissue differentiation, and responses to light and gravity (Teale *et al.,* 2006). Plant growth-promoting rhizobacteria predominantly synthesize IAA as secondary metabolites through tryptophan dependent pathway (Ahmad *et al*., 2005; Mandal *et al*., 2007).

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Phosphate solubilization and IAA production by microbial isolates vary greatly among different species and strains and depend on the availability of substrate(s). As revealed by the previous studies, *Enterobacter* spp. is considered being efficient phosphate solubilizers as well as efficient IAA producers (Rosangela, 2012; Deepa *et al.,* 2010; Mirza *et al.* 2001). In the present study, *Enterobacter* species having the capacity to solubilize insoluble phosphates were isolated from green house soils in South Korea and their potential for IAA production under different culture conditions were investigated.

MATERIALS AND METHOD

Isolation of phosphate solubilizing bacterial strains

Phosphate solubilizing bacterial (PSB) strains were isolated from tomato growing rhizosphere soil samples collected from green houses at Chungchugnam-do province, Gongju-Gun area in South Korea. Serially diluted aliquots of soil samples were inoculated on NBRIP medium (National Botanical Research Institute Phosphate medium) containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999). The petri plates were incubated for 7 days at 30^0C and examined for colonies developing clear zone. Predominant colonies with conspicuous clear zones around them were further purified by re-streaking on the fresh NBRIP agar plates at 30^oC. Thirty five bacterial strains that exhibited relatively large clear zones on the agar plates were selected as phosphate solubilizing organisms and their pure cultures were maintained in a glycerol suspension (30% v/v) at -80^oC until use.

Screening of isolated phosphate solubilizing bacterial isolates for IAA production

Nutrient agar plates containing 0.1% tryptophan were inoculated individually with isolates in aseptic conditions for screening of isolated strains for IAA production. Each inoculated plate was overlaid with an 82 mm diameter filter paper immediately after inoculation, and incubated until formation of colonies with 0.5 to 2 mm diameter. After removing the filter papers, the plates were treated with Salkowski's reagent (2% 0.5M $FeCl_3$ in 35% perchloric acid) and kept at room temperature (30°C) until the development of color. IAA producing bacteria were identified by the formation of a characteristic pink to red halo surrounding the colony. Diameter of each halo was recorded after 30 min. The strains distinguished with large pink color halo on agar plates were selected the further studies. Out of 35 phosphate solubilizing isolates, three strains with distinguished performance (highest IAA production and phosphate solubilization) were selected, for further studies

Identification of the selected bacterial strain

The partial sequencing of 16S rRNA for the selected bacterial strains was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'-GGTTACCTTGTTACGACTT -3'). The online program BLAST was used in identifying the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using CLUSTAL X program (Thompson *et al*., 1997), which involved sequence alignment by neighbor joining method (Saitou and Nei, 1987) and maximum parsimony using the MEGA4 program (Kumar *et al*., 2001). Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Assay of inorganic phosphate solubilization

The inorganic phosphate solubilization was assayed using liquid NBRIP medium for 7 days. A sample (10 ml) of each cultured and control were taken each day after the inoculation and centrifuged at 8000 rpm for 15 min. The clear supernatant was used in determining the pH and amount of phosphorous released into the medium.

Optimization of IAA production

IAA production of the strains was assayed under different incubation period, media, temperature, and different tryptophan concentration in the medium to optimize their potential for IAA production. The effect of incubation period on IAA production was tested by growing the strain on culture medium (NB-Nutrient Broth medium with 0.2% tryptophan) for 60 hrs. The optimum tryptophan concentration for the maximum IAA production was assayed by adding different tryptophan concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1%) to culture medium. The effect of temperature on the IAA production was determined by incubating culture medium (NB medium with 0.2% tryptophan) at different temperatures ranging from 25-40^{o}C.

In all cases, a sterilized uninoculated medium was served as the control. Sample of cultured and control were taken into centrifugation tube and centrifuged 10 min at 10000 rpm. The clear supernatant was used to determine IAA production as described by Gutierrez *et al.* (2009). For this, 1 ml of clear supernatant of was mixed with 4 ml of the Salkowski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.05 M $FeCl_3$ solution). The mixture was incubated in the dark at 37^{0}C for 30 minutes. Development of pink color indicated the IAA production and optical density was taken at 535 nm using UV spectrophotometer. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA obtained in the range of 10-100 µg/ml.

