



Original Research Article

Optimization for production of Indole acetic acid (IAA) by plant growth promoting *Streptomyces* sp VSMGT1014 isolated from rice rhizosphere

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ABSTRACT

Keywords

Rice rhizosphere, *Streptomyces* sp, Indole acetic acid, plant growth promotion

The present study focused on the the characterization of Indole acetic acid (IAA) production under *in vitro* condition. A total of 90 actinomycetes was isolated from rice rhizosphere, which was collected from rice fields in the southern districts of Tamil Nadu, India. All isolates were screened for antagonism towards phytofungal pathogens such as *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium udum* and *Alternaria alternata*. Out of 95 isolates, 65 were found to be producing IAA was confirmed by colorimetric method. Isolate VSMGT1014 produced IAA in the ISP-2 medium supplemented with 0.5% L - tryptophan in the amount of 15.96 µg/ml. The ISP-2 medium was recorded as the best medium for production of IAA, where the maximum IAA production was recorded at 30 °C and pH 8 for the production of 4.76 µg/ml and 26.63 µg/ml respectively. The specific spot was found from the extracted IAA has similarity to authentic IAA with the same Rf value of 0.92. High performance liquid chromatography (HPLC) was carried out compared with authentic IAA at 17.5 min retention time was recorded. The selected strain VSMGT1014 could significantly enhance the growth of rice plants compared with that of non- inoculated plants. The results showed that an increase in root and shoot length 52% and 54.55% respectively compared to control. Overall, the results of this study indicate that potential of VSMGT1014 as a significant plant growth substance producer and good inoculants for the growth of rice seedlings.

Introduction

Rhizosphere is a wealthy location of microbes, ecological niche and should be explored for potential plant growth promoting rhizobacteria (PGPR), which developing as bio- inoculants for interact

with plant roots and enhancement of yield of crop plants. There are several plant growth promoting rhizobacterial (PGPR) inoculants that seems to promote plant growth through different mechanism such as

plant growth hormone production, nutrient acquisition and plant disease suppression. PGPR are inhabiting rhizosphere are capable of producing plant growth regulators such as auxin, gibberellins and ethylene. Indole acetic acid is a naturally occurring auxin which involves in cellular development and physiological processes in plants. Different soil microorganisms including bacteria (Stein et al., 1990), fungi (Finnie and Van Staden, 1985) and algae (Rifat Hayat et al., 2010) are capable of producing physiologically active quantities of auxins, which may exert prominent effects on plant growth and development. The application of single and combined application of microbes could increase plant growth of cotton due to result of slightly deleterious effect of strain causing increased root leakage/damage, which allows a greater population of aggressive rhizosphere and root colonizers such as *Trichoderma viride* and *Pseudomonas fluorescense* (Shanmugaiah et al., 2009).

Actinomycetes are Gram positive filamentous bacteria which generally inhabit soil and rhizosphere and can colonize the internal tissues of plants without causing any evident damage or morphological changes in plants (Hasegawa et al., 2006). Members of the genus *Streptomyces* characteristically produce extensively branched substrate hyphae from which long, branched aerial hyphae arise (Williams et al., 1989). Production of IAA varies among different species of *Streptomyces* and is also influenced by culture conditions, growth stage, and availability of substrate (Flaishman et al., 1996). Numerous plant-associated bacteria produce auxin and related indole compounds (Morris, 1986). Indole -3- acetic acid (IAA) is the main member of the auxin. Microorganisms inhabiting rhizosphere of plants and utilize the rich source of substrates from the roots

and are expected to synthesize and release auxins as secondary metabolites (Strzelczyk and Pokojaska- Burdziej, 1984). IAA is a naturally stirring in plants and it control many physiological processes like cell enlargement and tissue differentiation, responses to light and gravity, similarly it stimulates spore germination and mycelia elongation in the *Streptomyces* sp (Matsukawa et al., 2007). Several *Streptomyces* sp such as *S. olivaceoviridi*, *S. rimosus* and *S. rochei* from the tomato rhizosphere have the ability to produce IAA and improve plant growth by increased seed germination, root elongation and root dry weight (El-Tarabily, 2008).

