

Original Research Article

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## Microbial Characterization and Anti-microbial Properties of Cowhorn Silica Manure Controlling Rice Pathogens

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### ABSTRACT

#### Keywords

Cow horn silica, BD 501, Antimicrobial property, *Bacillus amyloliquefaciens*, Biodynamic agriculture.

#### Article Info

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The biodynamic agriculture emphasizes the use of cowhorn silica manure (BD 501) for the improvement of growth and enhances quality and yield of crop plants. In this study, the microbial load in BD 501 manure was enumerated and evaluated for its antagonistic properties against selected rice pathogens. Three predominant bacterial isolates, BD (A)S1, BD(A)S2 and BD(A)S3 were isolated, purified, characterized and identified through microscopical, biochemical and 16S rDNA sequencing methods. These bacterial isolates were identified as *Bacillus spp.* based on the microscopic observation, Gram staining and biochemical tests. At species level, BD (A)S1 and BD(A)S2 isolates were identified as *Bacillus amyloliquefaciens* and isolate BD(A)S3 was identified as *Bacillus toyonensis*. These 3 isolates were tested for their antimicrobial activity against rice pathogens such as *Rhizoctonia solani*, *Pyricularia oryzae* and *Xanthomonas oryzae*, the causal organism of sheath blight, blast and bacterial blight of rice plants respectively. The bacterial isolate *Bacillus amyloliquefaciens* recorded for a strong antifungal activity against *R.solani* and moderate activity against *Pyricularia oryzae*. This study provides a basis for confirming/ understanding the beneficial effect of BD 501.

### Introduction

Silicon (Si) is a beneficial element for plant growth. It can stimulate photosynthesis by improving leaf erectness, decrease susceptibility to disease and insect damage, prevent lodging and alleviate water and various mineral stresses. Increase food production was achieved through introduction of high yielding varieties, judicious application of fertilizers, pesticides and appropriate irrigation has been introduced in agriculture. Increased use of chemical fertilizer and pesticides resulted in

deterioration of the soil fertility and biological activity leading to less productivity. Also the use of these chemical fertilizers and pesticides led to the emergence of many pests & diseases and these problems can be controlled by following an alternative sustainable farming methods like vedic farming, agnihotra, organic farming, natural farming, biodynamic farming, zero budget farming, natuo farming, Eco-Agriculture, Permaculture, Rishi Kheti, Sadhu Kheti.

Although, all these farming techniques differ slightly in precise meaning and emphasis, the underlying ecofriendly principles in raising healthy plants (Gupta *et al.*, 2014).

Biodynamic agriculture is a unique organic farming system that utilizes, specific eight different types of fermented herbal preparations (BD 500 to BD 507) as compost additives and field sprays. This method of BD agriculture to aid humus, organic carbon and soil fertility. (Koepf *et al.*, 1976). BD 500 is a fermented cow horn manure which improves the soil fertility and formation of a strong root system. BD 501 stimulates photosynthetic activity, improve leaf erectness and decrease the disease incidents (Koepf *et al.*, 1976; Spaccini *et al.*, 2012; Pathak and Ram, 2004).

The effect of BD system on soil physio-chemical, biological and yield attributes of selected crops were extensively investigated by Perumal *et al.*, 2001 and 2003; Perumal and Stalin, 2006; Birkhofer *et al.*, 2008; Joergensen *et al.*, 2010; Ngosong *et al.*, 2010 and Reeve *et al.*, 2010. The molecular properties, microbial diversity and bioactivity of BD 500 and Cow Pat Pit (CPP) were extensively studied by Stalin, 2009; Arunkumar, 2011; Spaccini *et al.*, 2012; Giannattasio *et al.*, 2013 and Radha and Rao, 2014.

Boggs (2010) reported that, BD 501 can induce systemic resistance in plants. Fateux *et al.*, 2005 extensively studied that the systemic resistance has been induced by many material such as soluble silica solutions.

