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OPTIMIZATION OF CULTURAL CONDITIONS FOR HIGH PRODUCTION OF PHOSPHATE SOLUBILIZATION BY FLUORESCENT *PSEUDOMONAS* SP.

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ABSTRACT
A total of ten fluorescent *Pseudomonas* sp. were isolated from apple rhizosphere of Himachal Pradesh. These were characterized on the basis of morphological, physiological and biochemical characteristics. The present investigation was undertaken to optimize culture conditions for high production of P-solubilising activity by two fluorescent *Pseudomonas* sp. An-2-nali and Pn-2-kho as cultural conditions play an important role in cellular growth and production of biological activities. From the results it is concluded that out of four media i.e. PVK, NBRI-P, NBRI-Y and Kings B, the maximum P solubilization (427.5 μg/ml by both An-2-nali and Pn-2-kho) was observed in NBRI-P after 48 hr. The result showed that the efficiency of a medium for supporting P-solubilization in terms of available phosphate (Pi) was of following order: NBRI-P (427.5 μg/ml) > NBRI-Y (395 μg/ml) > PVK (330 μg/ml) > King's B (260 μg/ml) in case Pn-2-kho and similar decrease was observed for isolate An-2-nali. The maximum P-solubilization was obtained at temperature 28 ºC (427.5 μg/ml by An-2-nali and 410 μg/ml by Pn-2-kho), pH 7 and at an incubation time of 48 hr. A decline in the yield of these activities was observed above and below these conditions.

INTRODUCTION

Microorganisms require carbon, nitrogen, phosphorus, sulphur, minerals, vitamins and other growth factors. They are sensitive to temperature, oxygen and pH in their environment (Jennings, 1995). Understanding which environmental factors are important and how these influence the production of secondary metabolic activities is important. The culture conditions of microorganisms play an important role in production of biological activities (Kotake *et al*., 1992). As the physiological and nutritional requirement of an organism is genetically predetermined, it is important to provide the appropriate carbon and nitrogen source and also the proper environment for optimal production of activity.

The challenge is faced to provide the organisms with conditions that allow expression of secondary metabolites and accumulations of these metabolites (Bushell, 1989 and Demain, 1992). A number of media are employed for expression of secondary metabolites by microorganisms and initial evaluations of media are usually made (Jennings, 1995). The microorganism may produce one metabolite on one medium and a totally different one on another medium (Oxford *et al*., 1935). So, the development of culture media, which increases secondary metabolite production by micro organisms, is very important.

Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants. Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in plants (Sagervanshi *et al*., 2012). The fluorescent *Pseudomonas* sp. are known to be efficient phosphate solubilizers (Gulati *et al*., 2007 and Sharma *et al*., 2014a). The production of P solubilizing activity has been found to be highly dependent on the cultural conditions. Each species or a strain has a characteristic minimum, optimum and maximum temperature. The optimal temperature for growth may not be that best suited to product formation especially where the product is predominantly non growth associated as in the case of many secondary metabolites (Woodruff, 1961). Thus considering the importance of above mentioned topics, in the present study an attempt has been made to screen indigenous fluorescent *Pseudomonas* sp. for phosphate solubilization and to optimize suitable cultural conditions to enhance its solubilization potential that could induce plant growth and can be used for bioformulation development.

MATERIALS AND METHODS

Isolation and identification of fluorescent *Pseudomonas* sp.

The isolation was carried out from rhizospheric soil samples of apple plants planted in Shimla distt. of Himachal Pradesh. The soil samples were collected in aseptic bags and immediately transported to laboratory under cold conditions (4ºC) for further processing. The fluorescent *Pseudomonas* sp. was isolated using spread plate method using specific King's B medium (King *et al*., 1954). The composition of the medium was (g/l^{-1}): Peptone, 20.0; K_2HPO_4, 1.5; $MgSO_4.7H_2O$,

1.5; Glycerol, 15.0mL. The colonies on KMB plates were observed and selected isolates were further subjected to confirmatory biochemical and physiological tests as prescribed in Bergey's Manual of Systematic Bacteriology (Palleroni, 1984). For identification of fluorescent *Pseudomonas* colonies on the King's B plates, standard microbiological tests were conducted viz., Gram's staining, pigment production, oxidase test, catalase test, gelatine liquification, denitrification test and growth at 4ºC and 41ºC (Reynolds, 2004). Pure culture of *Pseudomonas* sp. was obtained following successive selection of fluorescing colonies on King's B medium under UV light at 365 nm (Rachid and Ahmed, 2005).