Many bacteria isolated from the rhizosphere have the potential to synthesize IAA *in vitro* in the presence or absence of physiological precursors such as tryptophan (Davies, 1998). Tryptophan is believed to be the primary precursor for the formation of IAA in plants and rhizobacterium (Monteiro et al., 1988). Many commercial applications of PGPR are being tested and frequently successful, however, a better understanding of the microbial interactions that result in plant growth, increase will greatly increase in terms of success rate in field applications (Gerhardson, 2002). In recent days, there have been an increasing number of reports that terrestrial and entophytic actinomycetes are apparently beneficial to plants. They protect plants against phytopathogens by the production of antibiotics or chitinolytic enzymes to inhibit fungal pathogens (Shanmugaiah et al., 2008; Misk and Franco, 2011). Actinomycetes also directly promote plant growth by the production of phytohormones (auxin, cytokinins and gibberellins), siderophore production, phosphate solubilization of inorganic phosphate, fixing nitrogen and suppression of stress ethylene in plant by production of 1- amino cyclopropane -1- carboxylate

(ACC) deaminase activity (Misk and Franco, 2011; Sadeghi et al., 2012). However, plant growth promotion by antagonistic rhizobacteria have not been fully investigated, hence plant growth hormone and plant disease suppression mechanism for exploration is most urgent in recent days (Muller et al., 1989). In this present study, we investigate the optimization of plant growth promoting properties by terrestrial actinomycetes isolated from rice rhizosphere.

Materials and Methods

Isolation of actinomycetes

Rice rhizosphere soils were collected from Madurai, Tamil Nadu, India. The rhizosphere samples were transported to the laboratory in polypropylene bags and stored at 10 °C. Actinomycetes were isolated on International Streptomyces Project –2 (ISP-2) medium (Yeast extract- 4, Malt extract-10, Dextrose-4 and agar- 20 (g/L) (Shirling and Gottlieb, 1966) supplemented with 50 µg/ml of cyclohexamide, 50 µg/ml of nalidixic acid and incubated at 28 °C for 7–10 days. After ten days of incubation actinomycetes colonies on the agar plates were picked on the basis of their morphological character and purified on ISP-2 agar medium.

Screening and identification of actinomycetes

All the isolated actinomycetes were screened for antagonism (Harikrishnan and Shanmugaiah, 2013) against plant fungal pathogens such as *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium udum* and *Alternaria alternata*. The selected isolate was identified based on the physiology, morphology, biochemical and scanning electron microscope observation. For scanning

electron microscope (SEM) the isolate was grown on the cover slides, air-dried in a desiccator and mounted on stubs, sputter-coated with gold and viewed (FESEM, Supra 55, Carl Zeiss, Germany).

Screening of isolates for IAA production

IAA production was determined based on the method described by Patten and Glick (2002) with slight modifications. *Streptomyces* sp 100 µl spores were inoculated in ISP-2 broth supplemented with 0.2% filter sterilized (0.2µm membrane filter, Whatmann) L- tryptophan solution and incubated at 28°C in a rotary shaker at 120 rpm for 7 days. After 7-days of incubation, the culture was centrifuged at 11, 000 rpm for 15 min. One milliliter of supernatant was mixed with 2 ml of Salkowski reagent (1ml of 0.5M FeCl₃ in 50mL of 35% HClO₄) and incubated for 1hr. Development of pink colour indicated the production of IAA. The quantification of IAA was read at 530 nm in a UV- Vis spectrophotometer. A standard curve was plotted for quantification of IAA solution and uninoculated medium with a reagent as a control. The amount of IAA in the culture was expressed as µg/ml.

Optimization of parameters for IAA production

The production of IAA was optimized for selected isolate VSMGT1014 by one factor at a time was employed in this study.

Effect of L-tryptophan concentration

The effect of L-tryptophan concentrations on IAA production was studied using ISP-2 medium supplemented with L-tryptophan at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 1, and 1.5% followed by a pH 7.0. The culture was incubated at 37 °C in a shaker at 120 rpm for 7 days.

Effect of incubation time

The effect of incubation time for IAA production by *Streptomyces* sp. VSMGT1014 was grown in 50 ml of ISP-2 broth supplemented with 2 µg/ml L-tryptophan at pH 7.0 and incubated at 37 °C in a shaker at 120 rpm for 7 days. IAA production was assayed by incubating *Streptomyces* culture under optimum conditions up to 8 days. Production of IAA and residual L- tryptophan was measured at every 24 h interval.

Selection of culture media

IAA production by VSMGT1014 was optimized with different medium such as ISP1, ISP2, ISP5, ISP7, ISP 8, Bennett's broth and Starch casein broth. The production of IAA was observed after 6 days of incubation at 37°C in 120 rpm.