In this present study, microbial population in BD 501 manure was analysed and evaluated the predominant bacteria present in BD 501 for their antagonistic activity against plant pathogens.

## **Materials and Methods**

### **Production of Preparation Cow Horn Manure (BD 501)**

Silica quartz was collected from Sevapur, Karur District, Tamil Nadu, India and made into powder form. The 25 g of fine silica powder (< 1mm) was moistened with 50 ml of water to make a (stiff) paste and stuffed into cow horn. The horns were then buried 30 cm depth in a soil pit, 1 inch apart with base downwards, added with 50% compost and soil during the month of April for 120 days by following method described by Procter (1997). The BD 501 manure was periodically withdrawn for total microbial count. The soil was analysed for microbial properties before and after preparation of manure.

### **Enumeration, Isolation and Screening of Bacteria**

The enumeration and isolation of one gram of BD 501 was added into a Erlenmeyer flask containing in 10 mL of sterile distilled water and kept under shaking condition for 1 hr. The suspension was diluted serially 10 fold and plated on nutrient agar plates and incubated at room temperature overnight (Waksman, 1952). Based on colony morphology and number of similar colony present, the predominant colonies were isolated and purified by quadrant streaking and stored at 4°C for further studies. The purified colonies were examined after 24 hr under phase contrast microscope (100 X) for the cell and spore morphology.

### **Morphological and Biochemical Tests for Characterization of Isolated Bacteria**

Morphological and biochemical test was carried out following the techniques outlined in Bergey's manual (1974). Three isolates

from BD 501 were short listed for evaluation. They were characterized for gram staining, indole production, methyl red, Voges-Proskauer (V-P), Utilization of Citrate, Gelatin Hydrolysis, Hydrolysis of Urea, Utilization of Arginine dehydrolase, Catalase and Triple Sugar Iron (TSI) test.

### **Identification of Bacteria by 16s rDNA Sequence Analysis**

The three isolate were identified through 16S rDNA sequencing methods. The near full length 16SrDNA gene sequences of the bacterial strains were sequenced at Gujarat State Biotechnological Mission (GSBTM), Gujarat, India. The sequences were deposited in the GSBTM database; accession numbers are given in Table 4.

### **Evaluation of Isolated Strains for Anti-microbial Activity against Plant Pathogens**

The bacterial isolates characterized as members of the genus *Bacillus* were screened for their antagonistic activity against three phytopathogenic microorganisms, *R.solani*, *P.oryzae* and *X.oryzae*, causative organism of the sheath blight, blast and bacterial blight of rice. The pathogenic culture *X.oryzae* was obtained from Shri AMM Murugappa Chettiar Research Centre culture collection, *R. solani* was obtained from CAS Botany (University of Madras) Tamil Nadu, India and *P.oryzae* from Tamil Nadu Rice Research Institute (Tamil Nadu Agricultural University) Aduthurai, Tamil Nadu, India.

All 3 strains were screened for the antimicrobial activity against rice pathogens using dual culture technique (Vidhyasekaran *et al.*, 1997) for fungal and well diffusion technique for bacterial pathogen.

## **Results and Discussion**

### **Initial and Final Characteristics of Experimental Pit Soil**

The soil from the pit was analysed for the total microbial population before burying and after harvest of manure and the results were provided in the Table 1. The microbial load of soil has increased in the soil after harvested the BD501 manure. Bacterial population in soil recorded  $22.00 \times 10^6$  CFU  $g^{-1}$  and  $24.00 \times 10^6$  CFU  $g^{-1}$ ,  $14.00 \times 10^4$  CFU  $g^{-1}$  and  $18.0 \times 10^4$  CFU in fungal and  $6.00 \times 10^3$  CFU and  $11.00 \times 10^3$  CFU in actinomycetes respectively in before burring and after harvest of manure in soil. The result indicates that the microbial population has been. This might be due to the increase in microbes that utilize or solubilize the silica.

