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Biocontrol Activity of *Bacillus subtilis* Isolated from Cow Dung Against Plant Pathogenic Fungi

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ABSTRACT

Plant diseases cause considerable losses in crop production and storage. Nowadays, growers still rely heavily on chemical pesticides to prevent, or control these diseases. However, the high effectiveness and ease of utilization of these chemicals can result in environmental contamination and the presences of pesticide residues on food, in addition to social and economic problems. Consequently, there is an increasing demand from consumers and officials to reduce the use of chemical pesticides. In this context, biological control through the use of natural antagonistic microorganisms has emerged as a promising alternative. Indeed, these bio pesticides present many advantages in term of sustainability, mode of action and toxicity compared to chemical pesticides. However, in the present study the cowdung sample was collected from in an around sundarakkottai, Mannargudi Taluk, Thiruvarur district, Tamil Nadu. The totally, there is an increasing demand from consumers and officials toreduce the use of al microbial population in the cowdung was determined by serial dilution techniques. The isolated bacterial colonies are identifying as Bacillus subtilis totally four Bacillus subtilis isolates were identify and among the four strain only. Three strains possess antagonistic activity against the fungal pathogen isolated from Rhizosphere soil sample. The isolated fungal pathogens are Aspergillus niger, Aspergillus flavus and Fusarium oxysporum. The antagonistic activity was carried out by dual culture plate method and liquid broth method.

Keywords: *Cow dung, pathogenic fungi, microbial population, Rhizophere.*

INTRODUCTION

In India, cow dung is accepted as a purifier and has an important role in preserving environment. Besides being used as a fuel, it also finds use as a disinfectant in homes. Burning of cow dung is thought to repel mosquitoes. It also has significant role in crop growth as manure because of humic compounds and fertilizing bioelements present in it[1]. The low C : N ratio in cow dung manure is an indication that it could be a good source of protein for the microbes involved in the decomposition of organic matter[2]. It is also a component of panchagavya; it is a term used in Ayurveda to describe five important substances obtained from cow, namely, urine, dung, milk, ghee and curd.

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A number of formulations mentioned in Ayurveda describe the use of panchagavya components either alone or in combination with drugs of herbal, animal or mineral origin. Cow dung showed positive response in suppression of mycelial growth of plant pathogenic fungi like *Fusarium solani*, *F. oxysporum* and *Sclerotinia sclerotiorum*[3]. Cow dung extract spray was also reported to be effective for the control of bacterial blight disease of rice and was as effective as penicillin, paushamycin and streptomycin[4]. Cow dung is excreted by bovine animal species which are herbivores. It consists of undigested residues of consumed matter which has passed through the cow's gastrointestinal system. Cow dung is widely studied for its use as organic agricultural fertilizers and extensively explored for its potential as alternative fuel or biogas due to its high methane content [5.] However, there is lack of research on the microbial diversity and other potential applications of cow dung[6].

The primary reason for the lack of knowledge regarding the composition of the cow dung microbiome relates to the difficulty and expense of methods used to evaluate those populations[7]. Culture based methods are extremely time consuming and to date we have only been able to culture approximately 1% of the bacteria present in animal gut[8]. Metagenomics is the culture-indepent analysis of mixture of microbial genomes (metagenome) using an approach based either on expression (functional analysis) or sequencing (sequence-based analysis). Metagenomic analysis involves isolating DNA from an environmental sample, cloning the DNA into a suitable vector, transforming the clones into a host bacterium and screening the resulting transformants[9]. Cowdung (CD) is a mixture of dung and urine, generally in the ratio of 3:1. It contains crude fibre, crude protein, cellulose, hemicellulose and 24 types of minerals such as N,K,S, traces of P, Fe, Co, Mg, P, Cl, Mn, etc[10]. It is normally used as an organic fertilizer for enhancing soil fertility, as a source of fuel, for dressing seeds, plastering cut ends of vegetative propagated sugarcane, dressing plant wounds, sprinkling diluted suspension of CD on plant surface, etc. From ancient times several bacterial strains, mostly belonging to *Bacillus* spp., were isolated from CD. However, it was not clear whether the microflora of CD have a direct role in enhancing sprouting and seedling growth. Besides, although the potential of CD in enhancing soil fertility is known to Indian sub-continental farmers for centuries[11], little is known whether CD microorganisms mediate nutrients cycling such as sulphur(s) oxidation and phosphorus (p) solubilization in soil. There is circumstantial evidences to shown that microorganisms isolated from CD have industrial potential[12]. Reported that a fungus, *Trichoderma* isolated from CD had the ability to convert cellulosic to ethanol. It envisages that the microorganisms in CD may have the ability to produce enzymes and others biomolecules. From these above point of views, the following were conducted to demonstrated the beneficial activities of CD and CD microflora: (1) study the microbial load of fresh and aged CD, (2) explore the antimicrobial activity of selected microorganisms (*Bacillus subtilis* strains isolated from CD) against *Fusarium oxysporum* , *Aspergillus niger* and *Aspergillus flavus* isolated from the Rhizosphere soil samples.