Effect of temperature and Ph

The optimum pH for the production of IAA by *Streptomyces* sp VSMGT1014 was determined with different pH value 2, 3, 4, 5, 6, 7, 8, 9 and 10. Similar experiments were performed to assess the effect of temperature by the above said culture and flasks were incubated with a different temperature at 25, 30, 35 37, 40 and 45°C.

Extraction of crude IAA

The extraction of Indole acetic acid from *Streptomyces* sp. VSMGT 1014 was carried out by the normal solvent extraction method (Shanmugaiah et al., 2010). One ml spores of *Streptomyces* sp VSMGT1014 were used to inoculate 1litre medium of ISP-2 broth supplemented with 0.5µg/mL filter sterilized L- Tryptophan and incubated at 30 °C for 5 days on a shaker (200 rpm). After 5 days of incubation, cells were separated from the

supernatant by centrifugation at 10, 000 rpm for 30 min. The supernatant was acidified to pH 2 with 1N HCl and extracted twice with ethyl acetate. Extracted ethyl acetate fraction was evaporated in a rotator evaporator at 40 °C. The extract was dissolved in 1ml of methanol and kept at – 20 °C.

Detection of IAA by thin layer chromatography

The extracted methanol fraction of crude compounds was performed using pre-coated silica gel TLC plates of grade F₂₇₄ (E-Merck, Germany) to detect IAA compounds produced by *Streptomyces* sp. VSMGT 1014. The crude extract was spotted with capillary tube and solvent front was allowed to run for approximately 80% of the plate. The crude was eluted with butanone-ethyl acetate-ethanol-water (3:5:1:1) solvent system. Spots with R_f values coincide with that of authentic IAA were identified under UV light (254 nm) and by spraying on the plates with Ehmann reagent (Ehmann, 1977).

Detection of IAA by High performance Liquid Chromatography (HPLC)

HPLC analysis of IAA was carried out on a C18 column (5µm; 25 x 0.46cm) by using HPLC grade acetonitrile- water system containing 0.1% trifluoroacetic acid was programmed over 30 min at a flow rate of 0.5mL/min with UV detector at 220 nm at 40 °C. Mobile phase consisted of methanol and water (80: 20 v/v) run at flow rate was analysed by comparison with the elution profiles of those authentic IAA injected separately.

Plant growth promotion

Plant growth promotion ability of the isolate VSMGT1014 was assessed *in vitro* in petri

plates. Rice seeds (IR-50) were surface sterilized with 0.1% (w/v) HgCl₂ for 5 min and thoroughly washed with sterile deionised water. Air dried rice seeds were soaked in 10⁸ culture suspension, culture filtrate of VSMGT1014 for six hours and then placed in petri plates. Culture and culture filtrate, sterile ISP-2 medium were included as control. After 10 days seeds germination, the root and shoot length, fresh and dry weight was measured.

Statistical analysis

Values were given as means \pm SD for triplicate samples.

Results and Discussion

Isolation and screening of actinomycetes

In this study, numerous morphologically different colonies were obtained from rice rhizosphere on ISP2 agar, 90 actinomycetes isolates were screened for antagonism against *R. solani*, *M. phaseolina*, *F. oxysporum*, *F. udum* and *A. alternata* (Figure 1). Among 90 actinomycetes, a total of 65 (72.2%) isolates have shown the antagonistic activity and the ability to produce IAA. Based on the potential antagonistic activity against fungal pathogens and superior IAA production, the isolate VSMGT1014 was selected for further studies. The potential antagonistic strain VSMGT1014 was identified as *Streptomyces* sp based on the physiological, biochemical and scanning electron microscopic observation (Figure 2).

Effect of L- tryptophan concentration on IAA production

Different concentrations of L- tryptophan between 0.1 to 1.5% were selected for the production of IAA. The spectrophotometric

analysis showed that gradual increase in the IAA production with respective L-tryptophan concentration. However, 0.5% L-tryptophan concentration in the medium showed maximum production of IAA. The maximum IAA production was observed as 15.96 μ g/ml when 0.5% L-tryptophan concentration was amended in the medium compared with known IAA standard (Figure 3).

Effect of pH and temperature on IAA production

The maximum IAA production 26.63 μ g/ml by *Streptomyces* sp VSMGT1014 was observed at pH 8. The result showed that a progressive increase in the production of IAA by VSMGT1014 from pH 2 – 8. In contrast, at pH 9 -10 were shown that drastic reduction of IAA production compared to control (Figure 4). Similarly the highest IAA production (4.76 μ g/ml) was recorded at 30 °C followed by 35 – 45 °C, whereas 3.90 μ g/ml was obtained for the control (Figure 5).

