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Address for Correspondence:

Dr. R. Balagurunathan
The Editor-JCPAM
Department of Microbiology
Periyar University
Salem - 636 011, Tamil Nadu, India
Phone: +91-427-2345779 Ext: 217
Email : rbalaperiya@gmail.com

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Synergistic and Antagonistic Interactions among Endophytic Bacterial Isolates of *Vigna mungo* (L.) Hepper

Poonam Dubey* and G.P. Gupta
Department of Botany & Microbiology, Gurukula Kangri Vishwavidyalaya
Haridwar- 249 404, Uttarakhand, India
**Corresponding Author: Poonam Dubey, Department of Botany & Microbiology, Gurukula Kangri Vishwavidyalaya, Haridwar- 249 404, Uttarakhand, India, Email: poonam.gkv@gmail.com*

ABSTRACT

Twenty bacterial isolates (VR1-VR20) were screened from root nodules of *Vigna mungo* on different growth media and their morphological, physiological and biochemical characterization was carried out. Their all characters along with carbon utilization properties were analysed by NTSYS-pc version 2.02e and MVSP version 3.21 software to get the Jaccard's coefficient dendrograms that classified the isolates into the three major groups, VR1-VR10 isolates in one group, VR15-VR20 isolates in second group and VR11-VR14 isolates in third group. Accordingly, these isolates were named as *Bradyrhizobium* sp. strains VR1-VR10, *Bacillus* sp. strains VR11-VR14 and *Pseudomonas* sp. strains VR15-VR20. The strains VR1, VR2, VR11 and VR13 showed synergism among them. Moreover, cell-free culture filtrate of *Bradyrhizobium* sp. VR2 positively affected the growth of *Bacillus* sp. VR11 when compared with control. Cell growth of *Bacillus* sp. VR11 gradually increased with incubation time up to 96 h suggesting their possible application as co-inoculants for plant growth promotion and/or biocontrol of soil-born phytopathogens.

Key words: Synergism; antagonisms; endophytic bacteria, *Vigna mungo*.

INTRODUCTION

Endophytes are the microorganisms that live within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence (Bandara *et al*., 2006). They inhabit majority of healthy and symptom-less plants. Moreover, diverse endophytic bacteria such as *Pantoea agglomerans, Enterobacter kobei, Enterobacter cloacae, Leclercia adecarboxylata, Escherichia vulneris, Bacillus,* and *Paenibacillus* and *Pseudomonas* sp. belonging to gamma proteobacteria have been isolated from root nodules of various plants (Benhizia *et al*., 2004; Zakhia *et al*., 2006). Endophytes also produce unusual secondary metabolites of plant importance (Taechowisan *et al* 2005); thus they have an excellent potential to be used as plant growth promoters with legumes and non-legumes (e.g. Antoun *et al* 1998; Bai *et al.* 2002).

The endophytic bacteria are known to show either synergism or antagonism among them. However, the ways how the interactions among endophytes influence the plant productivity have not been explained so far ? Some of the rhizobial isolates have been reported to display synergistic interaction with *Pseudomonas*. Samavat *et al.* (2011) applied singly or in combination with the culture filtrate of five rhizobia isolates to evaluate the potential of two isolates of *P. fluorescens* (UTPF 68 and UTPF 109) for enhancement of their biocontrol potential.

Interactions between endophytic bacteria strongly affect the 3ehavior of the interacting strains through production of more extracellular metabolites. However, it is unknown if such interactions also occur during competition for carbon within the plant tissue. The present work was aimed at the isolation, characterization and identification of endophytic bacteria from *Vigna mungo* and their synergistic or antagonistic effects *in vitro*.

MATERIALS AND METHODS

Isolation of endophytic bacteria from *V. mungo:* Healthy *V. mungo* seedlings were collected from the experimental plot, put into sterile polyethylene bags and brought to the laboratory. Bacterial strains were screened from root nodules as described by Vincent (1970) by transferring into the plates of different sterilized media such as CrYEMA medium, Bacillus agar medium, King's B medium and nutrient agar medium. The cultures were identified following the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) and compared against the standard cultures of *Bradyrhizobium* sp. NAIMCC-B-00262 [obtained from National Bureau of Agriculturally Important Microorganisms Culture Collection (NAIMCC), Azamgarh, U.P., India], *Bacillus subtilis* MTCC 441 and *Pseudomonas* sp. MTCC 129 [procured from Microbial Type Culture Collection Centre (MTCC), Institute of Microbiological Techniques (IMTECH), Chandigarh, India].

