Optimization of Indole-3-Acetic Acid Production by Diazotrophic *B. subtilis* DR2 (KP455653), Isolated from Rhizosphere of *Eragrostis cynosuroides*

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Abstract*—Diazotrophic rhizobacteria, trigger and enhance plant growth as well as yield through various mechanisms, so their use can reduce the application frequency of chemical fertilizers. Indole-3-acetic acid (IAA), a most common natural auxin influences several physiological processes of the plant's health. The present study is aimed to optimize the conditions for IAA production, along with assay for plant growth promoting traits of *Bacillus subtilis* DR2 (KP455653), which is a diazotrophic Gram positive, rod bacterium, isolated from rhizosphere of road side weed, *Eragrostis cynosuroides* from Danapur, Patna, Bihar, India. The screening for IAA production was done in JNFb$^-$ broth with tryptophan (1 g.l^{-1}) and without tryptophan at pH 5.8, 30±2 ℃ temperature and 48 h incubation. 137.81 µg.ml^{-1} and 100.26 µg.ml^{-1} IAA was produced in Trp$^+$ and Trp$^-$ media, respectively. Under various optimized conditions, maximum IAA was produced at 96 h incubation (137.81 µg.ml^{-1}), 35 ℃ temperature (141.92 µg.ml^{-1}), pH 7 (158.79 µg.ml^{-1}), mannitol as carbon (160.85 µg.ml^{-1}) and ammonium sulfate as nitrogen (162.93 µg.ml^{-1}) sources with tryptophan at final concentration of 1.2 µg.ml^{-1} (168.09 µg.ml^{-1}), which enhanced the production by 1.2 fold. The findings suggest that *B. subtilis* DR2 is a potent organism to be used as biofertilizer.

***Index Terms*— DR2, IAA, PGP, rhizobacteria**

I. Introduction

Rhizosphere, the narrow zone nearby the root system is much richer than the surrounding bulk soil, constituting a sink for carbon and other energy sources, supporting intense microbial growth and activity, due to presence of various organic compounds, released through exudation, secretion and deposition. The microbial community may be neutral, detrimental or beneficial for plant growth [1]. They promote plant growth directly or indirectly, along with enhancement of soil fertility by various growth promoting activities, so referred as plant growth promoting rhizobacteria, i.e., PGPR [2]. The PGPRs have emerged as the best alternative of hazardous chemical fertilizers for sustainable and eco-friendly agriculture, because they are diazotrophs converting N_2 into ammonia to be used by plants and also trigger plant growth via production of phytohormones, viz., IAA, gibberellic acid, cytokinins and ethylene. 80% of diazotrophic indole producing rhizobacteria promotes plant growth directly via phosphate solubilization, production of plant enzymes, HCN, antibiotics, siderophores for sequestering of iron and by lowering ethylene concentration via ACC deaminase activity [3]. IAA acts as an important signal molecule in the regulation of plant development by initiation, cell division and cell enlargement [4]. The amino acid L-tryptophan, serves as a physiological precursor for biosynthesis of auxins in microbes and plants [5], [6]. Bacteria synthesize auxins to perturb host physiological processes for their own benefit by altering the auxin pool, depending upon the amount of IAA produced. Therefore, it becomes necessary to identify and incorporate those efficient bacterial strains, which reside in the rhizosphere of plants, utilize the rich source of substrates, released from roots and are expected to produce auxins as secondary metabolites [7]. Several soil bacteria, particularly those belonging to the species of *Bacillus* and *Pseudomonas* have remarkable abilities to synthesize various beneficial substances, along with potent PGP activities. Amongst them, spore forming Bacilli are considered to be better, as they are more resistant to all the adverse situations, like temperature, chemicals, etc. [8]. The widespread occurrence of *Eragrostis cynosuroides* on the road side virgin land, prompted us to explore its rhizospheric population for (i) novel sources of indole producing diazotrophic bacteria (ii) assessment for PGP properties and assay and optimization of indole producing ability of *Bacillus subtilis* DR2 (KP455653) under different cultural conditions for agriculture and commercial purposes.