MATERIALS AND METHODS

SAMBLE COLLECTION

Different sample of cow dung were collected from different areas of Sundarakottai, Thiruvarur District , Tamilnadu, aseptically in sterile poly bags and transported to Microbiology laboratory of the PG and Research Department of Microbiology for the evaluation of microbial analysis.

PREPARATION OF COW DUNG SUSPENSION

Cow dung suspensions were prepared by serial dilution method. The collected and labelled, 1gm of cow dung sample were mixed in 10ml sterilized phosphate buffer and vigorously shaked in vortex for 2 minutes for proper mixing in of sample. Before plating, all the samples were incubated at 37°Cfor 30-40 minute in an incubator for activation of microorganism. After

incubation dilutions of each sample were prepared by using standard dilution method with the help of sterilized pipette. In this method, phosphate blanks were prepared, each contain 9ml of sterilized phosphate buffer. The labeled tubes were placed in test tube standard solution was transferred aseptically in test tube number 1, and further 1ml of sample was transferred to number 2 and same procedure was repeated for each dilution.

ISOLATION AND PURIFICATION OF *BACILLUS*

The different bacterial cultures were purified by using streak plate method on Nutrient agar medium. Using sterilized inoculating loop, slightly picked up the colony from the spread plate dragged the loop over the surface of another plate in a zigzag motion. Sterilized the loop over the flame, turned the plate to 90 and dragged the loop over the area streaked before in similar manner. Again sterilized the loop over the flame in the same process was repeated again, all the plates were incubated for 24 hours. The isolated colonies were in the third sector. This method was repeated several times until purified colonies were obtained. The purified bacterial cultures were maintained over Nutrient agar slant.

CHARACTERIZATION AND IDNTIFICATION OF *BACILLUS* SPECIES

After the pure culturing method, the isolated colonies of microorganisms were observed for colony morphology determination; colour, shape, size, surface, edges, margins and elevation. These cultures were identified by different staining such as Gram's staining, endospore staining etc.

IN VITRO ANTAGONISTIC TEST

DUAL-CULTURE-PLATE METHOD

The mycelia of *F. oxysporum*, *Aspergillus niger* and *Aspergillus flavus* were dual-culture plated with either *B. subtilis* BS1 or *B. subtilis* BS3 (to test for antagonism) as described in this project. One 10-mm disk of pure culture of the fungus was placed at the center of a petriplate (10cm) containing PDA. A circular line made with a 6-cm-diameter petriplate dipped in a suspension of B. *subtilis* strain(1×10^6 CFU ml^{-1}) was placed surrounding the fungal inoculum. Plates were cultured for 120 h at 30°C and fungal growth (the diameter of the lawn produced by the pathogen) was measured and compared to control growth, where the bacterial suspension was replaced with sterile distilled water. Each experiment considered a single *F. oxysporum*, *Aspergillus niger* and *Aspergillus flavus* isolated and was run in duplicate and repeated at least three times. Results are expressed as the mean percentage inhibition of growth of the corresponding *F. oxysporum*, *Aspergillus niger* and *Aspergillus flavus* in the presence of either of the *B. subtilis* isolates (BS1or BS4).

