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Research Article

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Study of phosphate solubilising *Enterobacter cloacae* sub sp. *cloacae* strain YCA for production of plant growth promoting substances

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ABSTRACT

Phosphates solubilising bacterial spp. isolated from saline soil near Kolhapur city were tested for IAA production using pure tryptophan. All isolates were then screened for production of IAA using natural sources of tryptophan such as soybean flour, pumpkin seed powder and watermelon seed powder. IAA production was tested qualitatively and quantitatively. Most effective phosphate solubilising bacterial spp. was identified as Enterobacter cloacae subsp. cloacae YCA on the basis of its morphological, biochemical characteristics and 16s rRNA gene sequencing. The isolate was found to give high yield of IAA (0.89 mg/ml) from watermelon seed powder and it was also found to produce other plant growth promoting substances such as siderophore, ammonia, organic acids and hydrogen cyanide. The isolate was moderate halophile as it tolerates NaCl and KCl up to 6%. The amount of soluble phosphorus was also found to increase over initial level in the pot where isolate was inoculated. Results of pot trials using saline and fertile soil indicate the potential use of our isolate in enhancing plant growth.

Keywords: *Indole-3-acetic acid, Nutrient broth, Kartznelson and Bose medium, Siderophore, Saline soil.*

Abbreviations: *Indole-3-acetic acid (IAA), Nutrient broth (NB), Kartznelson and Bose (KB) medium.*

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion. A large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have reported as PGPR to enhance plant growth[1]. PGPR influence plant growth by two ways; indirectly and directly. Indirectly through production of substances inhibitory to phytopahogens and directly through phosphate solubilisation, nitrogen fixation, production of phytoharmones, siderophores etc[2].

IAA (indole-3-acetic acid) is the member of the group of phytohormones and is generally considered as the most important native auxin[3]. It functions as an important signal molecule in the regulation of plant development including organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, and gene regulation[4]. Phosphate solubilising bacteria are known to solubilise insoluble phosphate and make it available to plants[5]. They are also capable of producing phytoharmones like IAA. Most bacteria use tryptophan as a precursor for IAA production[1]. This study was aimed to assess the potential of 10 phosphate solubilising bacterial isolates in producing IAA by replacing tryptophan with natural precursors such as soybean flour, watermelon seed powder and pumpkin seed powder.

MATERIAL AND METHOD

Chemicals

Orthophosphoric acid, ethyl acetate, picric acid, sodium carbonate and other chemicals were obtained from Himedia (India). All chemicals used were of highest purity and of analytical grade.

Collection of sample

Soil samples were collected from different areas nearby Kolhapur city. The collected samples were stored at controlled conditions until use.

Enrichment and isolation of phosphate solubilising bacteria

Soil samples were inoculated in sterile KB broth and incubated at 28^0C for 5 days. Repeatedly three transfers were given. Bacterial cultures from final enriched broth showing clear zone on KB agar medium indicates their ability to solubilize phosphate. These isolates were purified and coded. All cultures were maintained on respective slant media and stored at 4^0C till further use.

Screening of isolates for IAA production from tryptophan

Isolates were screened for IAA production from tryptophan by inoculating in NB containing 0.1% tryptophan. Production of IAA in culture filtrate was detected colorimetrically at 540nm using orthophosphoric acid and Salkowski's reagent[6].

Screening of isolates for IAA production using natural sources of tryptophan

Positive cultures were then screened for their ability to produce IAA in presence of natural precursors such as soybean flour, watermelon seed powder, and pumpkin seed powder instead of tryptophan.

2.6 Optimization of natural precursor concentration for IAA production

Effective isolate was inoculated in sterile NB containing various concentrations of natural precursors like 2%, 4%, 6% and 8% and incubated at 28^0C for 6 days and IAA produced was estimated using Salkowski's reagent.

Characterization and identification of potential isolate

Phenotypic characters such as morphology (Gram staining and motility) and biochemical characteristics (sugar fermentations, Voges-Proskauer, citrate utilisation, arginine hydrolysis, indole production, methyl red and lysine decarboxylase tests) of potential isolate were studied by using standard procedures as per Bergey's manual.

The 16s rRNA gene of the efficient isolate was sequenced. The nucleotide sequence was determined by automated sequencer. The nucleotide sequence was submitted to NCBI. It was aligned and analyzed by using BLAST program. The homologous sequence was used for phylogenic analysis. The phylogenic tree was constructed with Clustalx software[7].