### **Preparation and Time Scale Analysis of BD 501**

BD 501 manure taken out from pit and was periodically analyzed up to 120 days for microbial population. Microbial analysis has shown that the manure was rich in bacterial population followed by fungi and actinomycetes. Maximum bacterial population was observed ( $1.50 \times 10^6$  CFU  $g^{-1}$ ) in 90<sup>th</sup> day whereas, fungi ( $4.00 \times 10^4$  CFU  $g^{-1}$ ) and actinomycetes ( $20.0 \times 10^2$  CFU  $g^{-1}$ ) found to be maximum in 120<sup>th</sup> day of the manure preparation.

### **Isolation of Bacteria from BD 501**

Three predominant bacteria from Biodynamic manure, BD 501 were isolated from nutrient agar plate. Many reports are available on the characterization of BD500 and Cow Pat Pit (CPP) (Perumal and Stalin, 2006; Stalin, 2009; Arunkumar, 2011; Giannattasio *et al.*, 2013). Stalin 2009 has

reported that CPP manure contains a high bacterial load ( $4.8 \times 10^6$ CFU g<sup>-1</sup>) and *Bacillus subtilis* was a predominant bacteria present in CPP manure. In another study, Arunkumar (2011) has studied microbial load and communities present in BD500 and BD preps. Likewise, Giannattasio *et al* (2013) has reported that BD500 has a microbial load of  $2.38 \times 10^8$  CFU g<sup>-1</sup>. It is likely that microbes present in the manures protect the plants from pests and diseases and favor the growth of the plants

**Morphological and Biochemical Tests for Characterization of Isolated Bacteria**

The bacterial isolates isolated from the manures were characterized by microscopical examination, Gram staining and several biochemical tests. All the isolates showed rod shape and were Gram positive. The results of the biochemical tests

were provided in Table 3. The results of biochemical test showed that the isolates belong to genus *Bacillus*.

**16S rDNA Sequencing of Bacterial Isolates from BD 501 Manure**

The 16SrDNA gene sequence of the bacteria revealed that strains BD(A)1 and BD(A)2 isolated from BD 501 belonged to *B.amyloliquefaciens*. Strain BD(A)3 belonged to *B.toyonensis* (Table 4).

The results are similar to the report by Radha and Rao *et al* (2014) who have characterized CPP and BD500 and found the presence of *Bacillus subtilis* and *Lysinibacillus xylanilyticus* in BD 500 and *Bacillus licheniformis* in CPP. Ours is the first report on the presence of *B.amyloliquefaciens* and *B.toyonensis* in BD 501.

**Table.1** Initial and Final Characterization of Manure Pit Soil of BD 501

<i>Parameters</i>	<b>Before preparation</b>	<b>After harvest</b>
<b>Bacteria (x 10<sup>6</sup> CFU g<sup>-1</sup>)</b>	22.00	24.00
<b>Fungi ( x 10<sup>4</sup>CFU g<sup>-1</sup>)</b>	14.00	18.00
<b>Actinomyctes (x10<sup>3</sup> CFU g<sup>-1</sup>)</b>	6.00	11.00

**Table.2** Periodical Characterization of BD 501 for Microbial Population

<b>Microbial population</b>	<b>Days of incubation</b>			
	<b>30</b>	<b>60</b>	<b>90</b>	<b>120</b>
<b>Bacteria (x10<sup>6</sup> CFU g<sup>-1</sup>)</b>	0.60	1.20	1.50	1.10
<b>Fungi ( x 10<sup>4</sup>CFUg<sup>-1</sup>)</b>	3.00	2.00	2.00	4.00
<b>Actinomycetes (x10<sup>2</sup>CFUg<sup>-1</sup>)</b>	10.0	12.0	15.0	20.0

**Table.3** Biochemical Tests of the Bacterial Strain Isolated from BD 501 Manure

Biochemical tests	BD(A)1	BD(A)2	BD(A)3
Gram staining	+(rods)	+(rods)	+(rods)
Catalase	+	+	+
Formation of Indole	-	-	-
Methyl Red Test	+	+	+
V-P test	+	+	+
Utilization of Citrate	-	-	-
Utilization of Urea	-	-	-
Utilization of Arginine dehydrolase	+	+	+
Hydrolysis of Gelatin	-	-	-
Utilization of Iron	+	+	+

