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Original Article

Isolation and optimization of IAA producing *Burkholderia seminalis* and its effect on seedlings of tomato

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Abstract

The present study was carried out to isolate Plant Growth Promoting Rhizobacteria (PGPR) strain from rhizosphere. Isolated strain was identified as *Burkholderia seminalis* using 16s rDNA. This strain was subjected to screening for indole acetic acid (IAA) production followed by optimization using statistical approach namely response surface methodology. IAA production from *B. seminalis* in nutrient broth supplemented with tryptophan was confirmed through thin layer chromatography (TLC). The effect of cell free culture medium (CFCM) obtained from isolated strain was examined on tomato seeds with Murashige and Skoog 2 (MS2) medium and it had a positive impact on germination of seeds. Further the IAA produced by *B. seminalis* in nutrient broth was purified and different concentrations were checked on tomato seed. It was observed that the germination index was similar to the control but the lateral roots development increased in the presence of 1 and 3 mg/mL of IAA in the medium.

Keywords: indole acetic acid, Murashige and Skoog 2, tomato seedlings, germination index, rhizosphere

1. Introduction

The world population keeps increasing and this maintains the demand for high crop yield to provide food for all. For better crop yield, usage of chemical fertilizers and pesticides for various purposes also has been high. Though satisfactory crop yield is obtained by the application of chemical fertilizers and pesticides, their use brings out certain disadvantages. These include pollution of large water resources, destruction of microorganisms, acidity of the soil and reduction in soil fertility etc. In recent years, scientists have focused their attention towards the potential of beneficial microbes such as Plant Growth Promoting Rhizobacteria (PGPR) for sustainable agriculture. Application of PGPR for better crop yield has increased tremendously in various parts of the world. Plant Growth Promoting Bacteria (PGPB), inhabiting the rhizosphere of the plants, are known to have a positive effect on plant growth. PGPR are group of bacteria that actively colonize the plant root region and increase plant growth and yield (Davies, 1995). PGPR possess several physiological features such as phytohormone, siderophore, HCN (antifungal) production, phosphate solublization, nitrogen fixation, denitrification and 1-amino-cyclopropane-1-carboxylate deaminase production. These characteristics are directly involved to the plant growth promotion (Iqbal and Hasnain, 2013). Till now various bacteria strain such as *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been isolated and identified as plant growth promoters (Khin *et al.*, 2012). Apart from several other beneficial traits, PGPR are known to produce phytohormones which are responsible for enhancing plant growth. Indole acetic acid (IAA) is one of the most physiologically active auxins, which can be produced by several microorganisms including PGPR through the L-tryptophan metabolism

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pathway. IAA is known to control organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, gene regulation and responses to light and gravity (Teale *et al.*, 2006; Lambrecht *et al.*, 2000). IAA, produced by PGPR are known to increase the root length, which gives larger root surface, which allows plants to get more access to nutrients from the soils (Boiero *et al.,* 2007). Optimization of IAA production can be done by varying the precursor (L-tryptophan) and other factors responsible for IAA production. In a traditional experimental design approach, one factor of the system is varied keeping other factors fixed during each run and is often termed as "one factor at a time" approach, thus increasing the experimental runs to a high value (Plackett and Burman, 1946).

Response Surface Methodology (RSM) is a combination of mathematical and statistical techniques which is useful for modeling and analyzing problems in which a response of consideration is influenced by a number of variables and the objective is to optimize this response (Natarajan and Aravindan, 2012)

The aim of the present study was to isolate and screen IAA producer PGPR, purify the IAA from broth and check different concentrations of IAA on the germination index of tomato seed. Response surface methodology was used to optimize the IAA production from isolated strain. Tryptophan, inoculum size and incubation days were used as variables for response surface methodology.

