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STUDY OF PHOSPHATE SOLUBILIZATION AND CELLULASE DEGRADATION ACTIVITY OF ANTIFUNGAL BACILLUS ISOLATES AGAINST FUSARIUM AND PYTHIUM SPECIES.

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Abstract:
Among '150' *Bacillus* isolates obtained from rhizosphere of healthy crop plants, eight isolates showing potent antifungal activity (percent growth inhibition >50%) against the phytopathogenic *Fusarium* and *Pythium* species were selected by dual culture (Co-culture) method, using PDA and PDB. These antifungal isolates were identified on the basis of morphological, cultural and biochemical characters as well as 16S r-RNA gene sequencing as *Bacillus thuringiensis*184, *B. subtilis*208, *B. thuringiensis*211, *B. cereus*220, *B. cereus*228, *B. subtilis*252, *B. subtilis*260 and *B. subtilis*288 and used to prepare biocontrol formulations with dried fecal pellets of sheep and goats. These *Bacillus* isolates were also studied for phosphate solubilization and cellulose degradation abilities using Pikovskaya (PVK) agar and Carboxyl methyl cellulose (CMC)- Peptone medium, respectively. Among the eight isolates, three isolates i.e. *Bacillus subtilis*208, *B. cereus*220 and *B. subtilis*252 were showed good phosphate solubilization ability whereas only *B. subtilis*252 showed considerable cellulase activity. We conclude that, application of these three isolates in soil will be significant not only as biological control agents of fungal diseases but also as plant growth promoting rhizobacteria (PGPR) to increase soil fertility, especially with respect to phosphate solubilization and cellulose mineralization.

Keywords: Antifungal *Bacillus*, Phosphate solubilization, Cellulase activity.

Introduction

India is an agro-based country and about more than 50% population is either directly or indirectly dependent on agriculture. Crop yield depends on mainly the soil fertility, control of crop diseases and weather. Indiscriminate use of chemical fertilizers and disease control agents has created many environmental and human health problems. Hence, use of biofertilizers and biological control agents of crop diseases has become an indispensable need of sustainable agriculture. Many species of microorganisms have been isolated from rhizosphere of healthy crop plants and disease suppressive soils and used to prepare biofertilizers and biocontrol formulations. Many rhizosphere microorganisms increase the soil fertility by conducting significant agrochemical processes such as mineralization of complex organic compounds, symbiotic and nonsymbiotic nitrogen fixation, solubilization of phosphorous compounds in soil, etc. [1,2]. These soil microbes are so called 'plant growth promoting microbes' (PGPM). Among the bacteria, species of Pseudomonas and Bacillus have been widely studied and developed as biocontrol agents, some of which also act as biofertilizers [3,4]. Phosphorus is the second most important mineral next to nitrogen required for the growth of plants and microorganisms. It constitutes about 3% cell dry weight mainly in nucleic acids and free nucleotides. However, a major quantity of soil phosphorus exists as organic compounds and insoluble inorganic compounds, such as iron and aluminium phosphates in acidic soils and calcium phosphates in alkaline soil, which are chemically locked for plants [5,6]. Microorganisms dissolve this phosphate mainly by producing organic and inorganic acids.

A tremendous amount of agricultural waste is added in soil which is essential to be mineralized by soil microbes. Cellulose, a homopolysaccharide of glucose contributes a major portion. Cellulose digesting microorganisms with the catalytic activity of cellulases are involved in this process. Cellulases is a multienzyme complex consisting of three enzymes- Endo-β-glucanase/ CMCase (EC 3.2.1.4), Exo-β-glucanase/ Cellobiohydrolase (EC3.2.1.91) and β-glucosidase/ Cellobiase [7]. Cellulolytic activity of soil microorganisms is not only significant for biodegradation of cellulosic wastes but also as one of the mechanism of antifungal activity against phytopathogens. The cellulolytic activity of microorganisms is involved in degradation of cell wall of fungal phytopathogens, especially that of 'Oomycetes' [8].

Our aim was to isolate Bacillus species from soil, with antagonistic potential against phytopathogenic Fusarium and Pythium species, phosphate solubilizing ability, and cellulose mineralization potential and to develop a 'all rounder' formulations applicable

in soil as biocontrol agents as well as biofertilizers.