Effect of different days and medium on IAA production

The effect IAA production was estimated up to 7 days, among the maximum IAA production was observed in 5th day incubation (7.7 μ g/ml) (Figure 6). Similarly out of seven different mediums tested, the highest amount of IAA production was recorded in ISP2 medium (9.26 μ g/ml). Whereas the lowest amount of IAA production was observed in ISP7 medium at 1.3 μ g/ml compared to control (Figure 7).

Detection of IAA on TLC and HPLC

The strain *Streptomyces* sp VSMGT1014 ability to produce IAA was confirmed by TLC and HPLC analysis. As shown in

Figure 8, when the TLC plate was treated with Ehmann reagent, the ethyl acetate extract from culture filtrate showed a clear pink colour spot on the TLC plate at the Rf value corresponding to standard IAA (0.9). Similarly, HPLC analysis was conducted to identify and quantify the IAA production precisely. As shown in Figure 9, ethyl acetate extract from the culture filtrate of the strain and corresponding reference authentic standard showed peak at the similar retention time (17.5 min).

Plant growth promotion of rice by *Streptomyces* sp VSMGT1014

The significant effect of plant growth promotion of rice seedlings by the isolate VSMGT1014 and culture filtrate of the same isolate has shown that, the substantial increase in seed germination (100%), root length (9cm, 5.98cm) and shoot length (10.28cm, 8.03cm) respectively (Table 1). Rice plant biomass significantly increased by the isolate VSMGT1014 in terms of dry weight and vigor index compared to control.

In this study, we examined the plant growth promoting activity by *Streptomyces* sp isolated from rice rhizosphere. Microorganisms from the rhizosphere region of various crops have shown high potential of auxin production (Sarwar and Kremmer, 1995). *Streptomyces* sp. constituted 50% of the population of terrestrial actinomycetes and 75% of antibiotics is produced by this genus. In the present study, we found that rhizosphere *Streptomyces* isolate VSMGT1014 has the capability to synthesizing IAA molecules which can directly help the host plants for the growth and differentiation. Moreover, this strain may involve in resisting the plants from harmful plant fungal pathogens.

In the present study, out of 90 actinomycetes,

65 were shown antagonistic potential and IAA production. Similarly, our results coincide with previous reports for production of IAA and antagonistic mechanism that five streptomyces strain which isolated from rice rhizosphere having biocontrol potential against *Fusarium* wilt disease in chickpea and also having Plant growth promoting traits (PGPT) such as IAA and siderophore production (Gopalakrishnan et al., 2011). Indole acetic acid production greatly varies with incubation period, medium composition, temperature and tryptophan concentration. The L- Tryptophan at 0.5% concentration was the best for IAA production by the isolate *Streptomyces* sp VSMGT1014, whereas at high concentration of tryptophan exerts an adverse effect on IAA production. Ahmad et al (2005) reported that rhizosphere *Azotobacter* sp. and *Pseudomonas* sp. produced to a high level of IAA when this bacterium was cultured in a nutrient broth amended with 2 to 5 µg/mL of tryptophan. The L- tryptophan as a physiological precursor for IAA production by microorganisms, because microorganism such as *Streptomyces*, *Pseudomonas* and *Bacillus* is capable of synthesis IAA by utilizing L- tryptophan through indole -3- pyruvic acid pathway (Patten and Glick, 1996; Shanmugaiah et al., 2008, 2009; Harikrishnan and Shanmugaiah, 2013, Charulatha et al., 2013).

Our result revealed that the maximum IAA production at pH 8). However, the below and above pH 8 the production of IAA was less, because *Streptomyces* sp population level is more in alkaline soil than the acidic soil (Shirokikh et al., 2007). The optimum IAA production was observed reaching after 96 h and then decreases slowly . A significant affiliation was observed between bacterial growth and IAA production from in our findings. Reduction in IAA

production in the later stages might be due to release of IAA degrading enzymes such as IAA oxidase, peroxidase by the bacteria (Hunter, 1989; Shanmugaiah et al., 2008). The effect of temperature on IAA production was studied for *Streptomyces* sp. VSMGT1014 produced the highest amount of IAA when it was grown using ISP2 broth at 30°C, which is suitable for growth and IAA production of this isolate. Similarly, our results were found in agreement with previous report, that temperatures in the range 25 – 30°C suitable for growth and

IAA production of *Streptomyces* sp. (Aldesuquy et al., 1998; Khamna et al., 2010).