2. Materials and Methods

2.1 Screening and isolation of IAA producing organism

Several soil samples were collected from in and around Bangalore. These soils were subjected to serial dilution and agar plating method for isolation of organisms. Dilution factors were taken ranging from 10^{-1} up to 10^{-10} and the diluted soil samples were spread on sterile nutrient agar plates and incubated at 37°C for 24 hours (Khan and Rizvi, 2011). The isolated pure cultures were tested for the production of IAA. The production of IAA was estimated using the Salkowski's reagent. The culture was centrifuged at 8000 rpm for 20 min. One ml of the supernatant was mixed with 4 ml of Solawaski's reagent. IAA estimation was done at 530 nm using UV-Vis spectrophotometer (Glickmann and Dessauxm, 1995).

2.2 Thin layer chromatography for confirmation of IAA production

The supernatant was acidified with 1N HCl (pH 2.5-3). After acidification the IAA was extracted twice with double volume of ethyl acetate. The extracted ethyl acetate was dried in a hot air oven at 50°C. The dried extract was dissolved in 500 µl of methanol (Patil, 2011). Confirmation of IAA presence from *B. seminalis* extracts was done by TLC. Ten µl extract along with IAA standard (procured from Sigma, USA) was spotted on silica gel 60F254 aluminum sheets (Merck, Germany). Benzene:n-butanol:acetic acid (70:25:5) was used as the mobile phase. The spots were identified under UV light (254 nm).

2.3 Identification of the organism

Bacterial strain was identified with 16s rDNA sequencing. The bacterial DNA was isolated and subjected to PCR using the universal primers "27F" and "1492R". An amplicon band of 500 bp was observed. The forward and reverse DNA sequencing was carried out using BDT v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyser. A BLAST was performed utilizing the 16s rDNA sequence with the database of the NCBI GenBank. Based on maximum identity score, the first ten sequences were selected and aligned using multiple alignment software programs Clustal W. distance matrix was generated using the Ribosomal Project database and a phylogenetic tree was constructed using MEGA 4 (Larkin *et al.,* 2007).

2.4 Production of IAA from *B. seminalis* and its effect on tomato seeds germination

IAA was purified from the culture supernatant with the help of thin layer chromatography (TLC). Purified spots from TLC were scraped and transferred into glass tubes and dissolved in nutrient broth medium. The tomato seeds were sterilized for 1 min in 70% ethanol, followed by incubation for 10 min in 10% NaOCl and rinsing with sterilized water five times. Sterilized seeds were placed in 1% water agar and kept for 3 days at 4°C for vernalization. After vernalization the seeds were transferred to the sloped agar surface of Murashige and Skoog 2 (MS2) medium containing 2% sucrose and 1.5% agarose. 5µl (5 mg/mL) purified IAA from the culture supernatant was put on to the sterile disks and the disks were placed onto the MS2 medium containing the tomato seeds (Anke *et al.*, 2007).

2.5 Experimental design; Response surface methodology (RSM)

Experimental design was formulated according to the Central Composite Design (CCD) of RSM using MATLAB software package for the three selected variables, viz. tryptophan, inoculum size and incubation days (Table 1). A set of 20 experiments was required with each variable having five levels. The relationship between the coded values and actual values, independent variable and the response were calculated according to a second order quadratic model (Table 2). The relative effects of two variables on response were examined from three dimensional contour plots (Dikshit and Tallapragada, 2014).

Table 1. Experimental range and levels of the independent variables

Variables with designate	Code	Actual factor level at coded factor levels of				
		-2	-1	0	1	2
Tryptophan	X_1	3	4	5	6	7
Incubation days	X_2	5	10	15	20	25
Inoculum size	X_3	1	2	3	4	5

Table 2. Central composite design (CCD) of factors for IAA production (mg/mL)