Production and extraction of IAA

The potent isolate was inoculated in nutrient broth containing 6% watermelon seed powder. After 10 days, the fermented broth was centrifuged at 10,000 rpm for 30 min. Supernatant was acidified to pH 2.5 to 3 with 1 N HCl and IAA was extracted by adding equal volume of ethyl acetate. Ethyl acetate fraction of IAA was then evaporated to dryness and solubilised with minimum volume of methanol[8]. Presence of IAA was confirmed by paper chromatography and TLC[9]. Quantification of IAA was carried out from standard graph obtained by using pure IAA.

Study of salt tolerance ability of the isolate

Salt tolerance ability of the isolate was studied by inoculating culture in KB agar medium containing different concentrations of various salts such as NaCl (1 to7%), KCl (1to7%), Na_2CO_3 (0.1to1%) and $NaHCO_3$ (0.1to1%).

Ammonia production

Bacterial isolate was tested for the production of ammonia in peptone water. Freshly grown culture was inoculated in 10 ml peptone water and incubated for 72 hrs at 28 ± 2°C. Production of ammonia was detected by addition of Nessler's reagent[10]. Development of brown to yellow colour indicates production of ammonia by isolate.

Hydrogen cyanide (HCN) production

Qualitative determination of HCN production by the efficient isolate was done by streaking the culture on nutrient agar medium supplemented with 4.4 gm glycine/lit. Whatman filter paper no. 1 soaked in sodium carbonate in 0.5% picric acid solution was placed on the top of the plate and plate was sealed with paraffin and incubated at 28^0C for 72hrs[11]. If Whatman filter paper becomes orange coloured it indicates production of HCN.

Siderophore production

The isolate was inoculated in NB and incubated at 28^0C for 24 hrs. 5% inoculum was inoculated in low iron modified broth medium and incubated at 28^0C for 4 days with constant shaking at 200 rpm on rotary shaker. After incubation broth was centrifuged and supernatant was analysed for presence of siderophore by CAS test[12]. 1 ml filtrate was added with 1-5 ml 2% $FeCl_3$ solution. Development of red colour of filtrate indicates presence of siderophore.

Organic acid production

The major mechanism for solubilisation of insoluble inorganic phosphates by micro- organisms is through production of organic acids. Hence, the organic acid production profile of the PSB was examined, by paper chromatographic technique[13]. One ml of 24 h. old culture of isolate was inoculated to 50 ml Pikovskaya's broth[14] and incubated at 28°C for 10 days. The broth was centrifuged at 10,000 rpm for 10 min. The supernatant so obtained was concentrated to nearly 1/10th of the original volume in a water bath maintained at 60°C. The concentrated material was then used for determination of organic acids by paper chromatography in comparison with standard organic acid[15]. Standards of organic acids were prepared at 20 mg/ml stock. About 10 µl of standards and 15µl of culture supernatant was spotted on Whatman No. 1 chromatographic paper and dried with a hair dryer. A descending chromatography was run using a solvent mixture of n-butanol, acetic acid and water in 12:3:5 ratios in a chromatographic chamber pre saturated with solvent for 6 h. The chromatogram was run for 16 h and air dried for 3 days. The air dried paper was sprayed with 0.04% bromocresol green. The paper was dried at room temperature. The Rf value of yellow spot of organic acid developed on a blue background was measured and compared with the Rf values of the standard organic acids for identification.

Study of effect of isolate on plant growth

Plate test

Sterile NA plate containing precursor was inoculated with culture and surface sterilized healthy jowar seeds were kept on the surface of agar. Control plate without culture was also seeded with surface sterilized jowar seeds and plates were incubated.

Pot test [10]

Pots were prepared for both saline and fertile soils separately as follows

Set I using fertile soil

Pot A : Only Fertile soil

Pot B : Fertile soil + culture

Pot C : Fertile soil + culture + precursor (watermelon seed powder)

Set II using saline soil

Pot D : Only saline soil

Pot E : Saline soil + culture

Pot F : Saline soil + culture + precursor (watermelon seed powder)

Seeds were surface sterilized and then sown in above pots. The pots were kept in sunlight and observed for seed germination and growth of seedlings. Seedling growth was measured daily.

RESULT AND DISCUSSION

The present study is done in order to isolate phosphate solubilising bacteria capable of producing large amount of indole acetic acid. Totally, 10 phosphate solubilising bacteria were isolated on KB agar.

After purification the isolates were coded as A, B, C, D, E, F, G, H, I and J. All isolates were studied for IAA production by Salkowski's method (Table no. 1). Out of 10 isolates, isolate J was selected as it produced maximum IAA (0.03mg/ml) within 6 days of incubation. Isolates F,E,I,G,C and A also produced IAA but in remarkably less quantity, while isolates B,D,H were unable to produce IAA even after 15 days of incubation.