Culture filtrate of *Streptomyces* sp. VSMGT1014 was used to extract IAA for characterization by TLC. Chromatograms of culture extract and standard IAA were sprayed with Ehrmann reagent, it seems that almost the same R_f values are in agreement with other reports (Xie et al., 1996; Sudha et al., 2012).

Fig.1 *In vitro* antagonism of actinomycetes against plant fungal pathogens

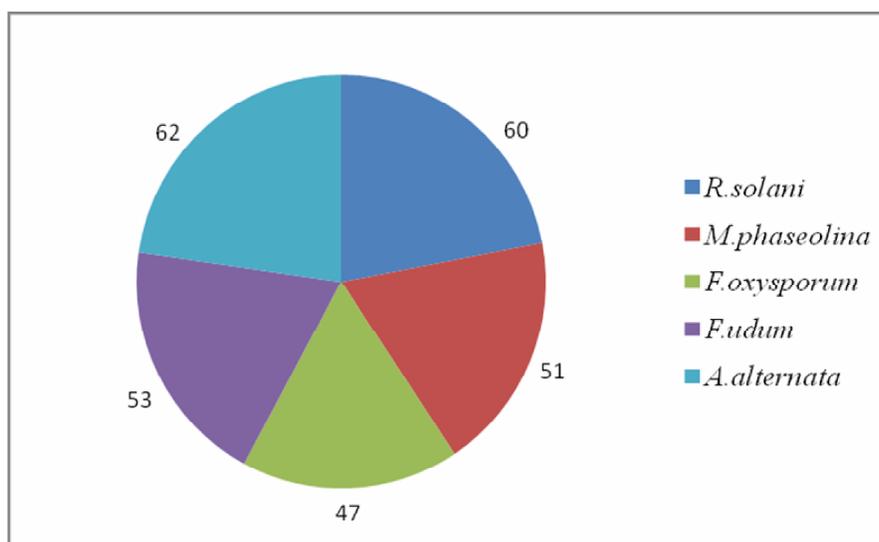


Fig.2 Scanning electron microscopic image of *Streptomyces* sp. VSMGT1014 (Magnification- 12.50KX)

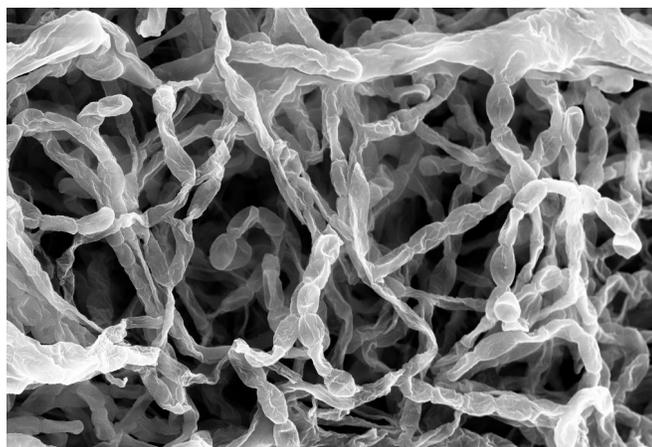


Fig.3 Effect of different concentration of L- tryptophan on IAA production by *Streptomyces* sp.VSMGT1014