**Table.4** Genomic Identity of the Bacterial Strains Isolated from BD 501 Manure

Sr. No.	Sample Id	Organism Name	Identity	Accession number
1	BD (A) S1	<i>Bacillus amyloliquefaciens</i>	99%	KT261793
2	BD (A) S2	<i>Bacillus amyloliquefaciens</i>	99%	KT261794
3	BD (A) S3	<i>Bacillus toyonensis</i>	99%	KT261795

#### Evaluation of Isolated Strains for Anti-microbial Activity against Plant Pathogens by Plate Assay Methods

The bacterial isolates were tested against rice pathogens such as *R.solani*, *P.oryzae* and *X.oryzae* in plate assay. Among the strains, *B.amyloliquefaciens* effectively controlled the growth of *R.solani* and latent the growth of *P.oryzae* mycelium and does not exhibit anti-bacterial activity against *X.oryzae*. *B.toyonensis* is having poor anti-microbial activity against rice pathogens.

Some authors have suggested that the use of anti-microbially active species and strains of the genus *Bacillus*, or the use of their metabolites as an alternative or supplementary method to chemical plant protection (Handelsman *et al.*, 1990; Sharga and Lyon, 1998). Many of these bacilli are

generally soil-inhabiting bacteria or exist as epiphytes and endophytes in the spermosphere (Walker *et al.*, 1998) and rhizosphere (Handelsman *et al.*, 1990). For this reason, *Bacillus* species are ideal candidates for use as biocontrol agents in seed treatment programs against soil-borne pathogens (Walker *et al.*, 1998). In our study, *B.amyloliquefaciens* isolates showed a good amount of antimicrobial activity which can be formulated as a biocontrol agent for rice plant pathogens which needs further study.

In conclusion, result on the identification and characterization of antimicrobial properties of bacteria from biodynamic manure BD 501 show that bacillus group of bacteria was predominant. This is a first report of the presence of *B.amyloliquefaciens* and *B.toyonensis* in BD

501 manure. The bacterial strain *B.amyloliquefaciens* was effectively controlling *R.solani*. This result might be first hand scientific understanding of BD 501 and the bacterial strain isolated from the manure can be deployed in industrial production as biocontrol agent.

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### References

Arunkumar, J. 2011. Utilization of alternative herbs and materials for the production of biodynamic manures and their efficiency on growth of selected plants. Ph.D thesis. University of Madras. India.

Bergry, D.H., Buchanan, R.E., Gibbons, N.E. 1974. Bergey's manual of determination bacteriology. Baltimore. Williams & Wilkins.

Birkhofer, K., Bezemer, T.M., Bloem, J., Bonkowski, M., Søren, C., Dubois, D., *et al.* 2008. Long-term organic farming fosters below and aboveground biota: Implications for soil quality, biological control and productivity. *Soil Biol. Biochem.*, 40: 2297–2308.

Boggs, L.C. 2010. The science behind biodynamic. *Organic Agri.*

Epstein, E. 1999. Silicon. *Annu. Rev. Plant Physiol. Pl. Mol. Biol.*, 50: 641–664.

Fautex, F., Remus-Borel, W., Menzies, J.G., Belanger, R.R. 2005. Silicon and plant disease resistance against pathogenic fungi. *FEMS Microbiol.*

*Lett.*, 249: 1–6.

Giannattasio, M., Elena, V., Flavio, F., Sara, A., Marina, Z., Fabio, S., Giuseppe, C., Piergiorgio, S., Andrea, E., Serenella, N., Valeria, R., Pietro, P., Spaccini, R., Pierluigi, M., Alessandro, P., Andrea, S. 2013. Microbiological features and bioactivity of a fermented manure product (Preparation 500) used in biodynamic agriculture. *J. Microbiol. Biotechnol.*, 23(5): 644–651.