***In vitro* characterization of fluorescent *Pseudomonas* sp. for phosphate solubilization**

The phosphate solubilizing ability of fluorescent *Pseudomonas* isolates were estimated in Pikovskaya's agar medium (Pikovskaya, 1948) using a known amount of inert phosphorus source (tricalcium phosphate). The composition of the medium was (g/l^{-1}): Glucose, 10.0; $Ca_3(PO_4)$, 5.0; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.2; $MnSO_4$, 0.1; $FeSO_4$, 0.0001; Yeast extract, 0.5; Bromocresol purple, 0.1. Phosphate solubilization was expressed in terms of mm diameter of yellow colored zone produced around the well. Quantitative analysis of solubilization of tricalcium phosphate in liquid medium was made as described by Bray and Kurtz (1945). The amount of soluble phosphorus was detected from standard curve of Potassium dihydrogen orthophosphate (KH_2PO_4).

Optimization of culture conditions for enhanced phosphate solubilizing activity

Effect of different media

Effect of different media on production of phosphate solubilizing activity were studied by growing selected *Pseudomonas* sp. in each media broth *i.e.,* Pikovskaya's (Pikovskaya, 1948), National Botanical Research Institute-P (NBRI-P) - Glucose, 10.0; $Ca_3(PO_4)_2$, 5.0; $MgCl_2.6H_2O$, 5.0; $MgSO_4.7H_2O$, 2.5; KCl, 0.2; NH_4SO_4, 0.1 (Nautiyal,1999); National Botanical Research Institute-Y (NBRI-Y) - Glucose, 10.0; $Ca_3(PO_4)_2$, 5.0; NH_4SO_4, 0.5; NaCl, 0.2; $MgSO_4.7H_2O$, 0.1; KCl, 0.2; $MnSO_4.7H_2O$, 0.002; $FeSO_4.7H_2O$, 0.02 (Nautiyal, 1999) and King's B. In each case 0.5 ml of inoculum of overnight grown culture of *Pseudomonas* sp. was used to inoculate 100 ml of each media in a 250 ml Erlenmeyer flask. Flasks were incubated at 28 ± 2ºC under shake conditions (100 rpm). Growth was observed as absorbance at 540 nm. Cultures were centrifuged at 10,000 rpm for 20 minutes at 4ºC and cell free culture supernatants were separated. Phosphate solubilizing activity was assayed qualitatively and quantitatively by their respective methods (Dickman and Bray, 1940; Bray and Kurtz, 1945; Olsen *et al*., 1954). Phosphate solubilizing activity was expressed in terms of mm diameter of pinkish/yellow zone produced around the well by 100 ìl of cell free culture supernatant. Quantitative estimation of phosphate solubilizing activity was done by using spectrophotometric assay at 660 nm as described by Bray and Kurtz (1945).

Effect of incubation time

Effect of incubation time on production of phosphate solubilizing activity was studied by growing *Pseudomonas* sp. for different time intervals of 24 hr, 48 hr, 72 hr, 96 hr. 0.5 ml of inoculum of overnight grown culture of each *Pseudomonas* sp. isolate was used to inoculate 100 ml of each media broth *i.e.,* Pikovskaya's, NBRI-P, NBRI-Y and King's B. Phosphate solubilizing activity was assayed by their respective well plate assay method. Plates were incubated at 28 ± 2 ºC for 48 h and were observed for yellow zone produced around the well by 100 ìl of cell free culture supernatant. Calorimetric estimation of phosphate solubilizing activity was done by using spectrophotometric method at 660 nm (Dickman and Bray, 1940; Bray and Kurtz, 1945; Olsen *et al*., 1954).