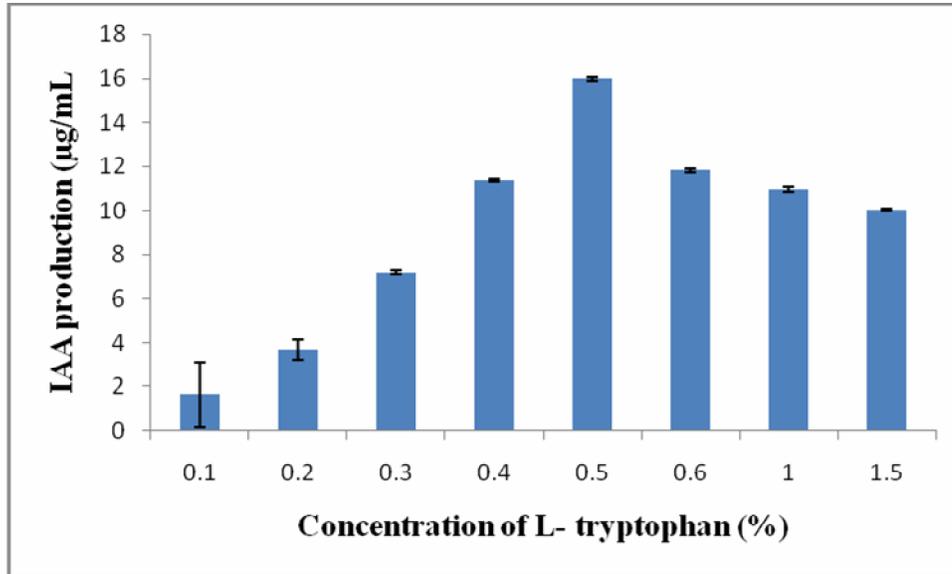


Fig.4 Effect of pH on IAA production by *Streptomyces* sp.VSMGT1014

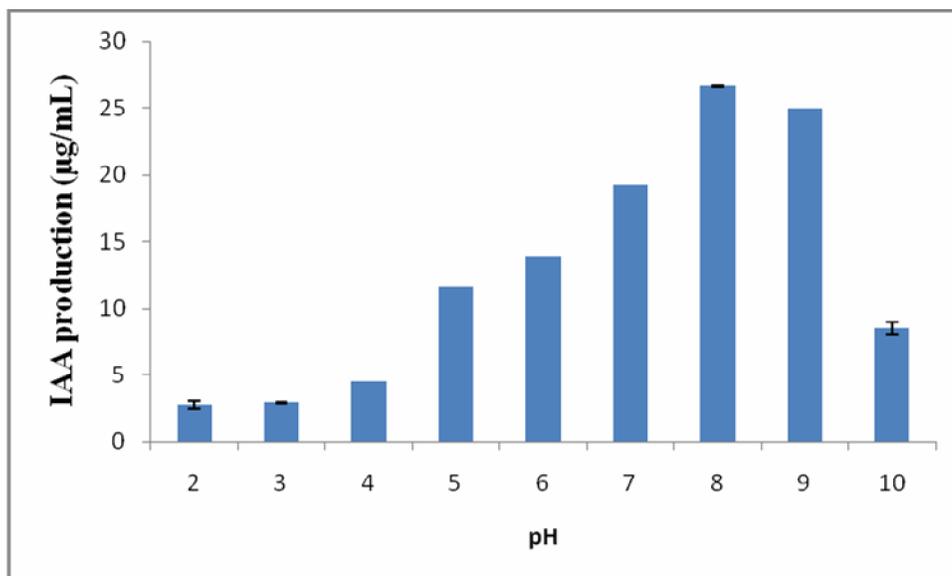


Fig.5 Effect of different temperature on IAA production by *Streptomyces* sp VSMGT1014