INTERACTION IN LIQUID BROTH

The interaction of *F. oxysporum*, *Aspergillus niger* and *Aspergillus flavus* with *B. subtilis* BS1and BS4 was studied in PD broth individually. Agar discs (5 mm in diameter) of *F. oxysporum*, *Aspergillus niger* and *Aspergillus flavus* were individually inoculated in 250-ml of PD broth. Suspensions of with *B. subtilis* BS1and BS4 (1×10^6 CFU ml^{-1}) were inoculated individually with PD broth. For each experiment, flasks in triplicate were incubated at 30°C for 5 days in an incubator under static conditions. Control cultures were grown without bacteria. Mycelial dry weights of the fungus grown in the presence or absence of *B. subtilis* BS1and BS4, individually, were determined by filtering out the spent medium using what man No.1 filter paper and drying the cell mass on the filter paper at 60°C for 3 days. Microscopic observation of the inhibition of *F. oxysporum*, *Aspergillus niger* and *Aspergillus flavus* by *B. subtilis* BS1was made using a light microscope.

RESULT AND DISCUSSION

In present study, different samples of cow dung were collected from different localities of Sundarakkottai, which were subjected for morphological and biochemical characterization. (Table 1, 2) These isolated bacterial strains were further evaluated for antagonistic activity against fungal pathogen causing plant disease. Finding of the present study were presented and discussed as follows.

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATED STRAINS

Microorganism produces colonies with characteristics which could be seen by naked eyes that are called as cultural characteristics. The cultural characteristics were observed on Nutrient Agar Medium plates after incubation. These morphological characteristics were observed in different from such as colony form, colony elevation, surface of the colony and colony colour. The collected samples of cow dung were enumerated for their microbial load of total bacteria. The maximum number of their microbial population was exhibited in dilution 10^{-4} which ranged from 55.5 $\times 10^{-4}$ to 190.4$\times 10^{-4}$cfu/ml and minimum concentration was exhibited in dilution 10^{-6}which ranged from 20.0$\times 10^{-6}$ to 53.6$\times 10^{-6}$. The morphological examinations of the isolates were determined procedure of basic stain; gram stain and endospore stain[13]. Out of four strains, three strains I_1, I_3 and I_5 were gram positive, cocci form and rest of the strain I_4 Is gram positive, bacillus form (Table 3). Among these isolated strains, only one strain I_4 shows endospore formation. Similar type of work was performed by also reported two isolates K2 and k4, both were gram positive microorganisms, capable of forming endospore[14].

Normally CD microflora contain abundant number of *Bacilli, lactobacilli* and cocci and some identified and unidentified fungi and yeast[15]. According to Kung[16], lower part of the gut of the cow contains various microorganisms including *Lactobacillus plantarum, lactobacillusacidophilus, B. subtilis, Enterococcus diacetylactis* etc. Other than these, the rumen of the cow contains various species of *Bacillus* and *Bifidobacterium* and yeast (commonly *saccharomyces cerevisiae)* for better rumen fermentation [17], which might be the initial microflora of CD. Normally aged CD gets invaded with several soil contaminants such as bacteria , fungi and actinomyces. In some cases, the cow is fed with feed admixed with *Trichoderma* formulation (for enhancing cellulose activity) to improve utilization of fibrous feed stuffs .This might be also the initial *Trichoderma* population in CD.

IDENTIFICATION OF FUNGI

In microscopic observation the fugal colonies are initially white and cottony. Often becoming pigmented rose or violet with age and developing mucoid areas when sporulating. Some species produce thick walled chlamydospores, which are solitary or in aggregates conidia are formed in silmy marshes were observed, so the isolates fungi are identified as *Aspergillus niger, Aspergillus flavus,* and *Fusarium oxysporum.*

***IN VITRO* STUDIES**

DUAL- CULTURE METHOD

The results of antagonism of BS1, BS2 and BS4 strains on the growth (*in vitro*) of *F. oxysporum*, *A. niger* and *A. flavus* are shown in (Table 5). The growth of *F. oxysporum*, *A. niger* and *A. flavus* in control samples (with out *B. subtilis*) was 7.4, 8.2 and 9.4 cm, respectively. On the fifth day of incubation, *F. oxysporum* growth was inhibited to 31.9% and 30.0% over the control by strains BS1 and BS4, respectively. Similarly the growth of *A. niger* and *A. flavus* was inhibited to 35.0% and 33.2% by strains BS1, BS2 and BS4, respectively. The BS3 and BS5 do not have antagonistic activity.