Statistical analysis

Values were given as means ± SD for triplicate samples. The data were subjected to analysis of variance (ANOVA) using SAS package (SAS, 1999). The Duncan's Multiple Range Test (DMRT) was employed to test the

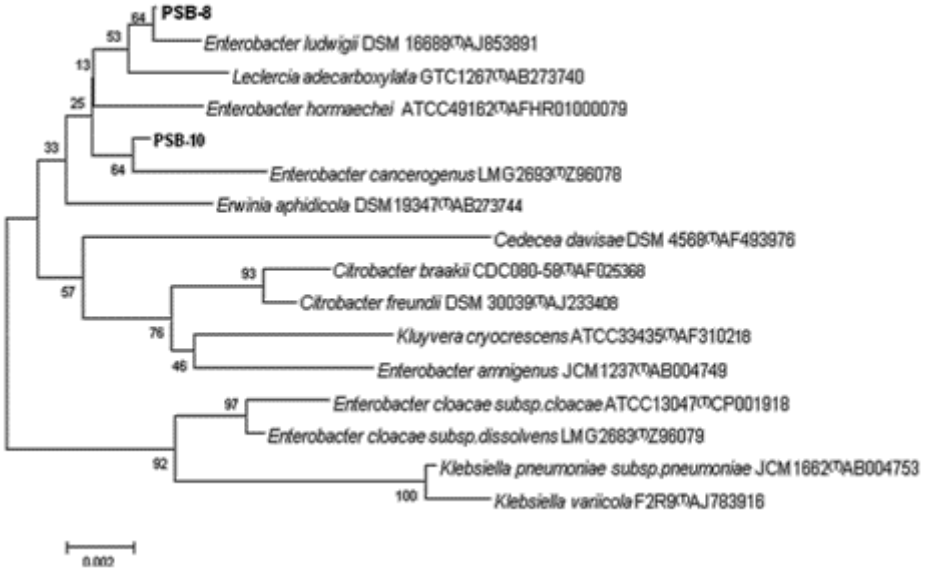

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Enterobacter ludwigii* and *Enterobacter hormaechei* strains with respect to related species. The scale bar indicates 0.002 substitutions per nucleotide position.

significance of treatment means at $P \leq 0.05$.

RESULTS

Isolation of phosphate solubilizing bacterial strains and screening for IAA production

Thirty five bacterial strains (PSB 1 to PSB 35) which exhibited clear zones on the NBRIP agar plates were selected as phosphate solubilizing organisms and they were screened for IAA production. All the tested phosphate solubilizing bacterial isolates showed positive response for IAA production with different concentrations as identified by characteristic red-pink halo. Out of 35 strains, 3 strains showed high production ability, 20 strains showed moderate while the rest showed low production ability of IAA.

Identification of the bacterial strain

According to 16S rRNA sequence analysis, the three strains which indicated highest IAA production showed close proximity with *Pantoea rodasii* LGM 26273, *Enterobacter ludwigii* DSM and 16688 *Enterobacter hormaechei* ATCC 49162. Out of three strains, the two *Enterobacter species* were selected for further study. Phylogenetic tree (Figure 1) shows the position of two strains with respect to the related species.

Assay of inorganic phosphate solubilization

Figure 2 and 3 represent the results of inorganic phosphate solubilization and the associated pH changes in the NBRIP medium by isolated *Enterobacter* strains during the 7 days incubation period. It was clear that both isolate significantly increased available phosphorus content in the medium and the highest phosphate solubilization (640 µg/ml) from *E. ludwigii* was recorded at day 2 of the incubation, whereas the highest phosphate solubilization (621 µg/ml) from the *E. hormaechei* was recorded at day 3 of incubation. The pH of the medium was reduced to 3.81 by *E. ludwigii* and 3.42 by *E. hormaechei* at the end of the incubation.

