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Studies on Indole Acetic Acid (IAA) production by *Azotobacter spp.* Isolated from soil samples

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Abstract
Azotobacter is considered as important fertilizing agents that contribute to the nitrogen availability and substitute's chemical fertilizer. Indole acetic acid (IAA) is the most common, naturally occurring, plant hormone of the auxin class. Screening of *Azotobacter* Isolate for IAA production and optimization studies of IAA production by isolate by observing effect of various parameter like temperature, pH, carbon source, nitrogen source, time and L-tryptophan concentration. In the study, the isolate A3, isolate A4 and isolate A7 showed maximum IAA production. Thus the isolate A3, isolate A4 and isolate A7 being beneficial in increasing crop production. Studies on optimization suggest that IAA production maximum at 37°C (temperature) for all 3 isolates at 96 Hrs of incubation time, at pH 2. pH 7 and pH 8 respectively. Mannitol and Sucrose found to be the best carbon source for IAA production and the Ammonium sulphate and urea found to be the best nitrogen source for IAA production. While at 1.5% Tryptophan, maximum IAA production for all 3 isolates.

Keywords: *Azotobacter*, Indole acetic acid (IAA), L-tryptophan

Introduction

Azotobacter is a free- living nitrogen fixing bacterium, these plant growth promoting rhizo bacteria which are the beneficial ones that stimulate plant growth by an array of mechanism (Raval and Desai, 2012) [19] but its distribution is affected by soil characteristics and climate condition (Brown and Burlingham, 1968) [4]. The ability of *Azotobacter* enables to fix N non- symbiotically has been widely studies. The occurrence of this organism has been reported in the rhizosphere of several crops such as rice (*oryza sativa* L.), maize (*zea mays* L.), sugarcane (*Saccharum officinarum* L.), Bajra (*pennisetum glaucum* L.), vegetables, and plantation crops (Mazid and khan, 2015) [8].

The important roles of *Azotobacter* to increase plant growth and productivity as well as reduce chemical fertilizer, and the prospective of *Azotobacter* to minimize the chemical fertilizer rates in food crop production (Reginawanti *et al*., 2020) [18]. Plant hormones regulate or influence a range of cellular and physiological process, such as cell division, cell enlargement, bud dormancy. Flowering fruit ripening, seed dormancy, seed germination and leaf abscission. Indole-3-acetic acid (IAA) is the main member of the auxin family that control many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity (Teale *et al*., 2006) [22]. Tryptophan is believed to be the primary precursor for the formation of IAA in plants and microorganism (Monteiro *et al*., 1988) [15]. Indole-3- acetic acid (IAA) is the common natural auxin that shows all auxin doing action and extensively affects plants physiology (Matre and Barate, 2018) [14].

Indole-3-acetic acid (IAA), a plant hormone compound, was a natural auxin produced by plants, algae, mosses, lichens and a diverse group of organisms. It was a metabolite derived from tryptophan (Trp) by many Trp-dependant and Trp independent pathways in plants and bacteria. Among IAA producing organisms, soil microorganism, especially bacteria which reside in soil rhizosphere or as free living bacteria in soil can also produce IAA (Nghia *et al.*, 2017) [16].

Indole-3-acetic acid (IAA, 3-IAA) is the most common, naturally occurring, plant hormone of the auxin class. IAA is produced in young leaves, stems and seeds from transamination and decarboxylation reactions of tryptophan. Effect of IAA on plants are significant and some of them are apical dominance (apex dominates the lateral meristems), phototropism, gravitropism, prevention of leaf and fruit abscission and induction of adventitious roots. Therefore IAA has profound influence on crops (Gordon and weber, 1951).

Indole acetic acid (IAA) is a naturally occurring auxin produced by PGPR through L-tryptophan metabolism pathway. IAA increases root growth and length, expands the root surface area, facilitates the absorption of soil nutrients by plants (Boiero *et al.*, 2007 [3]; Hariharan *et al.*, 2014 [6]; Padmavathi *et al.*, 2015). Some PGPR strains residing in the rhizosphere are known to produce IAA, Culture media conditions were optimized for maximum IAA production by adjusting various parameters such as the inoculation time, composition of media. L-tryptophan concentration, carbon, nitrogen sources, pH and temperature (Suliasih and Widawati, 2020).