Materials and Methods

Isolation and Identification of phytopathogenic fungal cultures-

Plant pathogenic fungal species were isolated from infected plant material by tissue segment method on PDA supplemented with streptomycin @ 25mg/lit. The fungal cultures were identified on the basis of cultural characters of one week PDA growth and microscopic characters by mounting with cotton blue. Phytopathogenic Pythium and Fusarium species were selected for further study [9].

Isolation and Identification of Bacillus species-

150 Bacillus species were isolated from rhizoplane soil samples of healthy crop plants and putatively identified to genus level on the basis of microscopic characters, Gram nature, motility by hanging drop technique and ability to form endospore by Schaeffer-Fulton's method [9].

Screening of antifungal Bacillus isolates-

Bacillus isolates with good antagonistic potential against Fusarium and Pythium species were selected by Dual culture (Co-culture) method, using PDA and PDB [9]. (Sandikar and Awasthi, 2009). Results were recorded in terms of diameter of inhibition zone (I.Z.) on PDA and percent growth inhibition (P.G.I.) in PDB, on 6th day [9,10].

Study of phosphate solubilizing abilities of antifungal Bacillus isolates

Eight potent antifungal Bacillus isolates obtained by primary and secondary screening were used to test phosphate solubilization ability. The active nutrient broth cultures were individually spot inoculated on Pikovskaya (PVK) agar (Hi-Media M520) plates, three cultures per plate at equidistance and incubated at 280C. Zone of clearance around the growth was observed up to 3 days [2].

Secondary screening of efficient phosphate solubilizers

The Bacillus isolates showing phosphate solubilization ability in primary screening were individually spot inoculated at the center of PVK plate and incubated at 280C. Diameter of clearance zone was measured successively after 24 h, up to 6 days. The P solubilization efficiency (PSE) was calculated on 6th day as the ratio of total diameter of clearance zone including bacterial growth (Z) and the colony diameter (C), multiplied by 100. PSE = Z/C×100 [2,5]. (Gothwal et al., 2006; Sandikar and Awasthi, 2008).

Study of cellulolytic activity of antifungal Bacillus isolates-

Induction of cellulase activity in cellulose broth-

Cellulases are the extracellular and inducible enzymes. 1ml active broth culture of each antifungal bacterial isolate was separately inoculated in 100ml of Carboxyl methyl cellulose (CMC)- Peptone medium (containing g/l of- Peptone- 10.0 Yeast extract 5.0, NaCl 5.0, KH2PO4 5.0 and Carboxyl methyl cellulose 10.0) in 250ml Erlenmeyer flasks [8,9]. (Singh et. al., 2001; Sandikar and Awasthi, 2009) and incubated on shaker incubator at 280C with 120rpm, up to a week.

Detection of cellulolytic activity by DNSA method-

3,5 dinitro salicylic acid DNSA (yellow) undergoes reduction in hot alkaline solution to 3-amino-5-nitrosalicylic acid (orange-red) in presence of the reducing sugars. The intensity of the coloured product correlates with the amount of reducing sugar produced and the extent of cellulolytic activity [11].

10ml of each CMC broth culture was pipetted successively on 3rd, 4th, 5th and 6th day under aseptic conditions and centrifuged at 10,000 rpm for 10min. 3ml culture supernatant and 1ml DNSA reagent was mixed in a test tube. A control (blank) tube containing 1ml reagent + 3ml sterile broth was also prepared. The test tubes along with control tubes were covered with marble and placed in boiling waterbath for 5min. The tubes were cooled to room temperature under tap water and optical density (OD) was read on spectrophotometer at 540nm, against the blank solution. The amount of reducing sugar produced was determined by using standard graph [8,11]. Cellulose degradation activity was also tested by mixing together all the eight antifungal Bacillus isolates.