Runs	X_1	Tryptophan (mg/mL)	X_2	Incubation days	X_3	Inoculum size	IAA estimated (mg/mL)	IAA observed (mg/mL)
1	-1	4	-1	10	-1	2%	0.38	0.3790
2	+1	6	-1	10	-1	2%	0.80	0.6990
3	-1	4	+1	20	-1	2%	1.10	0.9640
4	+1	6	+1	20	-1	2%	0.72	0.6340
5	-1	4	-1	10	+1	4%	0.21	0.1765
6	+1	6	-1	10	+1	4%	0.64	0.6565
7	-1	4	+1	20	+1	4%	0.98	0.9615
8	+1	6	+1	20	+1	4%	0.91	0.7915
9	-2	3	0	15	0	3%	0.50	0.5348
10	+2	7	0	15	0	3%	0.60	0.6848
11	0	5	-2	5	0	3%	0.20	0.1998
12	0	5	+2	25	0	3%	0.80	0.9198
13	0	5	0	15	-2	1%	0.73	0.8323
14	0	5	0	15	+2	5%	0.77	0.7873
15	0	5	0	15	0	3%	0.68	0.6516
16	0	5	0	15	0	3%	0.60	0.6516
17	0	5	0	15	0	3%	0.62	0.6516
18	0	5	0	15	0	3%	0.64	0.6516
19	0	5	0	15	0	3%	0.66	0.6516
20	0	5	0	15	0	3%	0.59	0.6516

Note: Experiments were carried in triplicates. Data represent the mean value. Standard deviation for estimated value is 0.015.

2.6 Statistical analysis

MATLAB® software Version 7.5.0.342 (R2007b) from The Math Works, Inc. was used for the regression and graphical analysis of the experimented data and for analyzing the response surface & contour plots. ANOVA was used to estimate the statistical parameters (p-value < 0.005).

3. Results and Discussion

3.1 Isolation and identification

Fifteen isolates were positive for IAA production; among them highest producer was chosen for further study. The culture was identified as *Burkholdoria* sp. based on the biochemical tests. The analysis of 16s rDNA fragment of the bacterium was identified as *B. seminalis* (Figure 1)

3.2 Confirmation of IAA production

IAA production from *B. seminalis* was confirmed through TLC. Extracted sample along with IAA standard were given the same retention factor (0.38) (Figure 2).

3.3 Production of IAA from *B. seminalis* and its effect on tomato seeds germination

Purified IAA, produced by *B. seminalis* was used in different concentrations to determine the germination index of tomato seeds. It was observed that 3 mg/mL of IAA concentration was optimum for lateral roots and shoot growth (Table 3). Obtained results showed that the roots of the seeds were stunted along with an increase in lateral root hairs when treated with the IAA (Figure 3). Treatment of seeds with purified IAA showed a positive influence on lateral root

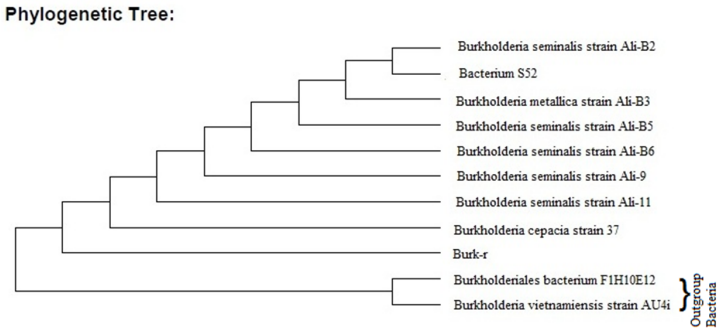

Figure 1. Phylogenetic analysis based on 16S rDNA gene sequences

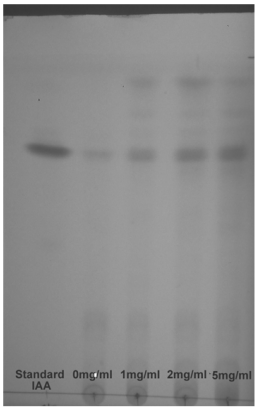

Figure 2. The TLC chromatogram depicting the bands of IAA standard and IAA produced by *Burkholderia seminalis* at different concentration of tryptophan.