IAA is considered to be the main biologically active plant hormone of the auxin class and is a product of L-tryptophan (Trp) metabolism (Zhao, 2010). IAA is a metabolite derived from Trp by many Trp-dependent and Trp-independent pathways in plants and bacteria (Matsukawa *et al*., 2007) [10].

Material and methods

- **Collection of soil samples**

The 25 soil samples were collected from different places of Akola region. The 10 gm of soil. Samples were collected from 15 cm depth with sterile spatula in a sterile polythene bags which were transported to laboratory and stored at 4°C further use.

- **Isolation of *Azotobacter spp*.**

1gm of soil sample was inoculated into the Nitrogen Free Mannitol broth and incubated at room temperature for enrichment upto 3 days. After 3 days the broth was inoculated on sterilized *Azotobacter* agar medium plates and further incubated for 3 days at room temperature. The isolated colonies were streaked on the Azotobacter agar slants and incubated. The pure cultures were maintained at 4°C till further use.

- **Identification and characterization of *Azotobacter***

Isolates were characterized by using Gram stain and colony characters (shape. Color, margin, nature of colony and texture). Determination of biochemical properties was conducted by using different biochemical tests such as citrate utilization, methyl red. VP, Indole, amylase, oxidase, urease and catalase. To study biochemical characteristics, such as acid and gas production through the fermentation of different sugars *viz*. - Sucrose, Lactose, Glucose, Mannitol and fructose were investigated by the isolates. To check cyst formation amongst isolates, the Burk's medium was prepared and inoculated with an isolates and incubated for 7 days at room temperature. After incubation these isolates were stained by crystal violet and observed under oil immersion objective for cyst formation.

- **Screening of isolates for production of Indole Acetic Acid**

The IAA production test was performed in Yeast Extract Mannitol broth with the addition of L-Tryptophan and this media was inoculated with isolates and then incubated at room temperature for 2 days. Non inoculated broth culture was kept as control. The broth was then centrifuge at 5000 rpm for 15 min and the supernatant was used for further study, the 1 ml of supernatant was then mixed with 2 ml of Salkowski's reagent (1.5ml of 0.5 M FeCl), con. H_2SO_4, 50 ml D.W). The mixture was kept in dark for 45 minutes at room temperature. Red colour developed was recorded and calculated with spectrophotometer at 530 am by using standard curve. IAA is quantitative method.

- **Prepration of standard curve for IAA estimation**

The different concentrations of IAA standards were prepared using standard IAA powder according to the standard protocol 100 µg/ml initial stock concentration of IAA was prepared by adding Img IAA in 10 ml acetone and mixed well. From the stock, series of standards i.e., 10, 20, 30, 40, 50, 60, 70, 80, 90 µg/ml was prepared. Then Iml of each standard, including blank was taken in a test tube and 2ml of Salkowski's reagent was added and optical Density was measured at 530 nm. Standard Curve was prepared by plotting absorbance at 530 nm against concentration of IAA solution.

Determination of effect of various parameters on IAA production

- **Effect of temperature on IAA production.**

Effect of temperature on LAA production was studied by inoculating the culture in the yeast extract mannitol broth with L-tryptophan. Incubating the production media at different temperatures as 10°C, room temperature, 37°C, 45°C for 72 hrs each. After incubation period the media centrifuged at 5000 rpm for 15 min and transferred 1 ml of supernatant with 2 ml of Salkowski's reagent. The IAA production was determined spectrophotometrically at 530 nm as previously described.

- **Effect of time on IAA production.**

The production media supplemented with L-tryptophan was incubated with culture at different incubation time as 24 hrs, 48 hrs 72 hrs and 96 hrs at 37°C, after that specific time period the media centrifuged at 5000 rpm for 15 min. The IAA production was determined spectrophotometrically at 530 nm as previously described.