Results

Table-1 Antifungal activity and Phosphate solubilization ability of *Bacillus* isolates-

***Bacillus* isolates**	**Antifungal activity against *Fusarium* species**		**Antifungal activity against *Pythium* species**		**PSE**
	I Z mm	**PGI**	**I Z**	**PGI**	
***B. thuringiensis*184**	32	73.63	34	72.08	00
***B. subtilis*208**	26	63.63	30	64.16	225
***B. thuringiensis*211**	24	51.81	21	51.66	00
***B. cereus*220**	30	72.18	31	70.08	280
***B. cereus*228**	28	62.50	28	61.66	00
***B. subtilis*252**	34	*78.63*	35	76.66	330
***B. thuringiensis*260**	22	50.45	22	50.91	00
***B. subtilis*288**	27	51.18	30	50.00	00

I. Z.- Inhibition zone on PDA and P.G.I.- Percent growth inhibition in PDB, on 6th day.
PSE- Phosphate solubilization efficiency on 6th day. All values are average of triplicates

Table-2 Cellulase activity of antifungal Bacillus isolates

***Bacillus* isolates**	**Glucose concentration (µg/ml)**			
	3rd Day	4th Day	5th Day	6th Day
***B. thuringiensis*184**	00	00	00	00
***B. subtilis*208**	00	00	06	08
***B. thuringiensis*211**	00	00	00	00
***B. cereus*220**	06	06	07	08
***B. cereus*228**	00	00	00	00
***B. subtilis*252**	20	32	45	68
***B. thuringiensis*260**	00	00	00	06
***B. subtilis*288**	00	00	00	00
Mixed culture	34	48	88	120

The values are average of triplicate tests.

Discussion:

Screening and Identification of antifungal *Bacillus* species

Eight isolates of Bacillus showing potent antifungal activity (percent growth inhibition > 50%) against the phytopathogenic Fusarium and Pythium species obtained by dual culture (Co-culture) method, using PDA and PDB were identified as Bacillus thuringiensis184, B. subtilis208, B. thuringiensis211, B. cereus220, B. cereus228, B. subtilis252, B. subtilis260 and B. subtilis288.

The antifungal activity of Bacillus isolates was found to vary with species to species as well as with the type of phytopathogen i.e. Fusarium and Pythium. Three isolates i.e. B. thuringiensis184, B. cereus220 and B. subtilis252 have shown very good antifungal activity i.e. PGI values >70% and diameter of inhibition zone >30mm. B. subtilis252 was proved to be the best isolate in this respect with PGI up to 78.63%. All these antifungal Bacillus isolates were used to prepare biocontrol formulations with dried fecal pellets of sheep and goats and proved successful, Sandikar and Awasthi (2009).

Study of Phosphate solubilization ability of Bacillus species

Among the eight isolates, three isolates i.e. Bacillus subtilis208, B. cereus220 and B. subtilis252 were showed good phosphate solubilization efficiency i.e. 225, 280 and 330 respectively (table-1). It is important to note that, among the three isolates which have shown maximum antifungal activity, two isolates i.e. B. subtilis252 and B. cereus220 also shown maximum phosphate solubilization efficiency (PSI) i.e. 330 and 280, respectively. Bacillus subtilis208, B. cereus220 and B. subtilis252 were proved successful to control Fusarium and Pythium infection and plant growth promotion in pot culture as well as field trials. Bacillus species were found as prominent phosphate solubilizers in rhizosphere of crop plants by Krishnaveni M. S. (2010). Manas et. al., 2012 isolated thermoresistant strains of Bacillus subtilis from cow dung with phosphate solubilization and IAA production abilities. Maheswar and Sathiyavani (2012) isolated efficient phosphate solubilizing Bacillus species from groundnut rhizosphere.

Cellulolytic activity of Bacillus species

Among the eight antifungal Bacillus isolates, only one i.e. B. subtilis252 showed considerable cellulase activity (table-2). The glucose concentration estimated by colourimetric method using the reagent DNSA found to increase successively on 3rd, 4th, 5th and 6th day as 20, 32, 45 and 68 µg/ml. A mixed culture of Bacillus however showed enhanced cellulolytic activity i.e. up to 120 on 6th day. Considerable cellulase activity was not observed in case of other antifungal and phosphate solubilizing Bacillus isolates. It may be due to the fact that, cellulose is a complex homopolysaccharide of glucose which is better degraded by combined biochemical activity called 'synergism' of mixed culture. Cellulose degrading bacteria have been isolated from soil, cow dung, gut of termites and compost (Saraswati et al., 2012).

We conclude that, application of bioformulations prepared using Bacillus subtilis208, B. cereus220 and B. subtilis252 not only act as biological control of fungal diseases of crops but also act as PGPR to increase soil fertility with respect to phosphate solubilization and cellulose mineralization. The isolate B. subtilis252 was found to be 'all rounder' in this respect.

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