LIQUID (PD) BROTH

The impact of *B. subtilis* BS1, BS2 and BS4 on the growth of *F. oxysporum*, *A. niger* and *A. flavus* was studied in liquid (PD) medium (Table 4). When *B. subtilis* strains BS1, BS2 and BS4 were inoculated along with *F. oxysporum*, the percentage inhibition was in the range of 48.5-54.6. Similar results were obtained in the study of interaction between *A.niger, A. flavus* and *B. subtilis* strains.

Light microscopic examination of *F. oxysporum* , *A. niger* and *A. flavus* collected after interaction with *B. subtilis* for 12 h showed that most fungal hyphae had lost their cytoplasmic.

TABLE 1: Biochemical characteristics of isolated microbes

S.NO	Tests	*Bacillus subtilis*
Microscopic observation		
1	Gram's staining	Gram positive
2	Shape	Rod
3	Motility	Motile
Biochemical characters		
4	Indole	-
5	Methyl red	-
6	Voges proskauer	+
7	Catalase	+
8	Oxidase	-
9	Citrate	-
10	Urease	-

Note: (Positive +, Negative -)

TABLE 2: Morphological characteristics of isolates from cow dung

Characteristics	Isolates				
	I1	**I2**	**I3**	**I4**	**I5**
Form of colony	Circular	Circular	Circular	Circular	Circular
Translucency and opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation of colony	Convex	Flat	Convex	Convex	Convex
Surface of colony	Smooth	Smooth	Smooth	Smooth	Smooth
Pigmentation	Creamy white	Yellow	Pink	White	Black
Cell shape	Coccus	Coccus	*Bacillus*	Coccus	Coccus
Spore stain	No	No	Yes	No	No

TABLE 3: Total microbial count in cowdung sample

S.No.	Dilutions	Method used	Total bacteria count				
			S1	**S2**	**S3**	**S4**	**S5**
1.	10^{-4}	Serial dilution method	190.4×10^{-2}	173×10^{-2}	60.5×10^{-2}	59.4×10^{-2}	55.5×10^{-2}
2.	10^{-5}	Serial dilution method	140.3×10^{-3}	90.5×10^{-3}	31.5×10^{-3}	30.8×10^{-3}	29.5×10^{-3}
3.	10^{-6}	Serial dilution method	80.5×10^{-4}	53.6×10^{-4}	50.5×10^{-4}	23.4×10^{-4}	20.0×10^{-4}
4.	10^{-7}	Serial dilution method	26.0×10^{-5}	24.3×10^{-5}	20.0×10^{-5}	15.5×10^{-5}	15.0×10^{-5}

TABLE 4: Antagonistic activity of *B. subtilis* against plant pathogens

Treatment	Fungal dry Mass(Mean±SD)
F.oxysporum (FO)	
1. FO	272 ± 11.0
2.FO+*B.subtilis* BS1	135± 9.5
3.FO+*B.subtilis* BS2	115±15.1
4.FO+*B.subtilis* BS4	108±10.0
A.niger (AN)	
1.AN	523 ± 10.1
2.AN+*B.subtilis* BS1	326 ± 5.1
3.AN+*B.subtilis* BS2	322 ± 13.2
4.AN+*B.subtilis* BS4	251 ± 11.3
A.flavus	
1.AF	431 ± 12.2
2.AF+*B.subtilis* BS1	428 ± 9.7
3.AF+*B.subtilis* BS2	242 ± 15.1
4.AF+*B.subtilis* BS4	235 ± 16.3

Note: FO: *Fusarium oxysporum*; AN: *Aspergillus niger* and AF: *Aspergillus flavus* (values are expressed as mean±SD).

CONCLUSION

In conclusion, CD traditionally used as organic fertilizer in Indian sub-continental farming for centuries. The addition of CD not only increases the mineral status of soil, but also enhances resistance of plant against pests and diseases[18.19], stimulates plant growth[20] and other beneficial activities such as S-oxidation and P-solubilization. Further studies are in progress to elucidate the mechanism underlying biocontrol and growth stimulation by *B. subtilis* strains isolated from CD as well as to develop biotechnological application of these microorganisms in fermentation industries.

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