- **Effect of pH on IAA production.**

The production media supplimented with L-tryptophan was adjusted for pH 2, 4, 7 and 8 by addition of IN HCL and IN NaOH. The sterilized media were then incubated with culture and after incubation production media centrifuged and supernatant mixed with Salkowaski's reagent. IAA produced was determined as per previous described.

- **Effect of L-tryptophan concentration on IAA production.**

The yeast extract mannitol broth was supplemented with different concentration of L tryptophan as 0.25, 0.5, 1.0, 1.5 and 2.0. It was inoculated with culture after autoclaving and incubated at 37°C for 72 hrs. After incubation IAA was determined at 530 nm by spectrophotometer.

- **Effect of carbon source on IAA production**

The production media supplemented with L-tryptophan and 1% of sugar like glucose, sucrose and mannitol were then inoculated with culture after autoclaving then incubated at 37 °C for 72 hrs. IAA was estimated at 530 nm by spectrophotometer as described previously.

- **Effect of nitrogen source on IAA production**

Effect of nitrogen source on IAA production was studied by inoculating the culture in the production media with 1% of nitrogen source like ammonium sulphate, potassium nitrate and urea. After autoclaving then incubated at 37°C for 72 hrs. IAA was estimated at 530 nm by spectrophotometer as described previously.

- **Extraction of IAA**

In 100 ml of tryptophan-fortified nutrient broth (1-5 mg/mL), IAA positive bacterial culture was inoculated and incubated at 28+2°C for a week by shaking at 150 rpm in incubator shaker. The bacterial cells were discarded and the supernatant alone was collected by centrifuging the culture at 10,000 rpm for 30 min. The supernatant was acidified from pH 2.5 to 3.0 using 1 N HCl and it was extracted twice with double the amount of ethyl acetate. The fraction extracted from ethyl acetate was evaporated to dryness at 40°C using a rotatory evaporator. Finally, the extract was dissolved with methanol and stored at 4 °C for further studies.

- **Characterization of IAA produced by isolates by FTIR (Fourier-transform Infra-red) analysis**

The FT-IR spectrum was recorded at the wavelength of 4000-400 cm-1. Characteristic peaks were obtained and the recorded report was analyzed. The extracted IAA Samples were mixed with Potassium bromide & grinded in a mortar and pestle, the IAA-KBr pellet was put in a Laboratory hydraulic press to Convert the pellet into discs, the discs were Set into the FT-IR machine and then IR-radiations were bombarded On to the sample, of 4000-400cm-1 wavelength to make the molecule of the sample to Vibrate

and on the basis of the molecular Vibrations and molecular stretching of the functional groups we get the IR spectra with characteristic peaks of different functional groups of the molecules.

Results and discussion

The isolate were determined for IAA production using salkowski's reagent The intensity of pink or red colour development at 530nm after addition of reagent was noted for each isolate. The results were noted for IAA production after 72 hrs at 37°C. The 3 isolates A3, A4 and A7 were selected for further study as the isolate showed maximum IAA production amongst all isolates. This is an accordance with result of Mankar and Barate (2019) [13] present results also favored by other former studies as M. E. Torbaghan *et al*., (2016) [21] and Nguyen khoi Nghia *et al.*, (2017) [16].

The effect of temperature studied shows that IAA production was maximum at 37°C for all 3 isolates after 72 hrs. At 37°C isolate A4 showed maximum IAA production followed by isolate A3 and isolate A7 also showed maximum IAA production (fig no 3) This is an accordance with result of Orhan (2016) [17] and Jayaprakashvel *et al*., (2014) [7] who reported 30°C to 37°C was optimum temperature for IAA production. This is in concordance with result of Mankar and Barate (2019) [13] who reported 45°C was optimum temperature for IAA production.