Characterization of the bacterial isolates: Morphological and physico-chemical characterizations of all bacterial isolates were done such as cultural characteristic, motility, Gram's staining (Dubey and Maheshwari, 2011), catalase activity (Graham and Parker, 1964), oxidase activity (Kovaks, 1956), gelatin hydrolysis (Sadowsky *et al*., 1983), urea hydrolysis (Lindstrom and Lehtomaki, 1988), citrate utilization (Koser, 1923), growth in presence of KNO_3 (El Idrissi *et al.*, 1996), poly hydroxy butyrate (PHB) accumulation (Navarini *et al*., 1992), MR-VP test, starch hydrolysis, and tolerance of 1-6% NaCl (EI-Idrissi *et al*., 1996). NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) Version 2.02e software (Rohlf, 1997) was used to obtain a dendrogram from all bacterial isolates and standard cultures of *Bradyrhizobium* sp. NAIMCC-B-00262, *Bacillus subtilis* MTCC-441 and *Pseudomonas* sp. MTCC-129 with UPGMA (unweighted pair-group method with arithmetic mean) clustering method based on Jaccards coefficient. This software analyses the cluster and establishes phylogenetic relatedness among all the bacterial isolates on the basis of physico-chemical characteristics performing UPGMA analysis.

Utilization of various carbon sources: Utilization of various carbon sources by bacterial isolates was done following El Idrissi *et al*. (1996). Himedia Carbohydrate^{TM} Kit (Himedia Laboratories Pvt. Ltd., Mumbai, India) was used to determine the 35 carbon source utilization that consisted of monosaccharides pentoses, monosaccharides, hexoses, disaccharides, trisaccharides, polysaccharides, organic compounds and sugar alcohol. Results of carbon utilization were analysed by using and MVSP (Multi Variate Software Package) version 3.21 software with UPGMA clustering method.

Interactions among the Bacterial Isolates *in vitro*: Interactions among the bacterial isolates were studied *in vitro* following the method of Pierson and Weller (1994). Bacterial lawn was prepared by seeding of nutrient agar plates separately with exponentially grown culture of VR1, VR2, VR11 and VR13. After 20 minutes, plates having lawn of VR1 strain were spotted by the freshly grown broth cultures of VR2, VR11 and VR13, separately. Similarly, plate containing lawn of VR2 strain was spotted by freshly grown broth cultures of VR1, VR11 and VR13. Similar step was also repeated with VR11 and VR13 strain. All the inoculated plates were incubated at 28±1^{o}C for 72 h along with a control plate (devoid any inoculum) and examined for zone of inhibition/non-inhibition around the spotted culture to record antagonistic or synergistic interaction between two bacterial strains.

Effect of cell-free culture filtrate (CFCF) of *Bradyrhizobium* sp. strain VR2 on growth of *Bacillus* sp. VR11 : Based on the results obtained from the above experiment, it seemed to find out the effect of cell-free culture filtrates of *Bradyrhizobium* sp. strain VR2 on growth of *Bacillus* sp. strain VR11 *in vitro*. *Bradyrhizobium* sp. strain VR2 was grown at 28±1^{o}C in YEM broth for 72 hours at 150 rpm on a rotary shaker following the method of Samavat *et al.* (2011). Similarly, *Bacillus* sp. strain VR11 was also grown in 250 ml Erlenmeyer flasks containing 50 ml Bacillus broth medium on a rotary shaker for 72 h. Culture of *Bradyrhizobium* sp. strain VR2 was centrifuged at 7,000 rpm to get cell-free culture filtrates. Thereafter, 2 ml cell suspension of *Bacillus* sp. strain VR11 and 4 ml cell-free culture filtrate of

Bradyrhizobium sp. strain VR2 were poured in a sterilized culture tubes containing 4 ml of Bacillus broth medium, in three replicates. The control set consisted on 4 ml Bacillus broth medium, 4 ml YEM broth and 2 ml of *Bacillus* sp. strain VR11 cell suspension. All the tubes were incubated at 25^{o}C on a rotary shaker. The growth of *Bacillus* sp. strain VR11 was measured after 24, 48, 72 and 96 h intervals in terms of turbidity (optical density) at 600 nm by using a spectrophotometer (Shimadzu, Japan).