II. Materials and Methods

A. Sample Collection and Isolation of Rhizospheric Diazotrophic Bacteria

Soil sample was collected in sterile plastic bags from the rhizosphere of *Eragrostis cynosuroides* growing on

Manuscript received September 25, 2017; revised November 25, 2017

doi: 10.18178/ijpmbs.7.2.20-27

the road side (devoid of fertilizer) of Danapur, Patna, Bihar, India, (25º 34' 56.2" N, 85º 2' 37.06" E) and processed within three hours. In this study, all the experiments were performed in triplicates. To isolate nitrogen fixers, soil sample diluted up to 10^{-6} dilution and spread on to nitrogen free JNFb$^-$ solid agar medium [9] and incubated at 30±2 ℃ for 4-5 days. Bacterial colonies appearing on the plates were purified and sub cultured repeatedly. Their diazotrophy, under anaerobic condition was confirmed on the basis of pellicle formation by growing on nitrogen free JNFb$^-$ agar medium (0.15% agar), without shaking. Preservation was done at 4 ℃ in JNFb$^-$ medium.

B. Assay for Plant Growth Promoting Traits

All the isolates were screened for PGP traits in terms of i) nitrogen fixation [10] and ii) IAA production [11]. The selected isolate was examined for i) phosphate solubilization [12], ii) siderophore production [13], iii) HCN production [14], iv) ACC deaminase activity [15] and v) antifungal activity [16] on the species of *Aspergillus* and *Fusarium*. Quantification was done only for nitrogen fixation [17] and IAA production [11].

1) Nitrogen fixation: Screening and estimation

Isolates were inoculated on nitrogen free malate media containing BTB (Bromo Thymol Blue) as an indicator [10] and incubated at 30±2 ℃ for 3-4 days. The blue color zone producers were marked as nitrogen fixers. Nitrogenase activity was estimated according to the method of [17] and acetylene reduction assay (ARA) as per the details of [18].

2) IAA: Production and estimation

Indole test was performed by inoculating the isolates into tryptone (1%) broth for 48 h at 30±2 ℃, followed by addition of kovac's reagent (1ml). Appearance of cherry red color ring confirms IAA production. For quantification, culture was grown in JNFb$^-$ broth media with 1g.l^{-1} L- tryptophan (Trp$^+$) and without tryptophan (Trp$^-$), pH 5.8 and incubated at 30±2 ℃ with shaking at 80 rpm for 3-4 days. The culture was centrifuged at 3000 rpm for 30 min. 1ml of supernatant was mixed with 2ml of Salkowaski reagent (1ml of 0.5M $FeCl_3$ mixed in 50ml of 35% $HClO_4$). The resulting mixture was left at room temperature for 25 min and the absorbance recorded at 530 nm.

C. Characterization: Phenotypic and Genotypic

The identification was done on the basis of phenotypic genotypic characters [18].

D. Optimization of Cultural Conditions for IAA

Effect of different incubation time (24, 48, 72, 96, 120 and 144 h) was studied in JNFb$^-$ Trp$^+$ (1g.l^{-1}) broth medium at pH 5.8 and 30±2 ℃. Thereafter, effect of various temperatures (25, 30, 35, 40, 45 and 50 ℃), pH (4, 5, 6, 7, 8 and 9) were investigated followed by optimization of C-sources (5g.l^{-1} malic acid, glucose, sucrose, mannitol, fructose and lactose), N-sources (0.1% w/v urea, $NaNO_3$, KNO_3, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$) and tryptophan concentrations (0.1, 0.2…...1.0, 1.2, 1.4 and 1.6 g.l^{-1}). Optimization was done with one variable at a time.

E. Statistical Analysis

The data obtained were statistically analyzed for social sciences (SPSS 16.0) software, and graphically represented as the mean ± standard deviation (n=3).