Seven isolates showing positive results were further tested for quantitative estimation of IAA in presence of natural sources of tryptophan. All isolates utilised watermelon seed powder and pumpkin seed powder for IAA production; however in presence of watermelon seed powder isolate J produced IAA rapidly and in more quantity (0.05mg/ml) than other isolates as depicted in table no. 2. However, soyabean flour was utilized by none of the isolates.

This potential isolate namely J, was identified by studying morphological and biochemical characteristics as per Bergey's manual. It was found to be Gram negative motile rod positive for glucose, mannitol, lactose, sucrose, citrate utilization, Voges-Proskauer and arginine hydrolysis test and negative for indole, methyl red and lysine decarboxylase test. These biochemical tests demonstrated only the genus *Enterobacter*. Conformation of this isolate was done by 16s rRNA gene sequencing. The nucleotide sequence was submitted to NCBI. It was aligned and analyzed and named as *Enterobacter cloacae subsp. cloacae YCA.* (Genbank accession no. BankIt 1742549 *Enterobacter* KM 186607). Its phylogenic position in relation to other species of same genus is illustrated in Fig.1.

The isolate when studied for IAA production using different concentrations of watermelon seed and pumpkin seed powder showed high yield of IAA (0.05mg/ml) at 6% watermelon seed powder. When concentration was increased above 6%, IAA production was found to decrease (Table no. 3). This decrease may be due to feedback inhibition[16].

Studies on optimization of incubation time showed a higher rate of IAA production on the 10^{th} day (0.89mg/ml) as shown in table no.4, in presence of watermelon seed powder at 6% concentration. IAA produced was extracted and identified by paper chromatography and thin layer chromatography by comparing with R_f value of standard IAA (Fig 2).

The isolate was moderate halophile as it tolerates NaCl and KCl up to 6%, $NaHCO_3$ up to 1% and Na_2CO_3 up to 0.5% as per Table no. 5.

It also produced ammonia, HCN, siderophore and organic acid. The produced ammonia helps in inducing plant growth as it is the most common assimilatory form of nitrogen. The production of defensive secondary metabolites like HCN and siderophore will suppress plant diseases thus indicating *Enterobacter cloacae subsp. cloacae YCA* as a bio-control agent. Since siderophore is secreted under iron deficient conditions, this ability of the isolate will help in providing iron to plants in utilizable form[17]. Organic acid secreted was detected as acetic acid which reduces the pH and contributes in phosphate mineralization. Thus enhances phosphate availability to plants[18].

The results of pot trials of set I showed that, shoot length of jowar seedlings in pot C was higher (30cm) in presence of isolate as compared to pot A (18cm) and pot B (20cm) on 29^{th} day (Fig. 5) (Table no. 6). Jowar seeds were not found to germinate in saline soil hence pot trials in saline soil were carried out using chavali seeds. For SET II, the isolate was also found to promote growth of seedlings in pot F (5.6cm), as compared to pot E (3.6cm) and pot D (dry) as shown in (Fig. 6) (Table no. 7). Soluble phosphorus content was found to increase in both pots of fertile as well as saline soils where isolate was inoculated over uninoculated control. (Table no. 8). Pot trials indicate that our isolate is beneficial for growth of plants in fertile as well as saline soil when applied with watermelon seed powder.

Table No.1: Screening and isolation of IAA producing PSB

PSB Isolates	Medium	Incubation time	IAA mg/ml
J	NB	**6 days**	**0.03**
F	with 0.1%	6 days	0.01
E,I,G,C,A	tryptophan	6 days	0.005
B,D,H		15 days	_

Table No. 2: Screening of isolates for IAA production using natural sources of tryptophan

Natural Precursors	PSB isolates	Incubation time	IAA in mg/ml
Soybean flour	J,F,E,I,G,C,A	15 days	-
Watermelon seed powder	**J**	**6 days**	**0.05**
	F,E,I,G,C,A	10 days	0.03
Pumpkin seed powder	J	6 days	0.01
	F,E,I,G,C,A	10 days	0.005

Table No. 3: Optimization of precursor concentration for IAA production using *Enterobacter cloacae* sub sp. *cloacae strain* YCA

Natural Precursor	IAA in mg/ml			
	Concentration of precursor			
	2%	4%	**6%**	8%
Watermelon seed powder	0.01	0.03	**0.05**	0.04
Pumpkin seed powder	0.01	0.02	0.03	0.01

Table No. 4: Study of incubation time for IAA production from *Enterobacter cloacae* sub sp. *cloacae* strain YCA in presence of watermelon seed powder