hairs and shoot height. This growth in lateral roots enabled plants to absorb more nutrients from the soil. *Pseudomonas putida* UB1culture filtrate treated seedlings of maize had also been reported to exhibit an increase in lateral roots (Bharucha *et al.,* 2013). Similar observations have been reported by Khin *et al.* (2012) for IAA producing bacteria for enhancing lateral roots. This indicates that other components present along with IAA in the culture filtrate may have either positive or negative influence on plant growth. However, the present study examined only the influence of purified IAA produced by *B. seminalis* for the germination of the seeds. Though germination index was not that significant the shoot height and number of lateral roots present were higher when compared to the control. This effectively demonstrates that, instead of the whole cell culture, Purified IAA from *B. seminalis* can be utilized for the germination and growth of the plants.

3.4 Optimization for the production of IAA by response surface methodology

For production of optimum IAA from *B. seminalis*, a statistical approach, namely Response Surface Methodology, was employed. A central composite design was utilized to study the interactions between the significant factors. It was also used to determine the optimal levels of the factors. Relationship of the three variables for IAA production utilizing RSM technique is explained in equation 1.

Table 3. The shoot height, root length and No. of lateral roots of tomato seedlings

Concentration of IAA	Shoot height (cm)	Root length (cm)	No. of lateral roots
CFCM	3.40	2.70	1
1 mg/mL	3.00	1.00	6
3 mg/mL	4.60	0.50	9
5 mg/mL	3.00	0.40	6

Note: Experiments were carried in triplicates. Data represent the mean value. Standard deviation for shoot height, root length and no. of lateral roots is 0.15, 0.17 and 0.25 respectively.

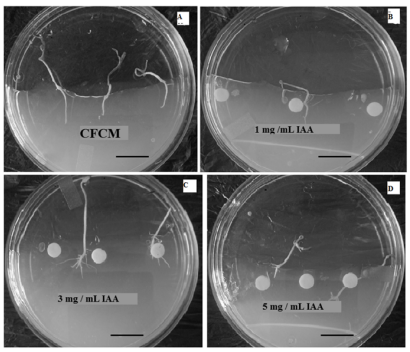

Figure 3. (A) Effect of the cell free supernatant on the germination of the tomato seeds, seeds in the CFCM plate showed the elongated roots. (B) The seeds exposed to purified IAA (1 mg/mL) from *Burkholderia seminalis* dissolved in nutrient broth showed that the germination of seeds is limited with only 1/3rd showing the germination. (C) IAA (3 mg/mL) from *Burkholderia seminalis* showed the stunted roots with highest number of lateral root hairs. (D) IAA (5 mg/mL) from *Burkholderia seminalis* showed stunted roots with few lateral hairs (Scale bar represents 1 cm of length).