III. Results and Discussions

B. Isolation and Characterization of Rhizospheric Diazotrophic Bacteria

Seven isolates (DR1-DR7) appeared on solid JNFb$^-$ media, which upon repeated sub culturing retained their growth without losing diazotrophy. Growth of the isolates in nitrogen free-semi solid medium (0.15% agar) resulted in the formation of pellicles with significant differences in their location below the surface of media indicating their diazotrophic property under semi anaerobic environment [19].

C. Assay for Plant Growth Promoting Traits

1) Nitrogen fixation: Screening and estimation

All the isolates tested positive for nitrogen fixation. (Table I). The zone of coloration was (20mm) in DR2, which is maximum amongst the positive isolates. Similarly, highest (60.23 nmol C_2H_4 mg^{-1} protein h^{-1}) nitrogenase activity was observed in DR2 (Fig. 1). The color zones (11-27 mm) have been reported by [10] in bacteria isolated from rhizosphere of sewan grass. The diazotrophy of isolates were further confirmed by appearance of blue zone in nitrogen free medium, followed by ARA. This finding is in conformity with the earlier reports of [20]. It has been argued that, nitrogenase activity is solely detectable upon growth in nitrogen free media, as it provides right niche for diazotrophic bacteria [19]. Thus, *B. subtilis* DR2 was identified as best nitrogen fixer.

TABLE I. Qualitative Assessment of PGP Activities in the Isolates (DR1-DR7)

Isolates	Nitrogen Fixation (JNFb$^-$ agar medium)	Phosphate Solubilization (PVK agar medium)	IAA Production (JNFb$^-$ with Tryptophan medium)	Siderophore Production (CAS medium)	HCN Production	ACC deaminase Activity	Antifungal Activity	
							Aspergillus sps.	*Fusarium* sps.
DR1	+	++	+	+	-	-	-	-
DR2	+++	+++	+++	+++	+++	+++	+	++
DR3	+	-	-	-	-	-	-	-
DR4	++	+	++	++	-	-	-	-
DR5	+	-	-	-	-	-	-	-
DR6	+	+	+	+	-	-	-	-
DR7	+++	++	++	+++	++	++	-	-

Low (+); Medium (++); High (+++); Negative (-)

2) IAA production: Screening and estimation

Out of seven isolates, five (DR1, DR2, DR4, DR6 and DR7) tested positive for IAA production. It was remarkable that in all the positive isolates, IAA production was significantly higher in Trp$^+$ as well as in

Trp^- media. Isolate *B. subtilis* DR2 recorded maximum indole in both Trp^+ (137.33 $\mu g.ml^{-1}$) and Trp^- (100.26 $\mu g.ml^{-1}$) media (Fig. 1). [1] reported IAA production in rhizospheric bacteria of banana, ranging from 89-108 $\mu g.ml^{-1}$ in 8 strains of fluorescent *Pseudomonas* and 50-51 $\mu g.ml^{-1}$ in two strains of *Bacillus*. However, [21] reported highest (11.49 $\mu g.ml^{-1}$) IAA in Trp^+ medium from the bacteria DPY-05 isolated from aerial roots of orchids, which is significantly lower than our findings. In the present work *B. subtilis* DR2 is also a rhizobacteria with significant IAA producing capability. In plants and bacteria, tryptophan has been identified as main precursor for IAA biosynthesis by two pathways, i.e., Trp-dependent and Trp-independent. Production of IAA in both, Trp^+ and Trp^- media by *B. subtilis* DR2, suggests the presence of both pathways in the organism. Production of IAA in Trp^- medium indicates the genetic makeup of the test organism, to be exploited for commercial use. Therefore, screening of organisms for their *in vitro* potential of auxin secretion could act as reliable tool for selection of efficient plant growth promoters.

On the basis of optimum nitrogen fixing and IAA producing abilities, *B. subtilis* DR2 was selected for further investigation.