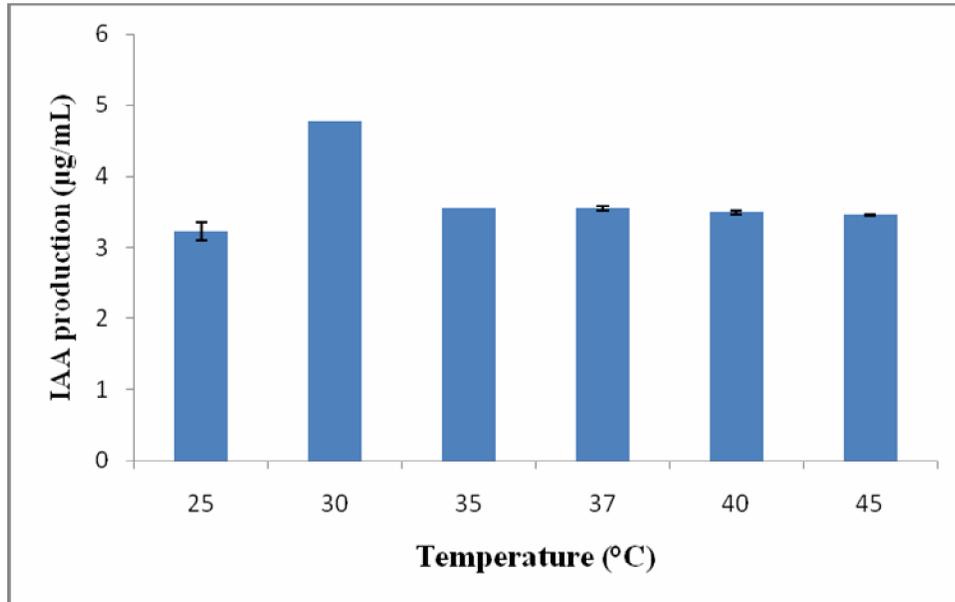


Fig.6 Time course study of IAA production by *Streptomyces* sp.VSMGT1014

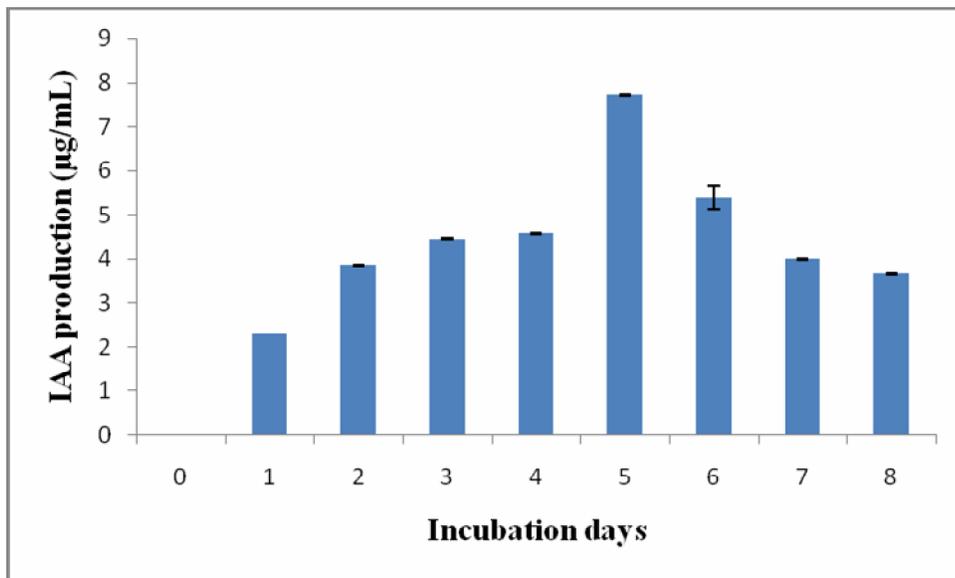


Fig.7 Production of IAA in different cultural media by *Streptomyces* sp VSMGT1014

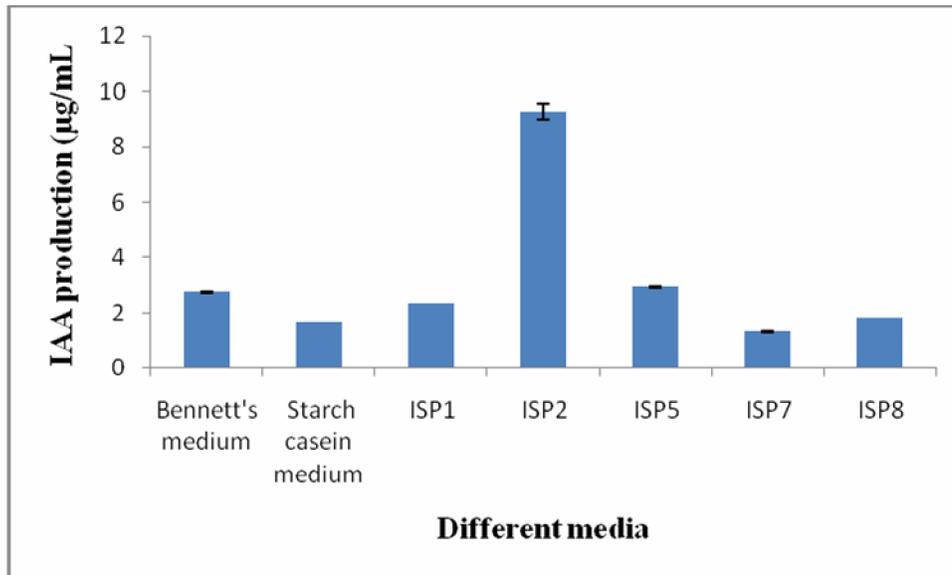


Fig.8 Detection of IAA production in thin layer chromatography by *Streptomyces* sp. VSMGT1014 compared with authentic IAA (1- Authentic IAA; 2- IAA from VSMGT1014)