Optimization of IAA production

Both strains exhibited more or less similar response to different media as shown in Figure 4. NB medium was the best medium for IAA production (142 µg/ml in *E. ludwigii* and 139 µg/ml in *E. hormaechei*) followed by NBRIP medium (58 µg/ml in *E. ludwigii* and

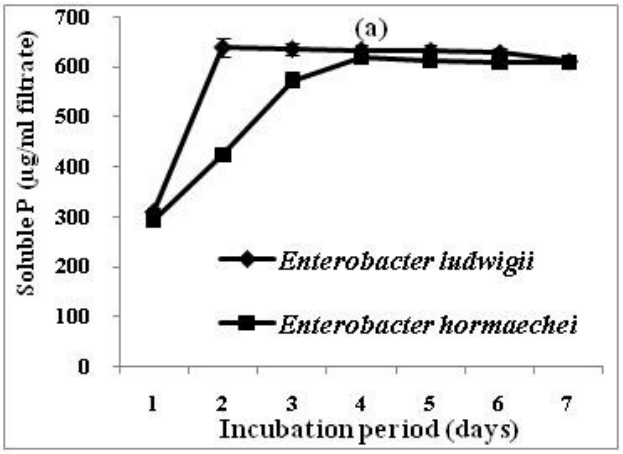

Figure 2. Phosphate solubilization by *Enterobacter ludwigii* and *Enterobacter hormaechei* strains. Values are the means (n = 3) ± standard deviation.

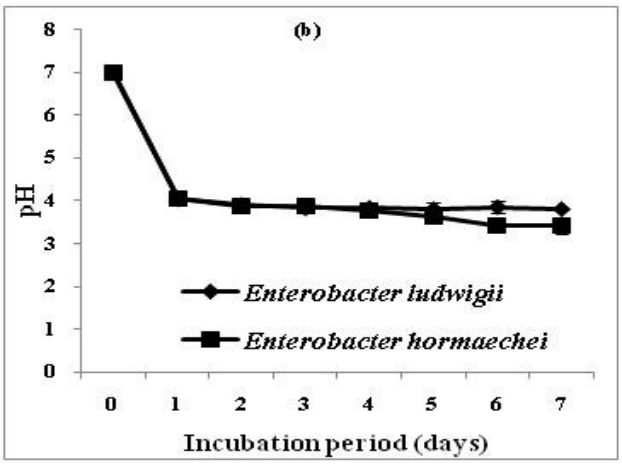

Figure 3. Changes of pH in NBRIP culture medium containing *Enterobacter ludwigii* and *Enterobacter hormaechei* strains. Values are the means ($n = 3$) ± standard deviation.

57 µg/ml in *E. hormaechei*). However the strains exhibited higher growth with other two mediums.

As shown in Figure 5, IAA production increased gradually with increase in incubation period up to 48 hours in *E. ludwigii* (136 µg/ml) and up to 24 hours in *E. hormaechei* (162 µg/ml) and showed a decline afterwards. IAA production reached to the maximum at the stationary phase (after 48 hours in *E. ludwigii* and after 24 hours in *E. hormaechei*) of the growth.

Effect of tryptophan concentration on IAA production was assessed by adding different tryptophan concentrations (ranging from 0.1 to 1%) to the culture medium. As shown in Figure 6, IAA production was dramatically increased with increasing amounts of tryptophan up to 0.8% in *E. ludwigii* (240 µg/ml) and up to 0.6% in *E. hormaechei* (332 µg/ml), thereafter a slight reduction was observed. Both strains showed more or less constant growth pattern with different tryptophan concentrations.

Strains recorded the highest IAA production (133 µg/ml in *E. ludwigii* and 135 µg/ml in *E. hormaechei*) at 30°C followed by 25°C (123 µg/ml in *E. ludwigii* and 57 µg/ml in *E. hormaechei*). The IAA production was significantly decreased when temperature increased from 30 to 35°C (Figure 7).

DISCUSSION

A large number of bacteria which could enhance plant growth have been reported for last few decades. They include species of *Pseudomonas, Azospirillium, Arthrobacteria, Klebsiella, Enterobacter, Alcaligenes, Arthrobacteria, Bacillus and Serratia* (Okon and Labandera-Gonzalez, 1994). Out of them *Enterobacter* species have been identified as efficient phosphate solubilizers who possess the ability to produce IAA also (Rosangela, 2012; Deepa *et al.,* 2010; Mirza *et al.* 2001).