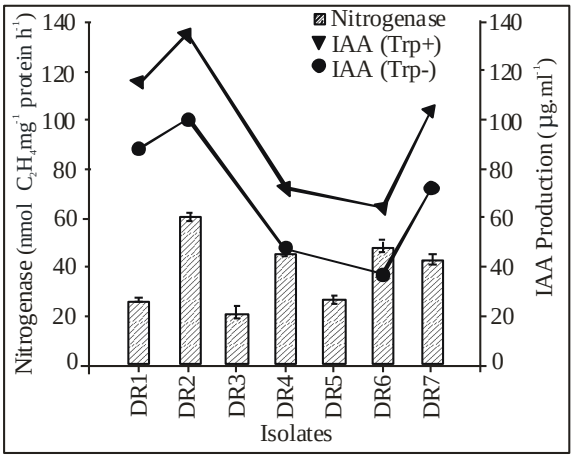

Figure 1. Bacterial isolates showing Nitrogenase activity (nmol $C_2H_4mg^{-1}$ protein h^{-1}) and IAA production ($\mu g.ml^{-1}$).

3) Phosphate solubilization: Screening and estimation

The DR2 gave positive test for phosphate solubilization in term of halo zone (15 mm) on PVK agar plate (Table I). In our earlier publication [18] DR2 emerged as the most efficient phosphate solubilizer with 48.16 $mg.l^{-1}$ as soluble phosphate under optimized production conditions of 96 h of incubation at 30 ℃. pH 7.0, glucose and ammonium sulfate as carbon and nitrogen sources, respectively. The most efficient and dominant solubilizer belongs to genera *Bacillus* and *Pseudomonas* [22]. Moreover, amount to be solubilized depends on efficiency of strains. Phosphate solubilization is done by mono- and tri- carboxylic acids, mono- and di-carboxylic hydroxyl acids and some uncommon acids, which are secreted by various phosphate solubizing bacteria [23], [24]. Organic acids lower pH as they dissociate in a PH dependent equilibrium into their respective anions and protons [25].

4) Siderophore production

Siderophore production in DR2 was confirmed by the development of orange halo zones (31mm) (Table I). Reference [26] reported maximum siderophore production in *Pseudomonas* species, Ar-3-kul (20 mm) and Pn-1-kul (21mm) isolated from apple and pear, respectively. Siderophore producing microorganisms have biocontrol abilities, acting as chelator by binding to the available form of iron (Fe^{3+}) in the rhizosphere, making it unavailable to the phytopathogens [27].

5) HCN production

The production of HCN by DR2 is evidenced by the change in color of filter paper as deep brown (Table I). The color intensity is indicator of amount of HCN produced. Our findings are similar to those of [26], who reported maximum production of HCN, where color intensity ranged from yellow to brown in five bacterial isolates of apple and pear. Mechanisms controlling plant pathogens through HCN production by rhizobacteria includes, induction of plant resistance, blocking of cytochrome oxidase activity, increase in nutrient availability [18], [28], [29].

6) ACC deaminase activity

DR2 was found to be positive for the production of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) and utilized the 1-aminocyclopropane-1-carboxylate (ACC) as a sole source of N in minimal medium (Table I). 2-5% of rhizobacteria are PGPR, which solubilise phosphate, zinc and alleviate the various plant stresses by secreting ACC, thereby increasing plant growth, biomass and yield [30].

7) Antifungal activities

DR2 was able to inhibit the growth of *Aspergillus* sp. (8mm) and *Fusarium* sp. (9mm), showing immense antifungal activity (Table I). So, it could be recommended as biocontrol measure.

D. Characterization: Phenotypic and Genotypic

The isolate DR2 was identified as *Bacillus subtilis* DR2 and deposited in the gene bank, NCBI with accession no. DR2 KP455653 [18]. Reference [31] characterized genus *Bacillus* as growth promoter, because they produce auxins and gibberellins along with ability to fix nitrogen and solubilize phosphate, whereas according to [32], the most efficient and frequently encountered phosphate solubilizing bacteria belongs to the genus *Bacillus* or *Pseudomonas*.