Medium	Incubation time (days)	Optical density (at 540nm)	Concentration of IAA (mg/ml)
NB with 6%Watermelon seed powder	6	0.11	0.05
	7	0.13	0.11
	8	0.20	0.29
	9	0.37	0.72
	10	**0.43**	**0.89**
	11	0.39	0.79
	12	0.25	0.42

Table No. 5: Salt tolerance ability of *Enterobacter cloacae* sub sp. *cloacae* strain YCA

Name of salt	Concentration of salt in %						
	1	2	3	4	5	6	7
NaCl	+	+	+	+	+	+	-
KCl	+	+	+	+	+	+	-

	0.1	0.3	0.5	0.7	0.8	0.9	1
Na_2CO_3	+	+	+	-	-	-	-
$NaHCO_3$	+	+	+	+	+	+	+

\+ Growth
\- No growth

Table No. 6: Effect of *Enterobacter cloacae* sub sp. *cloacae* YCA on the growth of jowar plants in fertile soil

Time (days)	Fertile soil only	Fertile soil + isolate	Fertile soil + isolate + precursor
	Height of seedlings in cm		
3rd day	1.0	1.0	1.0
5th day	1.0	1.5	2.9
7th day	5.0	3.0	3.0
9th day	4.6	5.0	6.0
11th day	6.5	8.0	8.0
13th day	7.0	11.0	12.0
15th day	9.0	15.0	16.0
17th day	10.0	16.0	18.0
19th day	11.0	16.5	19.0
21th day	11.3	17.0	20.0
23th day	13.0	17.5	23.0
25th day	15.0	18.0	26.0
27th day	17.0	19.0	29.0
29th day	**18.0**	**20.0**	**30.0**

Table No. 7 Effect of *Enterobacter cloacae* sub sp. *cloacae* YCA on the growth of Chavali plants in saline soil

Time (days)	saline soil only	Saline soil + Isolate	Saline soil + Isolate + Precursor
	Height of seedlings		
6th day	1.0 mm	1.0 mm	2.0 mm
8th day	3.0 mm	6.0 mm	1.2 cm
10th day	5.0 mm	9.0 mm	2.0 cm
12th day	9.0 mm	1.2 cm	3.2 cm
14th day	1.1 mm	1.3 cm	3.3 cm
16th day	1.2 mm	2.0 cm	4.0 cm
18th day	Dry	2.4 cm	4.4 cm
20th day	Dry	2.7 cm	4.9 cm
22st day	Dry	3.1 cm	5.2 cm
24nd day	**Dry**	**3.6 cm**	**5.6 cm**

Table No. 8 Effect of *Enterobacter cloacae* sub sp. *cloacae* YCA on the soluble phosphorus content of soil

	Fertile soil	Saline soil
Initial phosphorus	2 kg/acre	3 kg/acre
	After 30 days	
Soil + Isolate	3 kg/acre	6 kg/acre
Soil + Isolate + Precursor	**5** kg/acre	**7** kg/acre

Fig.1: A tree showing phylogenetic affinity of the potent isolate [i.e. *Enterobacter cloacae subsp. cloacae* YCA] to other members of Genus: *Enterobacter*