In the study effect of pH on IAA production was studied. The isolate A3 showed maximum IAA production at pH 7 and the isolate A4 showed maximum IAA production at pH 2 and isolate A7 showed maximum IAA production at pH 8 (fig no 4). This is accordance with result of Kamble and Galerao (2015) who also reported maximum IAA production at pH 7. This is in concordance Matre and Barate (2018) [14] who reported at pH 9 maximum IAA production. Barucha *et al*., (2013) who also reported maximum IAA production at pH 7.5. Torbaghan *et al*., (2016) [21] also reported as acidic pH medium was found to be unfavorable for IAA production.

The effect of three different carbon source like Glucose, Sucrose and Mannitol at 1% concentration was also studied. Isolate A4 and isolate A7 shows Mannitol was found to be the best carbon source which gave maximum production of IAA and the isolate A3 show sucrose was found to be best carbon source which gave maximum production of IAA (fig no 5). This is supported by Mukhtar *et al*., (2018) [12] who reported that mannitol was a best carbon source for IAA production. According Mankar and Barate (2019) [13] mannitol was a best carbon source for IAA production. Shilts *et al.*, (2005) and Mohite (2013) [11] also reported mannitol as best carbon source.

In the study effect of three different nitrogen source like ammonium sulphate, potassium nitrate and urea at 1% concentration was also studied. Isolate A3 and isolate A7 shows ammonium sulphate was found to be the best nitrogen source which give maximum production of IAA and for the isolate A4 shows urea was found to be the best nitrogen source which gives maximum production of IAA (fig no 6) This is an accordance with result of Mukhtar *et al*., (2018) [12] who reported that ammonium sulphate was a best nitrogen source for IAA production.

In the study effect of time on IAA production was also observed. The results were recorded for IAA production after each 24 hrs upto 96 hrs. It was found that IAA production was maximum at 96 hrs of incubation time for isolate A3, isolate A4 and isolate A7 (fig no 7). This is in agreement with other studies that also supported that 2-7 days required for IAA production. Anbumalar *et al*., (2018) [1]. Present result also favored by other former studies as Rajput *et al.*, (2018) [20] also reported the production of IAA after 72 hrs. L-tryptophan is precursor of IAA production.

The effect of different L- tryptophan concentration on IAA production was recorded. To check this different concentration of L-tryptophan 0.25%, 0.5%, 1%, 1.5% and 2% added to media. All three isolates was found that IAA production was maximum at 1.5% L-tryptophan concentration (fig no 8). This is an accordance with result of Matre and Barate (2018) [14] who reported that 1.5% and 2% showed maximum IAA production. Jayaprakashvel et al., (2014) [7] reported that IAA production maximum at 1% and 2% concentration of L-tryptophan. Mohite (2013) [11] also reported 0.1% and 1.5% showed maximum IAA production. Bharucha *et al*., (2023) [2] reported 0.2 as optimum concentration of Tryptophan for IAA production.

In the Current Study FI-IR spectroscopy was utilized for further characterization of bacterial IAA, it was compared with standard IAA. The IAA Showed the presence of -OH (acidic). NH-CH2, C-0 (keton) and possible C-C (aromatic) Stretching, which was interpretated from the spectra and the data obtain from the FT-IR, The bacterial IAA showed peak at 3852.98 which was a broad peak which is for-OH (acid group Stretching). A peak at wavelength 2930-96 confirms the presence of Vibration of aromatic C-C group. The bacterial IAA showed a broad peak at 3878.47 which confirms the presence of -NH broad peak Corresponding to NH group at 3370-38 Confirms the presence of NH the aromatic -C=O (Keton) confirming with the peak at 1421-60.This is an accordance with result of Lakshmanan *etal*., (2022) who reported that N-H group at 3855 cm-1, which confirms the presence of-NH broad peak, C=O (keton) group at 1700 cm-1, C-OH group at 1384 cm-1.