E. Optimization of Cultural Condition for IAA

1) Effect of incubation period

IAA production by *B. subtilis* DR2 in Trp^+ medium started after 24 h, reached maximum at 96 h and then declined gradually. In Trp^+ media, maximum (137.81 $\mu g.ml^{-1}$) IAA was produced at 96 h, which declined to 17.07 $\mu g.ml^{-1}$ at 144 h (Fig. 2). Reference [33] stated that the highest accumulation of IAA was observed after 96 h by *B. subtilis* WR-W2. Similarly, [34] also reported, the optimum IAA production after 96 h in strain *Streptomyces* sp. VSMGT1014 isolated from rice

rhizosphere. Our results are in agreement with their findings. However, in static culture, the ranges of optimum incubation period from 6 to 12 days have also been reported by [21]. The variations in incubation periods for maximum IAA production have been interpreted in terms of type (static/solid/broth) of culture, test organisms, attainment of stationary phase of growth, aeration, adsorption of growth regulators to the substrate particles, production of IAA degrading enzymes such as IAA oxidase and peroxidase, culture conditions, growth range, availability of substrates, variation in species level, etc., [19], [35], [36]. In our finding maximum IAA production at 96 h incubation, may be due to attainment of stationary growth phase and the decline after that period with several cited reasons in this discussion.

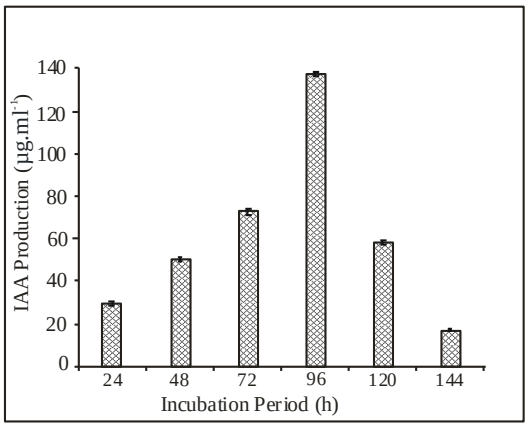

Figure 2. Effect of incubation preiod on IAA production (µg.ml^{-1}) of *B. subtilis* DR2.

2) Effect of temperature

The *B. subtilis* DR2 exhibited linear correlation with temperature upto 35 ℃ and then gradually declined. The maximum (141.92 µg.ml^{-1}) amount of IAA was recorded in Trp^{+} medium at 35 ℃ and minimum (20.88 µg.ml^{-1}) at 50 ℃ (Fig. 3). Optimum temperature 37 ℃ has been reported for IAA production for *Rhizobium* and *Bacillus* sp. [37] and in unidentified rhizobacteria isolated from aerial roots of epiphytic orchids [21]. However, 30 ℃ as optimum temperature for IAA production has been observed in *Acetobacter diazotrophicus* L1 isolated from sugarcane [35] and rhizospheric soil bacteria isolated from crop plants [38]. Reference [39], [40] have highlighted the importance of temperature in indole cell signaling, and this report confirms the earlier interpretations.

3) Effect of pH

One of the most important parameter for the growth of IAA producing organism and their metabolic activity is the pH of the production media [41]. In our investigation, maximum (158.79 µg.ml^{-1}) indole production was observed at pH 7 in Trp^{+} media (Fig. 3). pH 7 has also been reported to be suitable for maximum IAA production by *Pantoea agglomerans* PVM [42], which is similar to the present finding. Reference [21] also reached to our finding in rhizobacteria isolated from epiphytic orchids. pH 7.2 in *Rhizobium* strain VMA 301 for elaborated high levels of IAA production have been reported by [43]. However, in other publications, pH 6 has also appeared to be optimum in *Klebsiella* species isolated from the root nodules of *Vigna mungo* [44] and *Acetobacter diazotrophicus* L1 [35] for maximum indole secretion. In our finding high acidic and alkaline pH was not suitable for IAA production, which is supported by the previous findings. Moreover, as pointed by [45], the pH and temperature can affect the activity of enzymes involved in the biosynthesis of IAA.