Effect of different incubation temperature

The effect of different temperature *i.e.* 4°C, 28°C, 37°C and 50°C on phosphate solubilizing activity was studied by using best selected medium *viz*., NBRI-P (National Botanical Research Institute-P). 0.5 ml of inoculum of overnight grown culture of *Pseudomonas* sp. was used to inoculate 100 ml of media in a 250 ml Erlenmeyer flask. Flasks were incubated at different temperatures *i.e.* 4°C, 28°C, 37°C and 50°C for 48 hr. The growth was also observed as absorbance at 540 nm at different temperatures. Phosphate solubilizing activity was assayed qualitatively and quantitatively by their respective method (Dickman and Bray, 1940; Bray and Kurtz, 1945; Olsen *et al*., 1954).

Effect of pH

Effect of different pH on the growth and production of phosphate solubilizing activity was studied using the best selected NBRI-P medium of different pH *i.e.* 5, 6, 7 and 8 and keeping all other parameters constant *i.e.* temperature (28 °C), incubation time 48 hr. The growth was observed as absorbance at 540 nm at different pH. Phosphate solubilizing activity was assayed by well plate assay method and liquid assay method (Dickman and Bray, 1940; Bray and Kurtz, 1945; Olsen *et al*., 1954).

RESULTS AND DISCUSSION

In the present investigation, a total of ten bacterial isolates were isolated from apple rhizosphere. The results of the morphological, physiological and biochemical tests performed for the identification of the effective native isolates of fluorescent *Pseudomonas* sp. showed that all the isolates were non spore forming, fluorescent with transparent to translucent colonies (irregular to rhizoid colonies on nutrient agar plates) and produced similar results with regard to gram staining (negative), starch hydrolysis (negative), gelatin liquification (positive), catalase test (positive), oxidase test (positive) and fluorescent pigmentation (positive). According to Todar (2004), more than half of the *Pseudomonas* bacteria produce pyocyanin which is a blue-green pigment, while *Pseudomonas fluorescens* produces fluorescent pigment that is soluble and greenish. In this study, all the isolates produced blue-green fluorescent pigment on King's B medium under ultraviolet light at 365 nm. The biochemical tests *i.e* catalase test, oxidase test, gelatin liquification, starch hydrolysis, IAA production, siderophore production and hydrogen cyanide production further confirmed the isolates to be as belonging to fluorecent *Pseudomonas as* reported by earlier workers

Table 1: Effect of different media on the growth and production of phosphate solubilizing activity at 28° C temperature under shake condition for different time of incubation

Fluorescent *Pseudomonas* isolates	Medium	Growth[1] at 540 nm				Mean	Phosphate solubilizingactivity, yellow zone(mm dia)[2]				Mean	Phosphate solubilizingactivity , concentration (μg/ml)[3]				Mean
		24 hr	48 hr	72 hr	96 hr		24 hr	48 hr	72 hr	96 hr		24 hr	48 hr	72 hr	96 hr	
An-2-nali	PVK	0.621	0.662	0.620	0.591	0.623	15	28	42	55	35	325	410	362.5	385	370.6
	NBRI-P	0.498	0.712	0.621	0.602	0.608	25	50	73	88	59	340	427.5	365	380	378.1
	NBRI-Y	0.501	0.690	0.582	0.560	0.585	35	53	70	86	61	285	410	285	385	341.2
	King's	0.726	0.890	0.752	0.739	0.776	-	-	-	-	0	270	280	277.5	285	286.5
Pn-2-kho	PVK	0.560	0.704	0.642	0.639	0.636	14	25	40	59	34.50	260	330	265	300	288.7
	NBRI-P	0.522	0.804	0.768	0.765	0.714	28	52	75	89	61	362.5	427.5	415	420	406.2
	NBRI-Y	0.602	0.748	0.651	0.642	0.660	30	41	72	85	57	295	395	300	342	333.0
	King's	0.781	0.912	0.852	0.839	0.846	-	-	-	-	0	160	260	162.5	255	217.7
Mean		0.601	0.765	0.686	0.673		18.37	31.12	46.50	57.75		278.18	367.5	304.06	278.1	
$CD_{0.05}$																
T					.004					1.41					11.50	
I					.003					0.99					8.31	
T×I					.009					2.81					22.92	