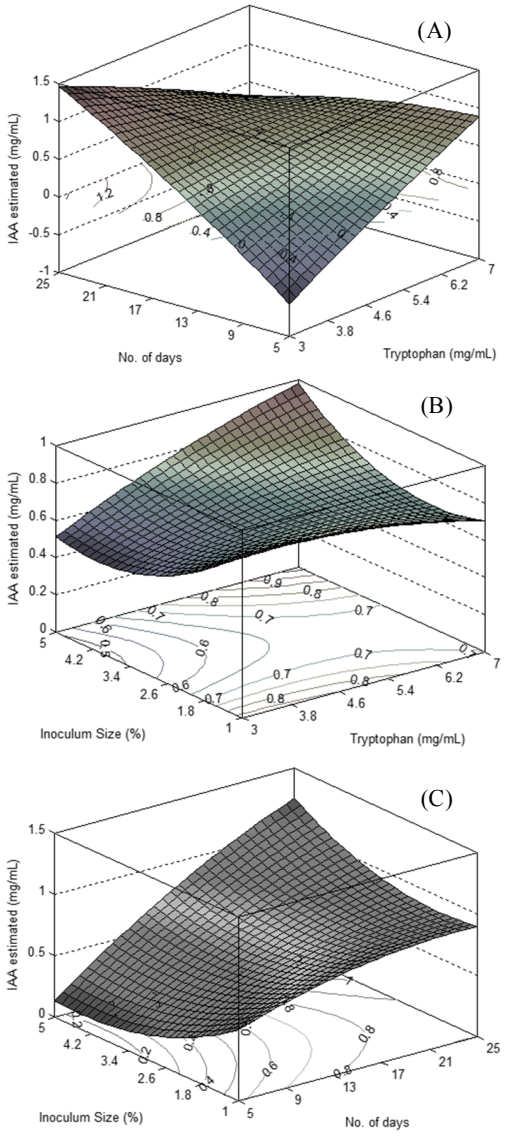

Figure 4. (A) 3D Response surface plot and contour plot showing the relative effect tryptophan concentration and incubation time on the production of IAA while keeping inoculum size at its central level. (B) 3D Response surface plot and contour plot of tryptophan levels versus inoculum size while keeping incubation time at its central level. (C) 3D Response surface plot and contour plots of inoculum size versus incubation time while keeping tryptophan concentration at its central level.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_1X_3 \\ b_6X_2X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2 \quad (1)$$

Substituting the coefficients from Table 4 in eq. (1), we get

$$Y = -1.5417 + 0.5095 \times X_1 + 0.1960 \times X_2 - 0.5985 \times X_3 \\ -0.0325 \times X_1 \times X_2 + 0.0400 \times X_1 \times X_3 + 0.0100 \\ \times X_2 \times X_3 - 0.0105 \times X_1^2 - 0.0009 \times X_2^2 + 0.0395 \times X_3^2$$

Where the output Y is the estimated IAA, X_1 is Tryptophan, X_2 is No. of incubation days and X_3 is the inoculum size. To study the interaction effects of the variables affecting the production of IAA the output was plotted in the form of 3D surface plots keeping one variable at constant (centralized) level and varying the other two independent factors. These 3D plots are shown in Figures 4A to 4C. Figure 4A shows the dependency of tryptophan concentration and incubation time on the production of IAA. At lower levels of tryptophan, the IAA production increases with an increase in the no. of days of incubation, with concentration of IAA peaking to 1.5 mg/mL. At higher concentrations of the tryptophan, the concentration of the IAA did not vary much (0.9 mg/mL). On the 5th day of incubation, the concentration of IAA increased with increasing tryptophan concentration, but as the incubation days increased the level of IAA slightly decreased. The highest amount of IAA produced was 1.5 mg/mL on 25th day with tryptophan concentration of 3.8 mg/mL.

The plot showing tryptophan levels versus inoculum size in Figure 4B indicates that production of IAA increased with increasing tryptophan levels and increasing inoculum size. However, no appreciable variation was noticed with either of these variables on the IAA production. The maximum IAA produced was 1 mg/mL at 5% inoculum size and 7 mg/mL of tryptophan concentration.

Figure 4C shows the 3D response surface plots of inoculum size versus incubation days. From 5th day to 25th day the IAA production increased slightly at lower levels of inoculum size but quite significantly at higher inoculum size. The IAA production decreased with inoculum size at lower incubation days. The highest concentration of IAA (1.3 mg/mL) was observed at 25th day of incubation with 5% of inoculum size.

B. seminalis had shown slow growth in the medium. IAA production by *B. seminalis* started after the 5th day of incubation and reached a maximum on the 25th day of incubation. The result suggested that IAA production was associated with growth of the microorganism, and maximum production was observed at the 25th day of incubation. Bharucha *et al.* (2013) has reported that IAA production increased linearly from 2 to 8 days and decreased later with a decrease in the growth of *P. putida* UB1. After the 25th day of incubation, there was a decrease in IAA production. This might be due to the release of IAA degrading enzymes such as indole acetic acid oxidase and peroxidase (Jeyanthi and Ganesh, 2013).