RESULTS

Morphological, physiological and biochemical characterization of bacterial isolates: Bacterial isolates VR1-VR10 that that grew on NAM, YEMA and CrYEMA were Gram-negative rods, non-endospore forming, motile, and positive for PHB accumulation, catalase activity, indole production and esculin hydrolysis. Isolates VR11-VR14 that grew on Bacillus agar medium were gram-positive, rod shaped, motile, and produced endospores (Fig.1). All the isolates were capsulated, accumulated PHB and showed positive result for urease and oxidase production, nitrate reduction, and H_2S production, and negative result for methyl red, and esculin hydrolysis (except VR13). The isolates screened on King's B medium were gram–negative rods, non-capsulated, non-endospore forming and motile bacteria. They were positive for catalase production, PHB accumulation, esculin hydrolysis. None of the isolates were able to produce H_2S. Except VR16, all isolates were fluorescent bacteria that produced fluorescent pigment (Table 1 and 2). On the basis of morphological, physiological and biochemical characteristics and carbon utilization by the isolates and standard cultures, the isolates VR1-VR10 were identified as *Bradyrhizobium* sp. strains, VR11-VR14 as *Bacillus* sp. strains, and VR15-VR20 as *Pseudomonas* sp. strains (Fig.2).

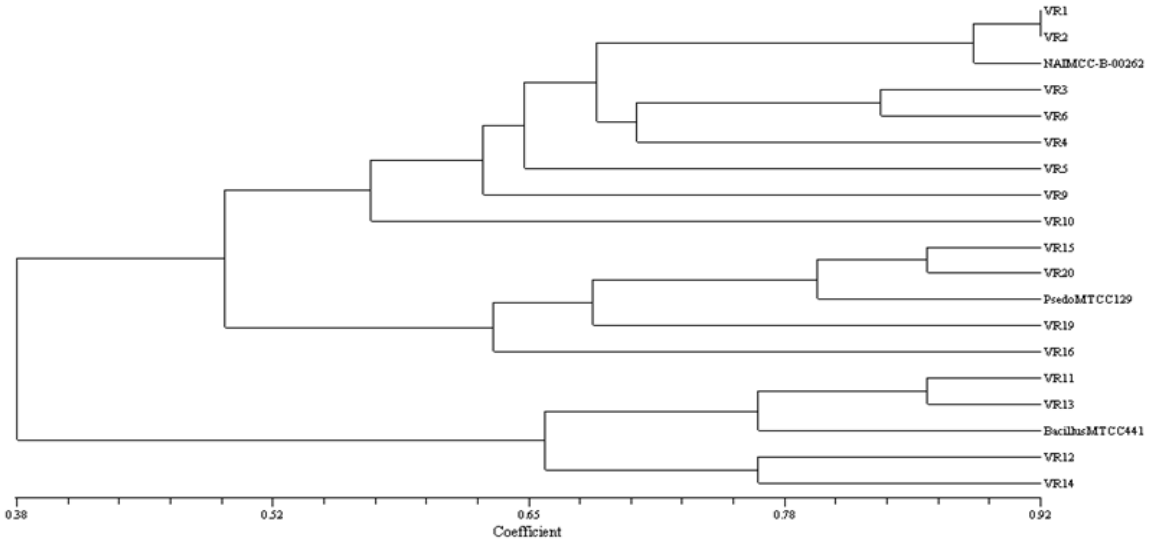

Fig. 1. Dendrogram was obtained from 19 bacterial isolates VR1–VR20 and standard cultures *Bradyrhizobium* sp. NAIMCC-B-00262, *Bacillus* MTCC441 and *Pseudomonas* sp. MTCC 129 with UPGMA (unweighted pair-group method with arithmetic mean) clustering method based on Jaccards coefficient by using physico-chemical characteristics.