Gupta, S., Kushwah, T., Yadav, S. 2014. Role of Earthworms in promoting sustainable agriculture in India. *Int. J. Curr. Microbiol. App. Sci.*, 3(7): 449–460.

Handelman, J., Raffel, S., Mester, E., Wunderlichand Guru, C. 1990. Biological control of dampingoff of alfalfa seedlings with *Bacillus cereus* UW85. *Appl. Environ. Microbiol.*, 56: 713–718.

Joergensen, R.G., Mäder, P., Fließbach, A. 2010. Long-term effects of organic farming on fungal and bacterial residues in relation to microbial energy metabolism. *Biol. Fert. Soils*, 46: 303–307.

Koepf, H.H., Pettersson, B.B., Schaumann, W. 1976. Biodynamic agriculture. The Anthroposophic Press, Spring Valley, NY.

Ma, J.F., Miyake, Y., Takahashi, E. 2001. Silicon as a beneficial element for crop plants. In: Silicon in Agriculture (Eds.) L. E. Datnoff, G. H. Snyder and G. HKorndorfer. Elsevier Science publisher, Amsterdam. Pp. 17–39.

Ngosong, C., Jarosch, M., Raupp, J., Neumann, E., Ruess, L. 2010. The impact of farming practice on soil microorganisms and arbuscularmycorrhizal fungi: Crop type versus long-term mineral and

- organic fertilization. *Appl. Soil. Ecol.*, 46: 134–142.
- Pathak, R.K., Ram, R.A. 2004. Successful Conversion of Conventional to Organic/Biodynamic: a Case Study. In: Establishing an Organic Export Sector. Production and export of organic fruit and vegetables in Asia. p. 61.
- Perumal, K., Chakrapathy, B., Vatsala, T.M. 2003. Influence of nutrient management on quality and yield of carrot. *J. Soil and Crops*, 13(2): 204–210.
- Perumal, K., Vatsala, T.M., Raj, M. 2001. Studies on microbial diversity of biodynamic preparations. *Biodyne*, 1(1): 3.
- Perumal, K., Stalin, V. 2006. Production of plant growth hormones and subtilin from organic and biodynamic manures. 18<sup>th</sup> world congress of soil science conference. 9–15.
- Proctor, P. 1997. *Grasp the nettle*. Random House New Zealand Ltd. New Zealand.
- Reeve, J.R., Carpenter-Boggs, L., Reganold, J.P., York, A.L., Brinton, W.F. 2010. Influence of biodynamic preparations on compost development and resultant compost extracts on wheat seedling growth. *Biores. Technol.*, 101: 5658–5666.
- Sharga, B.M., Lyon, G.D. 1998. *Bacillus subtilis* BS167 as an antagonist of potato black leg and soft rot bacteria. *Can. J. Microbiol*, 44: 777–784.
- Spaccini, R., Mazzei, P., Squartini, A., Giannattasio, M., Piccolo, A. 2012. Molecular properties of a fermented manure preparation used as field spray in biodynamic agriculture. *Environ. Sci. Poll. Res.*, 12: 232–253.
- Stalin, V. 2009. Physicochemical and microbiological characteristics of organic, biodynamic manures and their effect on growth attributes of selected plant. Ph.D thesis. University of Madras, India.
- Vidhyasekeran, P., Rabindran, R., Muthamilan, M., Nayar, K., Rajappan, K., Subramanian, N., Vasumathi, K. 1997. Development of a powder formulation *pseudomonas fluorescens* for control of rice blast. *Pl. Pathol.*, 46: 291–297.
- Walker, R., Dowell, A.A., Seddon, B. 1998. *Bacillus* isolate from the spermosphere of peas and dwarf French beans with antifungal activity against *Botrytis cinerea* and *Pythium species*. *J. Appl. Microbiol.*, 84: 791–801.
- Waksman, S.A. 1952. Principles of soil microbiology. Baltimore, 195–198.

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