L-tryptophan is considered to be a precursor to IAA production as its addition to medium enhances it. The auxin biosynthesis derived from L-tryptophan was reported to be enhanced several fold as per Khalid *et al.* (2004) who studied the effect of L-tryptophan concentration on IAA production. Bharucha *et al.* (2013) reported that variable amounts of auxins were produced by the rhizobacteria in vitro when culture media was supplemented with different concentrations of L-tryptophan. The auxin biosynthesis was reported to be further stimulated by variation of the culture media.

The regression coefficient results from the data of CCD experiments are shown in Table 4. Coefficient value significance is inversely proportional to the p-values and bears a direct effect with the magnitude of t-value. Hence from Table 4, it can be observed that the interaction of tryptophan and incubation time was the most significant factor (p-value = 0.00079). Linear effect was more significant for the incubation time (p = 0.0017) followed by inoculum size and tryptophan concentration, whereas the quadratic effect of inoculum size was more significant (p = 0.06788) than that of other factors.

The fitness of the model was determined by the co-efficient of determination R^2. A model having an R^2 value higher than 0.9 was considered as very high correlation between experimental value and predicted value from the model (Chen *et al.,* 2009). The R^2 value in this model was found to be 0.9013, which depicts that 90.13% of the total variation that occurred in the response value could be explained by the model and the remaining 8.87% is not explained by the model. P-value of less than 0.05 indicates that the model terms are significant. The ANOVA for the response surface quadratic model as shown in Table 5, the model was highly significant with the p value of 0.0006.

4. Validation of the Model

From CCD and RSM it was found that the optimum conditions for maximum yield of IAA from *B. seminalis* were at 3 mg/mL of tryptophan, 25 days of incubation and at 5% inoculum size. The IAA produced was 1.518 mg/mL, showing 4.6 fold increases from 0.33 mg/mL. These results were validated by carrying out the experiment at these conditions and the results showed a close match with the predicted values.

5. Conclusions

In the present study, cell-free culture medium (CFCM) and purified IAA from *Burkholderia seminalis* were examined for their effect on plant growth. It was observed that these have a strong effect on lateral root formation in tomato plants. The production of IAA was optimized using the statistical methodology, RSM. At optimized conditions, this strain produced sufficient amount of IAA, which can enhance plant growth. Hence, this strain can potentially be developed as a bio-inoculant for plant growth. This strain can be used as a bio fertilizer, which will help farmers to utilize the bacteria in the field and reduce their dependence on chemical fertilizers. These CFCM need to be further investigated under pot and field conditions to improve the growth and yield of tomato plants.

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Table 4. Regression coefficient results from the data of central composite designed experiments

Interactions	Co-efficients (b_0 to b_9 of eq. 1)	Standard error	t-value	p-value
Constant	-1.5417	0.9959	-1.5481	0.15264
Tryptophan (mg/mL)	0.5095	0.2430	2.0971	0.06238
No. of days	0.1960	0.0464	4.2215	0.00177
Inoculum size (%)	-0.5985	0.2322	-2.5776	0.02753
Tryptophan × No. of days	-0.0325	0.0069	-4.7442	0.00079
Tryptophan × Inoculum size	0.0400	0.0343	1.1678	0.26998
No. of days × Inoculum size	0.0100	0.0069	1.4597	0.17505
Tryptophan × Tryptophan	-0.0105	0.0193	-0.5411	0.60029
No. of days × No. of days	-0.0009	0.0008	-1.1881	0.26226
Inoculum size × Inoculum size	0.0395	0.0193	2.0468	0.06788

Table 5. Analysis of Variance (ANOVA) for response surface quadratic model

Sum of squares	Degree of Freedom	f-value	p-value	Mean Square	R^2	Adj R^2
0.0939	9	10.1476	0.0006	0.0094	0.9013	0.8125

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