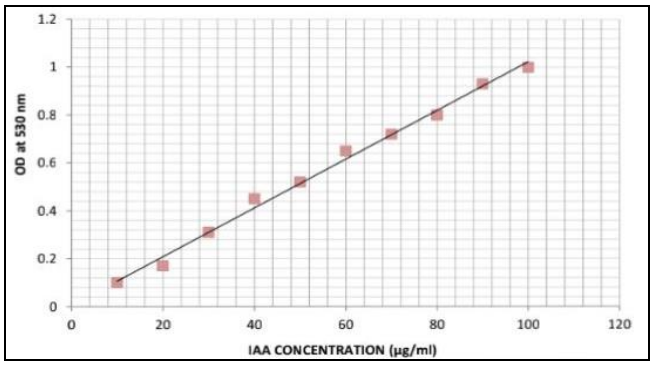


Fig 1: Stamdard graph for IAA concentration

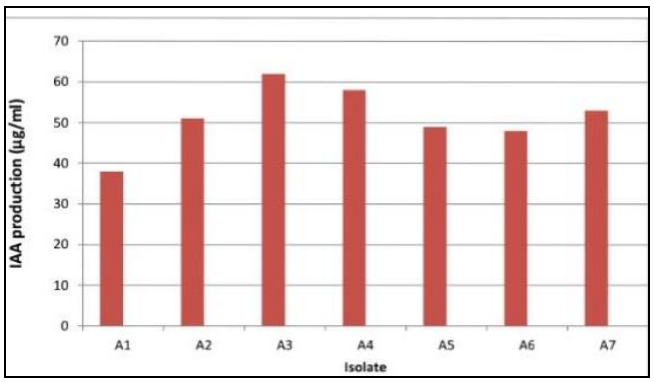


Fig 2: IAA production by Isolates

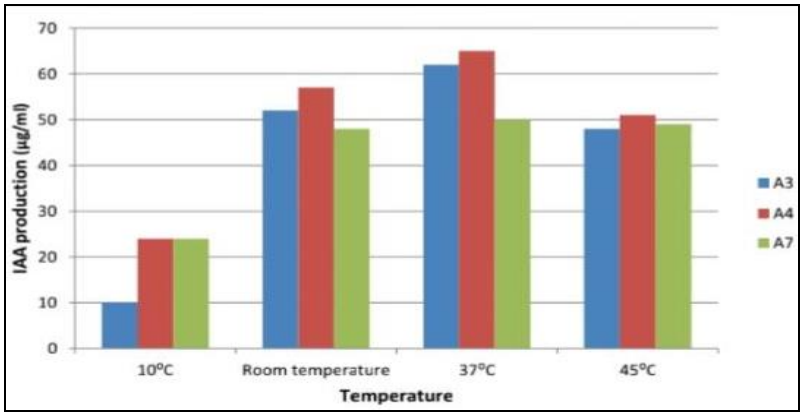


Fig 3: Effect of temperature on IAA production

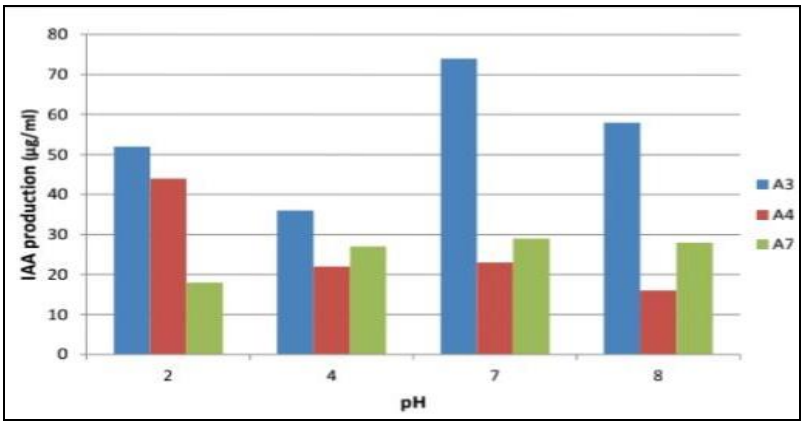


Fig 4: Effect of pH on IAA production

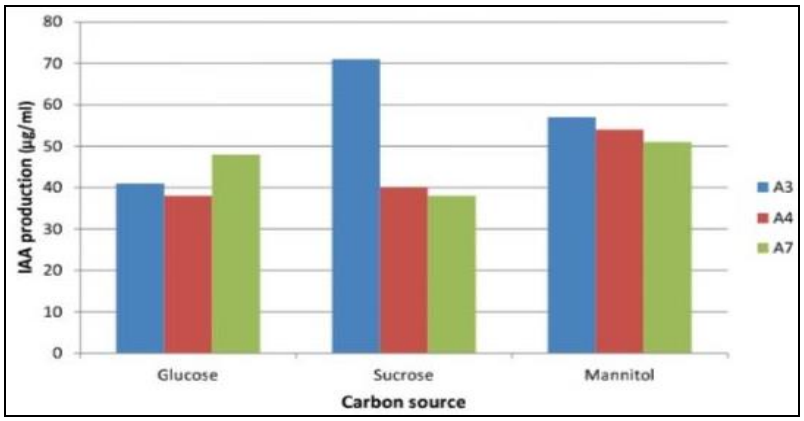


Fig 5: Effect of carbon source on IAA production

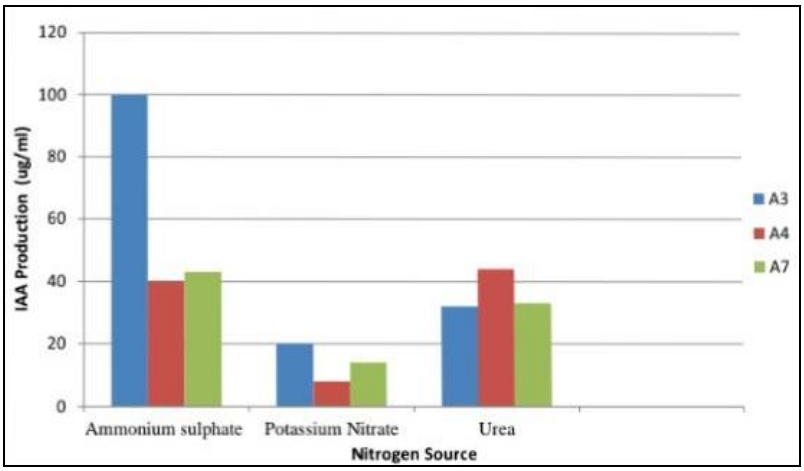