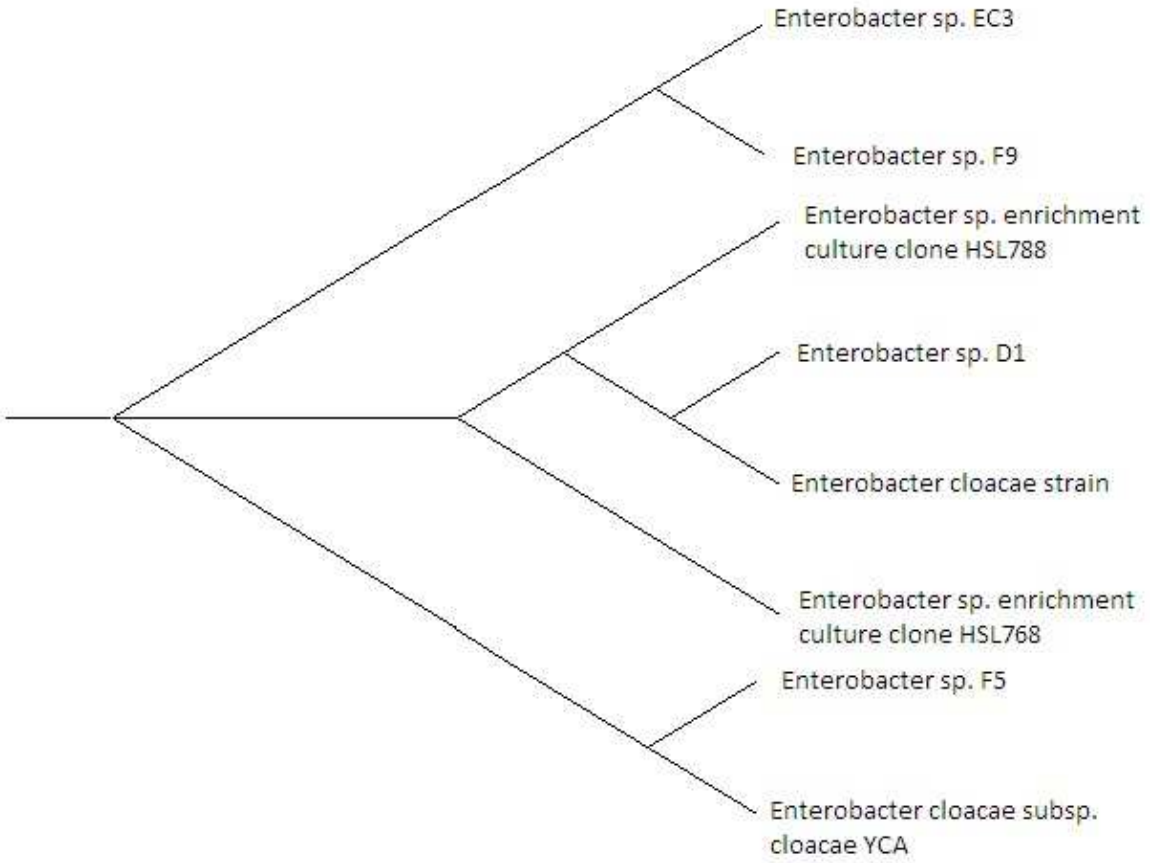

Fig.2: Silica gel plate showing pink spot of pure IAA and extracted IAA

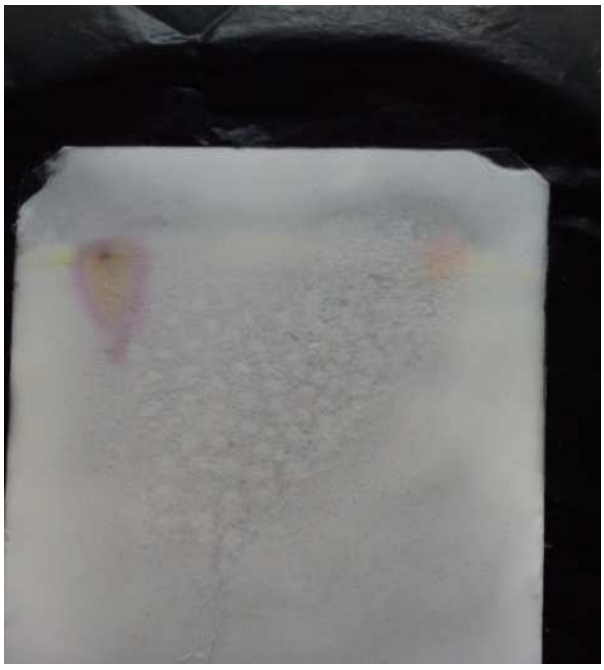

Fig.3: Plate test in presence of *Enterobacter cloacae* sub sp. *cloacae* strain YCA

Fig. 4: Plate test in absence of *Enterobacter cloacae* sub sp. *cloacae* strain YCA

Fig.5: Pot trials of fertile soils in presence of *Enterobacter cloacae* sub sp. *cloacae* strain YCA and watermelon seed powder

Fig.6. Pot trials of saline soil in presence of *Enterobacter cloacae* sub sp. *cloacae* strain YCA and watermelon seed powder

CONCLUSION

Amongst various natural precursors, watermelon seed powder in 6% concentration was found to be more effective for IAA production from *Enterobacter cloacae subsp. cloacae strain YCA* and yield obtained was 0.89 mg/ml after 10 days of incubation. This isolate was found to produce HCN, organic acid, ammonia and siderophore. It resulted in significant increase in shoot length in both saline and fertile soil in presence of precursor. The isolate was also found to be moderate halophile. The present study suggests that phosphate solubilization and IAA production in presence of easily available and cheap precursor i.e. watermelon seed powder is a beneficial aspect of *Enterobacter cloacae subsp. cloacae* strain YCA in enhancing crop yield. Thus using it as a biofertilizer along with watermelon seeds for fertile and saline soil will be an ecofriendly, economically feasible and promising alternative in the hands of farmers.

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