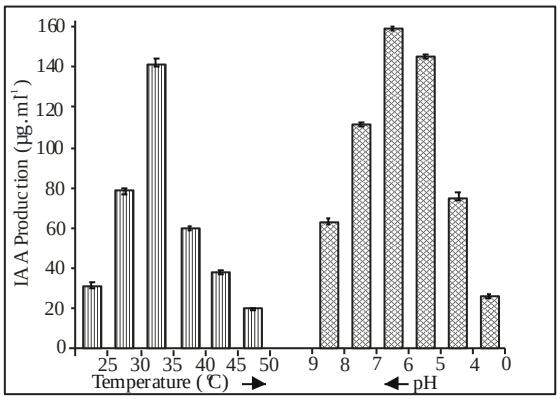

Figure 3. Effect of temperature and pH on IAA production (µg.ml^{-1}) of *B. subtilis* DR2.

4) Effect of carbon sources

The carbon sources supplemented in broth media provide energy and improves co-factor recycling in the cells [46], thus contribute to the overall efficiency of IAA biosynthesis [47]. In our investigation, presence of mannitol as C-source in the medium produced maximum (160.85 µg.ml^{-1}) indole followed by sucrose (148.44 µg.ml^{-1}) as compared to other carbon sources (Fig. 4). Different workers have optimized IAA production by different carbon sources as well as their combinations, eg., mannitol in *B. subtilis* WR-W2 [33], *Arthrobacter agilis* [6], sucrose in *Acetobacter diazotrophicus* L1 [35], mannitol and galactose [48] and mannitol and L-glutamic acid [49].

5) Effect of nitrogen sources

The effect of various inorganic nitrogen sources [$(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, KNO_3, NH_4NO_3, urea] in the Trp^{+} medium was evaluated. $(NH_4)_2SO_4$ produced maximum (162.93 µg.ml^{-1}) IAA, closely followed by NH_4Cl, NH_4NO_3, $NaNO_3$, KNO_3 and lowest (13.59 µg.ml^{-1}) in urea (Fig. 4). Our findings are supported by the statement of [49] that the nitrogen source present in the production medium affects IAA production. Different nitrogen sources have been used for various organisms by several workers for IAA optimization. Reference [35] reported NH_4Cl as most suitable for IAA production in *Acetobacter diazotrophicus*, whereas [38] found $NaNO_3$ for *B. megaterium*, KNO_3 and peptone for *Lactobacillus casei*, *B. subtilis and B. cereus*, while $NaNO_3$ and peptone for *Lactobacillus acidophilus*. However, in *Pseudomonas putida* UB1, maximum IAA was produced with $(NH_4)_2SO_4$ as nitrogen source [47], which is in conformity with the present finding. Stimulation of IAA biosynthesis in root nodule bacteria of various leguminous plants were observed, when the organic

nitrogen sources (L-asparagine and glutamic acid) were added [27].

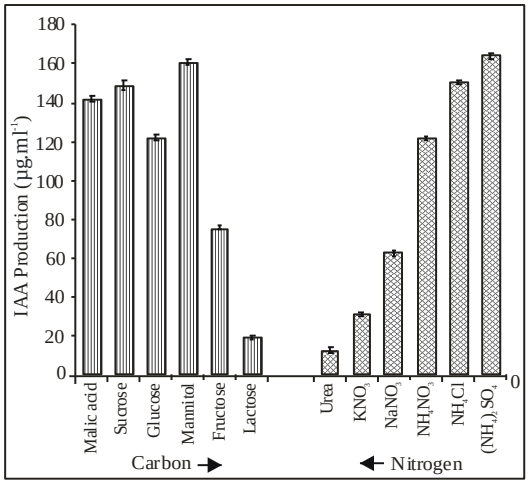

Figure 4. Effect of different carbon and nitrogen sources on IAA production (µg.ml^{-1}) of *B. subtilis* DR2