In the present study, isolated thirty five PSB and they were screened for IAA production. Based on the results of phosphate solubilization and IAA production, two *Enterobacter* strains with outstanding performances were selected for further studies. The strains were identified as *Enterobacter ludwigii* DSM and 16688 *Enterobacter hormaechei* ATCC 49162 according to the 16S rRNA sequence analysis. The relevent sequences were deposited in the NCBI Genebank under accession numbers KF836496 (*Enterobacter ludwigii*) and KF836497 (*Enterobacter hormaechei*).

The solubilization of inorganic phosphate occurs as a consequence of the secretion of low molecular weight organic acids by various soil bacteria (Zaidi *et al.,* 2009). Conversely, solu-

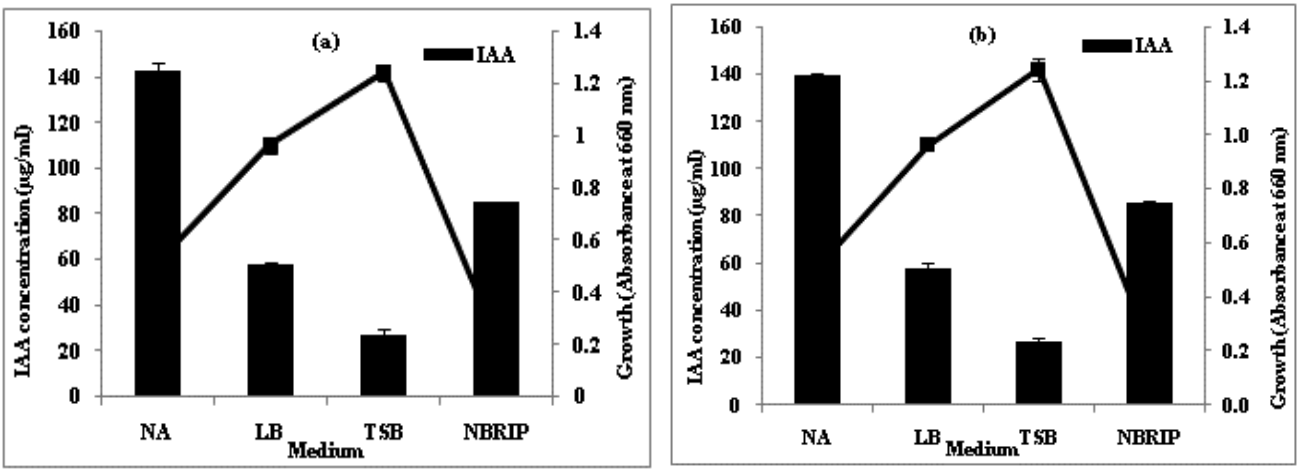

Figure 4. Effect of different medium on growth and IAA production of (a) *Enterobacter ludwigii* (b) *Enterobacter hormaechei*. Values given here are the means (n = 3) ± standard deviation.

bilization of organic phosphates occurs through phosphatases, catalyzing the hydrolysis of phosphoric esters (Glick, 2002; Jakobsen *et al.,* 2005). Therefore decreased pH in culture medium indicates the production of organic acid and phosphatase enzyme, the main mechanism responsible for phosphate solubilization (Chaiharn and Lumyong, 2011).

According to the previous reports, different mediums with different compositions have been tested for IAA production. In this regard, Shahab *et al.* (2009) and Chaiharn and Lumyong (2011) have used NB media and Moghaddam *et al.* (2012) have used LB medium as base medium for IAA production. For the present strain, NB medium was shown to be the best medium for IAA production.

Both *Enterobacter* strains showed growth-associated IAA production in NB medium and maximum production was observed after 48 and 24 hours of incubation respectively for *E. ludwigii* and *E. hormaechei.* Thereafter gradually reduction in growth and IAA production was observed. The increase of IAA production may be attributed to the greater availability of the precursor as reported by Patten and Glick (1996) who also observed increased IAA production up to 96 hours. Whereas the reduction in IAA production might be due to release of IAA degrading enzymes such as IAA oxidase, peroxidase by the bacteria as reported by Hunter (1989). Previous studies by Unyayar *et al.* (2000), Hansan (2002) and Swain *et al.* (2007) also observed the maximum IAA production during stationary phase of the growth.