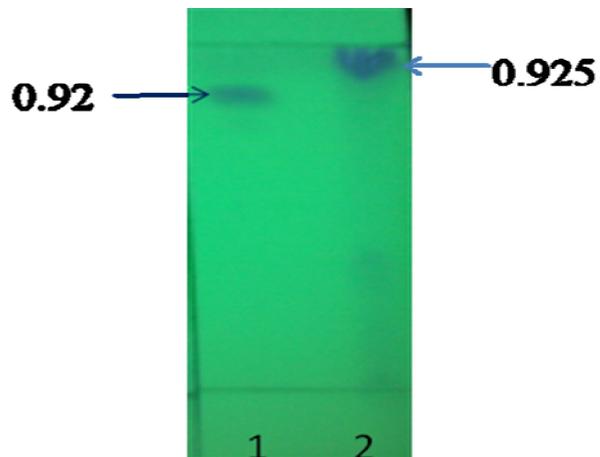


Fig. 9 HPLC profile of IAA production by *Streptomyces* sp. VSMGT1014 compared with authentic IAA (1-IAA from VSMGT1014; 2- Authentic IAA)

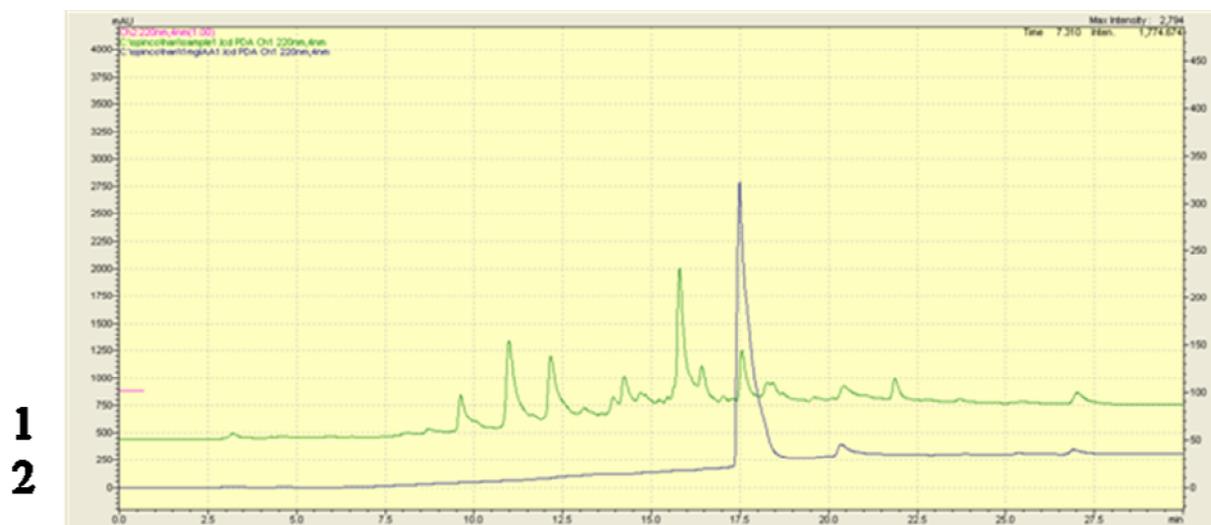


Table.1 Effect of rice plant growth promotion by *Streptomyces* sp VSMGT1014

Treatment	Germination %	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Vigour index
Culture	100	9.00±0.47	10.28±0.38	3.1±0.15	0.4±0.02	1929±82
Culture filtrate	100	5.98±0.27	8.03±0.67	3.6±0.5	0.29±0.01	1401±46
Control	80	3.35±0.27	3.63±0.47	2.3±0.15	0.11±0.02	698±72

Values are mean of triplicates with SD

In this study, we were benefited by the chromogenic stains, which further confirmed the results obtained from HPLC analysis for the production of IAA. In *in vitro* plant growth promotion of rice was proved by our strain VSMGT1014, through its ability to produce IAA and also the beneficial association with the host plants. Though bacteria and actinomycetes are known to exhibit several plant growth promoting traits, the production of phytohormones such as IAA and gibberellins

is considered to be one of the direct mechanism of plant growth promotion (Chanway, 2002). The results of our findings obtained through seed germination, root length, shoot length, fresh and dry weight significantly increases both culture and culture filtrate compare to control. The findings of the present investigation highlighted that the isolated strain has great potential to enhance soil fertility and plant growth promotion through the production of IAA. However, this assessment of plant

growth promotion further study needed under field conditions, it would be ideal in confirming the present findings and also in recommending the strain as bio- inoculants.

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