- Indicates no zone formation ; [1] Growth in terms of optical density at 540 nm at different time of incubation; [2] Phosphate solubilizing activity expressed in terms of mm diameter of yellow zone around the well on different media at 28ºC; [3] Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization ,which in turn represents μg/ml of available orthophosphate as calibrated from the standard curve of KH_2PO_4 (10-100 μg/ml)

Table 2: Effect of different temperatures on the growth and solubilization of tricalcium phosphate by selected fluorescent *Pseudomonas* sp. in NBRI-P medium for 48 hr

Fluorescent *Pseudomonas* isolates	Growth[1] at 540 nm Temperature (º C)				Mean	Yellow zone (mm dia)[2] Temperature (º C)				Mean	Pi (μg/ml)[3] Temperature (º C)				Mean
	4	28	37	50		4	28	37	50		4	28	37	50	
An-2-nali	0.062	0.782	0.642	0.122	0.40	-	36	35	-	17.75	40	427.5	385	20	218.1
Pn-2-kho	0.031	0.675	0.576	0.156	0.35	-	28	15	-	10.75	60	410	330	10	202.5
Mean	0.046	0.72	0.60	0.13		0	32	25	0		50	418.7	357.5	15	
$CD_{0.05}$															
T					.001					1.22					1.62
I					.002					1.73					2.29
T×I					.003					2.44					3.24

-Indicates no zone formation; [1]Growth in terms of optical density at 540 nm at different temperature; [2] Phosphate solubilizing activity expressed in terms of mm diameter of yellow zone around the well at different temperature; [3]Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization ,which in turn represents μg/ml of available orthophosphate as calibrated from the standard curve of KH_2PO_4 (10-100 μg/ml) at different temperatures.

Table 3: Effect of pH on the growth and solubilization of tricalcium phosphate by selected fluorescent *Pseudomonas* sp. in NBRI-P medium at 28ºC for 48 hr under shake conditions

Fluorescent *Pseudomonas* isolates	Growth[1] at 540 nm pH				Mean	Yellow zone (mm dia)[2] pH				Mean	Pi (μg/ml)[3] pH				Mean
	5	6	7	8		5	6	7	8		5	6	7	8	
An-2-nali	0.648	0.712	0.749	0.702	0.702	27	28	36	38	32.25	325	362.5	410	370	366.9
Pn-2-kho	0.689	0.758	0.894	0.764	0.776	30	30	36	37	33.25	295	340	385	365	346.2
Mean	0.668	0.735	0.821	0.733		28.50	29.0	36.03	37.50		310	351.2	397.5	367.5	
$CD_{0.05}$															
T					.001					1.73					1.62
I					.002					2.44					2.29
T×I					.003					3.45					3.22

[1]Growth in terms of optical density at 540 nm on NBRI-P media; [2] Phosphate solubilizing activity expressed in terms of mm diameter of yellow zone around the well on different pH at 28ºC; [3] Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization ,which in turn represents μg/ml of available orthophosphate as calibrated from the standard curve of KH_2PO_4 (10-100 μg/ml)

(Nathan *et al*., 2011; Tiwary and Balabaskar, 2012; Sharma *et al*., 2014b; Verma and Kaur, 2016). Rao *et al*., (1999) also identified *Pseudomonas* sp. on the basis of similar biochemical tests. Based on the colony morphology, microscopic observation, cultural, biochemical and physiological properties, the isolates were identified as belonging to genus *Pseudomonas* sp.

Culture conditions play an important role in cellular growth and also in production of biological activities by microorganisms. Although a good growth may occur in many media but secondary metabolites may only be produced in a specific medium. Sometimes a given organisms may produce one metabolite on one medium and a totally different one on another medium. Variation in the chemical composition of the medium and its relationship to yield different types of secondary metabolites is well known. Development of medium that produces high yields of desired secondary metabolite is still empirical to a considerable degree (Demain, 1973). Different organisms are able to grow in different types of growth media but the growth and biological activity may vary since organisms face different environmental factors affecting the production of biological activity by organisms The conducted experiments was focused on the identification of a suitable cultural conditions for two selected fluorescent *Pseudomonas* sp. *viz*.,

An-2-nali and Pn-2-kho that could induce higher levels of P solubilization.