Supplementation of culture media with tryptophan enhanced IAA production by both Enterobacter species (up to 0.8% and 0.6% respectively in *E. ludwigii* and *E. hormaechei*), which is in line with the findings of Spaepen and Vanderleyden (2011) and Sridevi and Mallaiah (2007). Similar to our results, Khalid *et al.* (2004), Patil *et al.* (2011) and Swain *et al.* (2007) also observed enhancement of IAA production in response to increased L-tryptophan concentration in the medium.

Both strains showed maximum IAA production and growth at 30°C, indicating that growth of bacteria is affected by high temperature as described by Malboodi *et al.* (2009). It is further confirmed by the present results with decreased bacterial growth and IAA production when incubated at high temperature over 30°C. However IAA production at varying temperature may depend on the bacterial species as well. According to Sudha *et al.* (2012), *Rhizobium* and *Bacillus* spp. recorded optimum IAA production at 37°C tem-

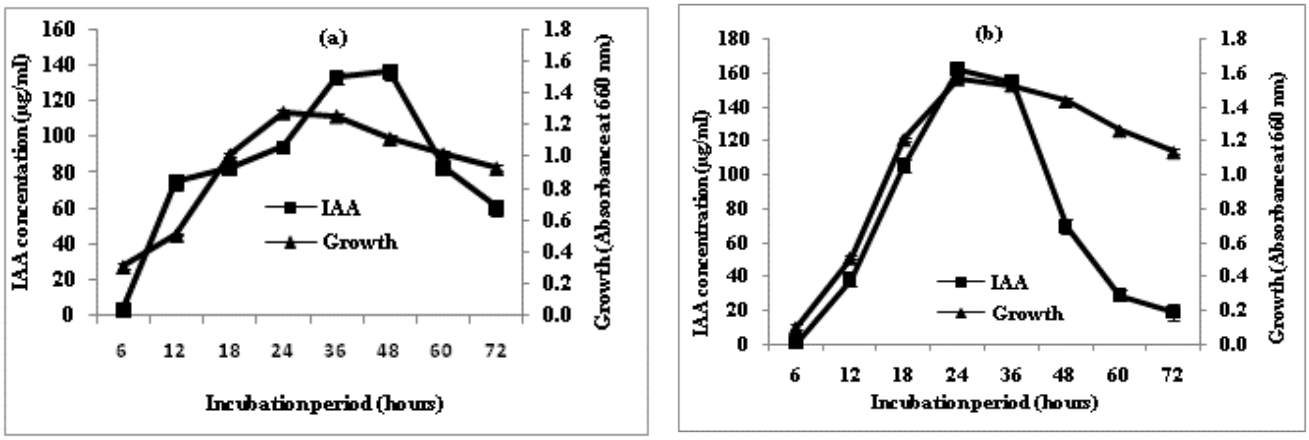

Figure 5. Effect of incubation temperature on growth and IAA production of (a) *Enterobacter ludwigii* (b) *Enterobacter hormaechei*. Values given here are the means (n = 3) ± standard deviation.

perature. Khamna *et al.* (2010) recorded optimum IAA production at 30°C temperature by *Streptomyces* sp. which is parallel to our findings.

CONCLUSION

According to the present findings, both *Enterobacter* strains have a great potential to be used as bio-fertilizer or bio-enhancer to enhance soil fertility and plant growth through phosphate solubilization and IAA production. However, further works under field conditions are required before being used in commercial scale as bio-inoculants.