6) Effect of L-tryptophan concentrations

The IAA production increased with increasing concentrations of tryptophan. Minimum (26.40 µg.ml^{-1}) and maximum (168.09 µg.ml^{-1}) were recorded at 0.1 and 1.2 g.l^{-1} of tryptophan, respectively, beyond which slight decline was observed (Fig. 5). Similar observations have been made, where 1.2 g.l^{-1} was optimum for IAA production in *Acetobacter diazotrophicus* L1 [35]. L-tryptophan acts as physiological precursor for IAA production by microorganisms. 80% of bacteria isolated from rhizosphere synthesize IAA through different pathways: (i) indole 3-acetamide (IAM), (ii) indole-3- pyruvic acid (IPA), (iii) tryptamine (TAM), (iv) indole-3-acetonitrile (IAN), (v) tryptophan side-chain oxidase (TSO), (vi) tryptophan independent pathways [50]. Microorganisms such as *Streptomyces*, *Pseudomonas* and *Bacillus* are capable of synthesizing IAA by utilizing L-tryptophan through IPA pathway [34], [51]. Bacteria also produce indole in absence of tryptophan, though, in lesser amount [39]. Enhanced production of IAA recorded in presence of tryptophan indicates that the organism utilizes tryptophan as a precursor for IAA biosynthesis.

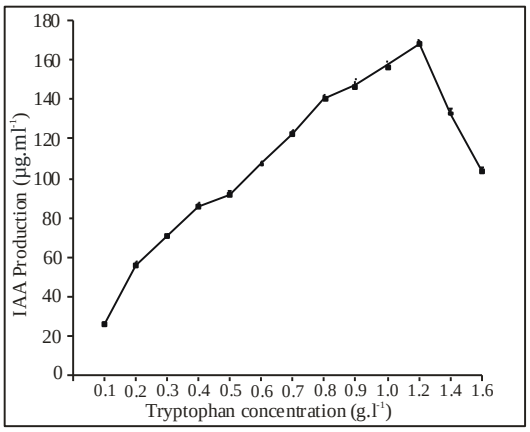

Figure 5. Effect of tryptophan concentration on IAA production (µg.ml^{-1}) of *B. subtilis* DR2.

IV. Conclusion

From this study, it is evident that rhizospheric soil of *Eragrostis cynosuroides*, a common weed can provide a rich source of plant growth promoting diazotrophic bacteria with inherent capacity to produce IAA in significant amount. Screening experiments were done to select the most active isolate for indole production along with other PGP activities like, N_2 fixation, phosphate solubilization, siderophore production, etc. *B. subtilis* DR2 emerged as novel IAA producer with efficient plant growth promoting traits. For maximum *in vitro* IAA secretion, media components, accompanied by physical parameters (incubation time, temperature, pH, C-source, N-source and tryptophan concentration) were optimized. Maximum IAA was estimated in JNFb^{-} media at 96 h incubation, 35±2 ℃ temperature, pH 7 in initial medium, supplemented with mannitol, ammonium sulfate and 1.2 g.l^{-1} tryptophan. The production has been apparently related to stationary phase of growth. The knowledge acquired in this study suggests that *B. subtilis* DR2 (KP455653) could be successfully used for large scale production of IAA by various fermentation processes and can also be used as biofertilizer, because of its several PGP activities.

Acknowledgment

The authors acknowledge kind support of ICAR, Pusa, New Delhi for facilitating the use of 5700 Nucon Gas Chromatograph (Nucon Engineers Ltd., New Delhi) and the Head, Department of Botany, Patna University, Patna for providing laboratory facilities.

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