Fig 6: Effect of Nitrogen source on IAA production

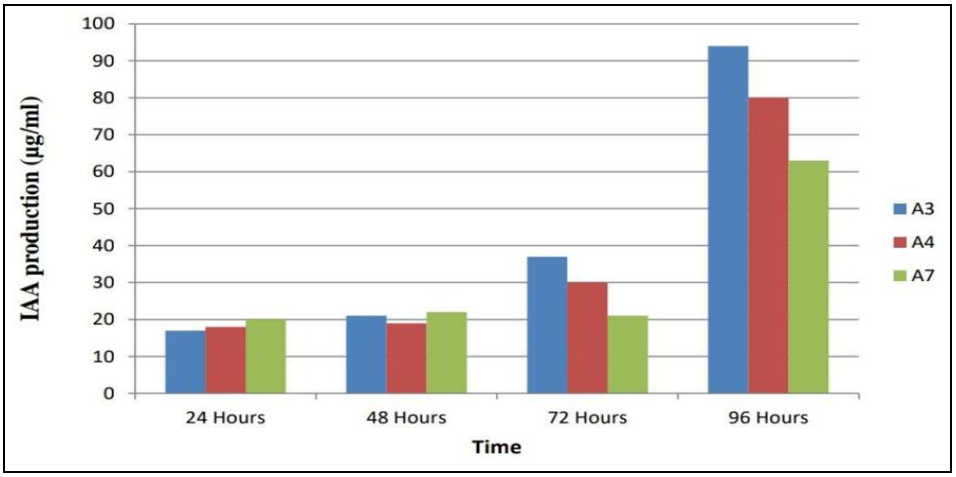


Fig 7: Effect of time IAA production by *Azotobacter* spp

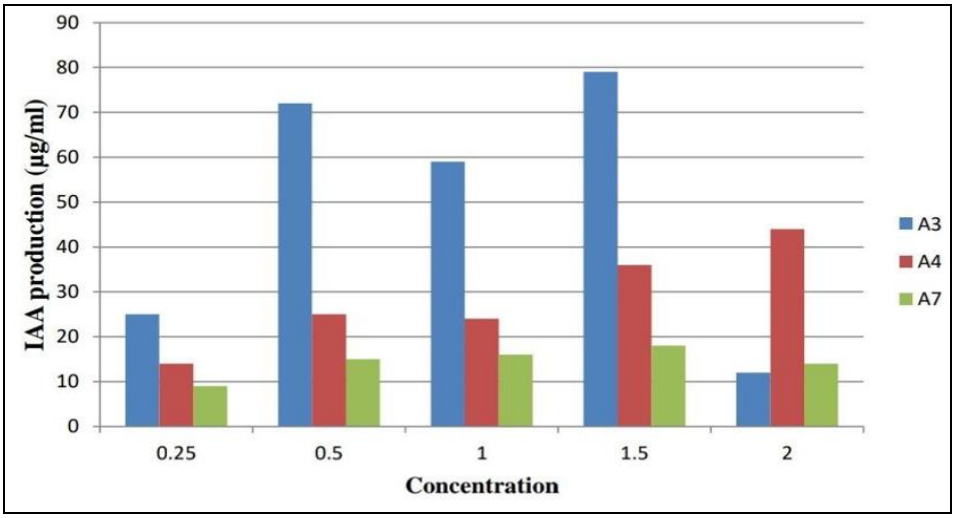
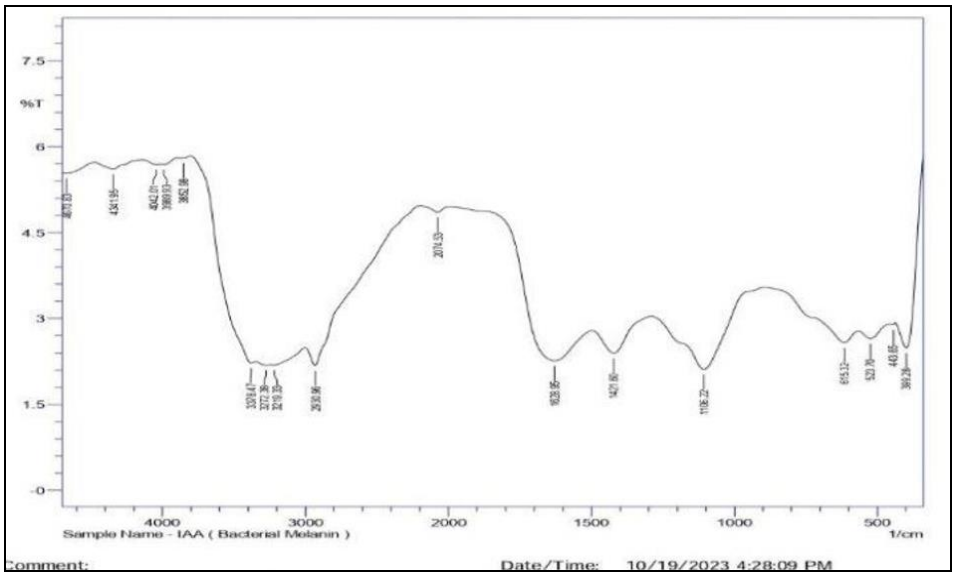


Fig 8: Effect of L-tryptophan on IAA production

FTIR of extracted IAA from isolates



Conclusion

In the study, the isolateA3, isolateA4 and isolateA7 showed maximum IAA production. Thus the isolate A3, isolate A4 and isolate A7 being beneficial in increasing crop production. Studies on optimization suggest that IAA production maximum at 37°C (temperature) for all 3 isolates at 96Hrs of incubation time, at pH2, pH7 and pH8 respectively. Mannitol and Sucrose found to be the best carbon source for IAA production and the Ammonium sulphate and urea found to be the best nitrogen source for IAA production. The 1.5% Tryptophan found best for maximum IAA production for all 3 isolates.

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