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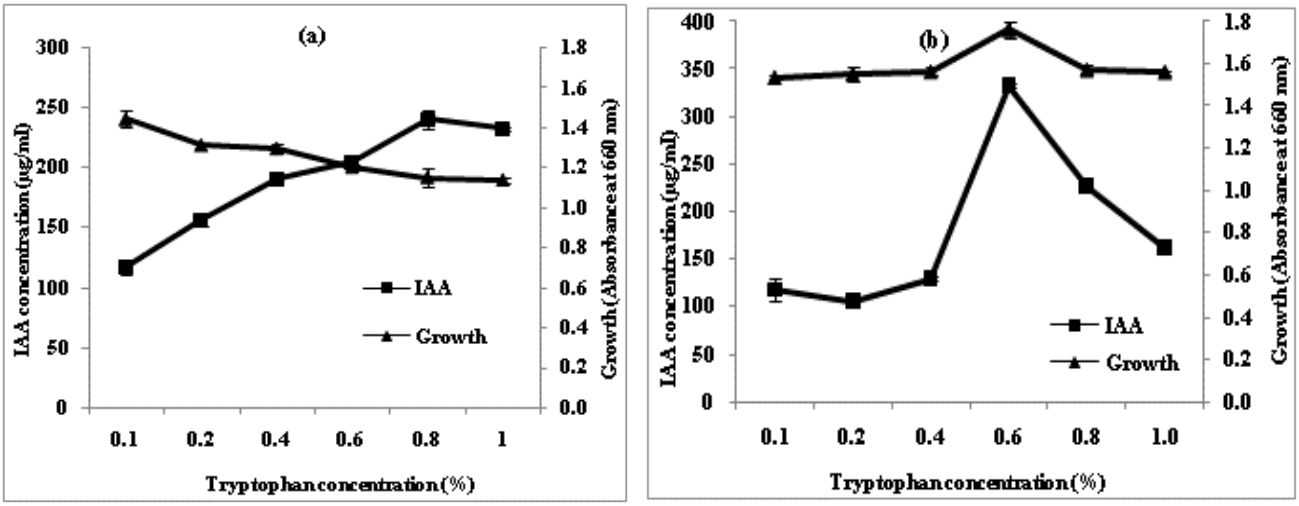

Figure 6. Effect of tryptophan concentration on growth and IAA production of (a) *Enterobacter ludwigii* (b) *Enterobacter hormaechei*. Values given here are the means (n = 3) ± standard deviation.

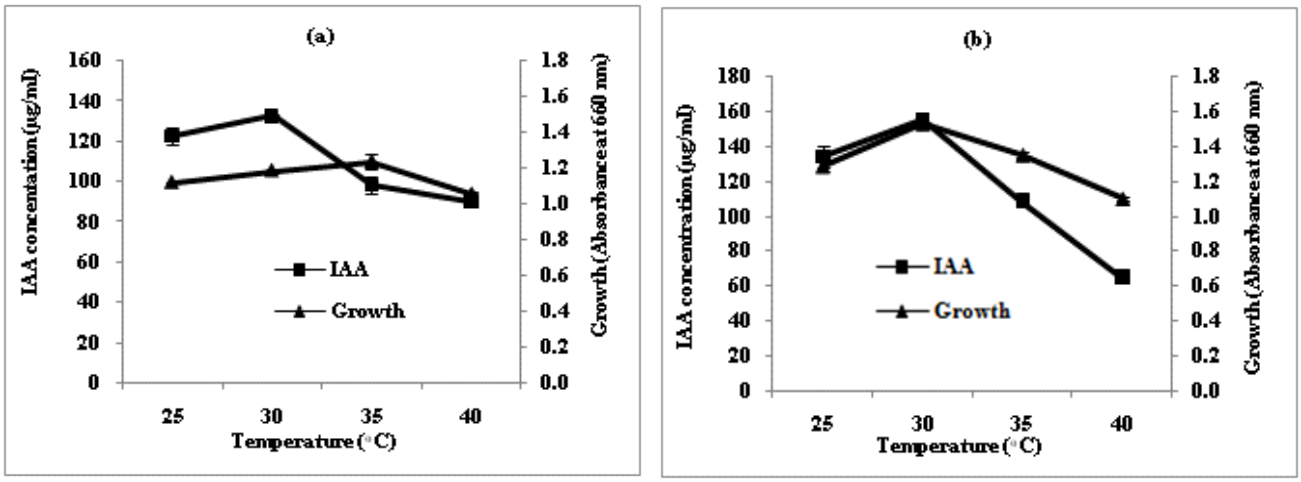

Figure 7. Effect of incubation temperature on growth and IAA production of (a) *Enterobacter ludwigii* (b) *Enterobacter hormaechei*. Values given here are the means (n = 3) ± standard deviation.

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