The growth and production of phosphate solubilizing activity by fluorescent *Pseudomonas* sp. An-2-nali and Pn-2-kho (Table 1) was studied under shake conditions (90 rpm) at different time of incubation (24 hr, 48 hr, 72 hr, 96 hr) in four cultural media (PVK, NBRI-P, NBRI-Y and King's B medium). The effect of different media and incubation time on phosphate solubilizing activity by fluorescent *Pseudomonas* sp. revealed that the maximum phosphate solubilisation was observed in NBRI-P medium. The maximum P-solubilization in terms of mm diameter was observed by Pn-2-kho (89 mm) after 96 hr of incubation whereas the maximum release of available phosphate (Pi) was observed by both the isolates viz., An-2-nali and Pn-2-kho (427.5 μg/ml) after 48 hr of incubation in NBRI-P medium. Similar results were observed by Thakur *et al*., (2014), who observed the highest P-solubilization in NBRI-P medium by isolate Pn-2-Panch (899 μg/ml) after 48 hr of incubation. The best media for the production of phosphate solubilizing activity in our study was found to be NBRI-P, which is supported by Nautiyal (1999) who also conducted experiment with PVK, NBRI-Y and NBRI-P medium for the selection of best medium for production of phosphate solubilizing activity. It was observed that glucose and $Ca_3(PO_4)_2$ were essential and yeast extract, ammonium sulphate were non essential component of the medium. Therefore the yeast extract was omitted from PVK medium to formulate a new medium which was devoid of yeast extract and was designated as NBRIY. In this medium ammonium sulphate was replaced by potassium nitrate to formulate NBRI-P medium. NBRI-P media has been used by Panhwar *et al*. (2012) for the isolation of P solubilizing bacteria from aerobic rice, who also concluded that highest P solubilizing activity was found in NBRI-P media. Lynn *et al*., (2013) also used NBRI-P media for characterization of P solubilizing strains from tomato. The result showed that the efficiency of a medium for supporting phosphate solubilizing activity in terms of available phosphate (Pi) was of following order: NBRI-P > NBRI-Y > PVK > King's B medium.

The production of phosphate solubilizing activity was observed at different temperature *i*.e. 4ºC, 28ºC, 37ºC and 50ºC. The maximum phosphate solubilizing activity was recorded at 28ºC (Table 2) for both the isolates and the maximum phosphate solubilizing activity in terms of available phosphate (Pi) was observed in An-2-nali (427.5μg/ml) where as in Pn-2-kho, it was 410μg/ml. However, decline in yield of these activities was observed above and below 28°C. The maximum phosphate solubilizing activity by isolates was observed at 48 hr of incubation. Our results collaborates with Thakur *et al*. (2014), who reported that the maximum P-solubilization activity was produced at 28ºC by all the *Pseudomonas* isolates and a decrease in yield of these activities was observed above and below 28ºC. Mishra *et al*. (2009) also reported that *Pseudomonas lurida* grew at temperature ranging from 4 to 30ºC, with a growth optimum at 28ºC. Production of more P-solubilizing activity at optimum temperature may be due to faster metabolic activity and increase in protein content and extracellular production in culture supernatant. From the results it could be observed that P-solubilizing activity in *Pseudomonas* isolates were not growth associated and corroborate with earlier report (Meyer and Abdallah, 1978).

The optimum pH of the medium for the production of phosphate solubilizing activity by fluorescent *Pseudomonas* isolates was determined by using National Botanical Research Institute-P (NBRI-P) media of different pH ranging from 5 to 8 at 28º C. The results (Table 3) showed that the optimum pH for the production of maximum phosphate solubilizing activity was pH 7. The maximum phosphate solubilizing activity in terms of available phosphate (Pi) was observed in isolate An-2-nali (410 μg/ml) at pH 7 and the minimum phosphate solubilizing activity was observed at pH 5 (325 μg/ml). In our study, pH 7 is found to be best for the growth as well as for P solubilizing activity which is also in collaboration with Thakur *et al*., (2014) and Jena, (2013) who concluded pH 7.0 as optimum for the P-solubilizing activity of the *Pseudomonas* isolates. These fluorescent *Pseudomonas* isolates from apple rhizosphere apart from possessing phosphate solubilising activity also possessed other plant growth promoting activities thus making them as a promising inoculant for other crops.

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