Table 1: Physiological and biochemical characteristics of different strains on YEMA/CrYEMA/ *Bacillus* agar/ King's B (KB) medium isolated from *V. mungo.*

1- Gram reaction, **2**- shape, 3- capsule, **4**-motility, **5**- endospore, **6**- PHB accumulation, **7**- catalase, **8**- urease, **9**- oxidase, **10**- starch hydrolysis, **11**- gelatin hydrolysis, **12**- indole production, **13**- growth on nutrient agar medium, **14**- growth on YEMA, **15**- growth on CrEMA, **16**- esculin hydrolysis, **17**- KNO_3 (8%) tolerance, **18**- Voges Proskaur test, **19**- methyl red , **20**- ONPG, **21**- citrate utlilization, **22**- H_2S prodyction, **23**- nitrate reduction, **24**- fluorescence, **25**- pigment; N- not tested.

Isolates	**Biochemical characteristics**																								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
VR1	-	Rod	-	+	-	+	+	-	+	-	+	+	+	+	+	+	-	-	-	+	+	-	N	N	N
VR2	-	Rod	-	+	-	+	+	-	+	-	+	+	+	+	+	+	-	-	-	-	+	-	N	N	N
VR3	-	Rod	+	+	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	N	N	N
VR4	-	Rod	+	+	-	+	+	-	+	+	-	+	+	+	+	+	-	-	-	+	-	-	N	N	N
VR5	-	Rod	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	-	N	N	N
VR6	-	Rod	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	N	N	N
VR9	-	Rod	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-	-	-	-	+	-	N	N	N
VR10	-	Rod	-	+	-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	-	-	N	N	N
VR11	+	Rod	-	+	+	+	+	+	+	-	-	+	+	N	N	-	-	-	-	+	-	+	+	N	N
VR12	+	Rod	-	+	+	+	+	+	+	+	-	-	+	N	N	-	-	-	-	-	-	+	+	N	N
VR13	+	Rod	-	+	+	+	+	+	+	-	-	+	+	N	N	+	-	-	-	+	-	+	+	N	N
VR14	+	Rod	-	+	+	+	+	-	+	+	+	-	+	N	N	-	-	-	-	+	-	+	+	N	N
VR15	-	Rod	-	+	-	+	+	-	+	+	+	+	+	N	N	+	-	+	-	+	-	-	-	+	+
VR16	-	Rod	-	+	-	+	+	+	-	+	-	+	+	N	N	+	-	-	-	+	+	-	+	-	+
VR19	-	Rod	-	+	-	+	+	+	+	+	-	+	+	N	N	+	+	-	-	-	+	-	-	+	+
VR20	-	Rod	-	+	-	+	+	-	+	-	+	+	+	N	N	+	-	+	-	+	+	-	-	+	+
Bradyrhizobium sp. NAIMCC-B-00262	-	Rod	-	+	-	+	+	-	+	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-
B. subtilis MTCC 441	+	Rod	-	+	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-	+	-	-	+	-	-
Pseudomonas sp. MTCC 129	-	Rod	-	+	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+	+	-	-	+	+

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Table 2. Utilization of various carbon sources by bacterial strain isolated from *Vigna mungo*.

Carbon sources	VR1	VR2	VR3	VR4	VR5	VR6	VR9	VR10	VR11	VR12	VR13	VR14	VR15	VR16	VR19	VR20	NAIMCC -B-00262	MTCC 441	MTCC 129
Monosaccharides pentoses																			
Xylose	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+
Ribose	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabinose	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
Monosaccharides Hexoses																			
Fructose	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Dextrose	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	-	-	+	+
Galactose	+	+	+	-	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Disaccharides																			
Lactose	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+
Maltose	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	-	+	-	-	-	+	+	+	-	+	+	+	+	-	+	+
Melibiose	-	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-
Sucrose	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
Cellobiose	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+
Trisaccharide																			
Raffinose	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+	-	-
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Polysaccharides																			
Inulin	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-
Organic compounds									-	-	-	-	-	-	-	-	-	-	-
Sodium gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Organic compounds																			
Glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONPG	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-
Esculin	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	-	+	-	-
Citrate	+	+	+	+	+	+	-	+	+	-	+	-	-	-	-	+	+	-	-
Sugar alcohol																			
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	+	-	-	-	+	-	+	+	+	+	-	-	+	+	-	+	+
Sorbitol	+	+	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Adonitol	+	-	+	+	-	+	+	+	-	+	+	+	-	+	-	+	+	-	+

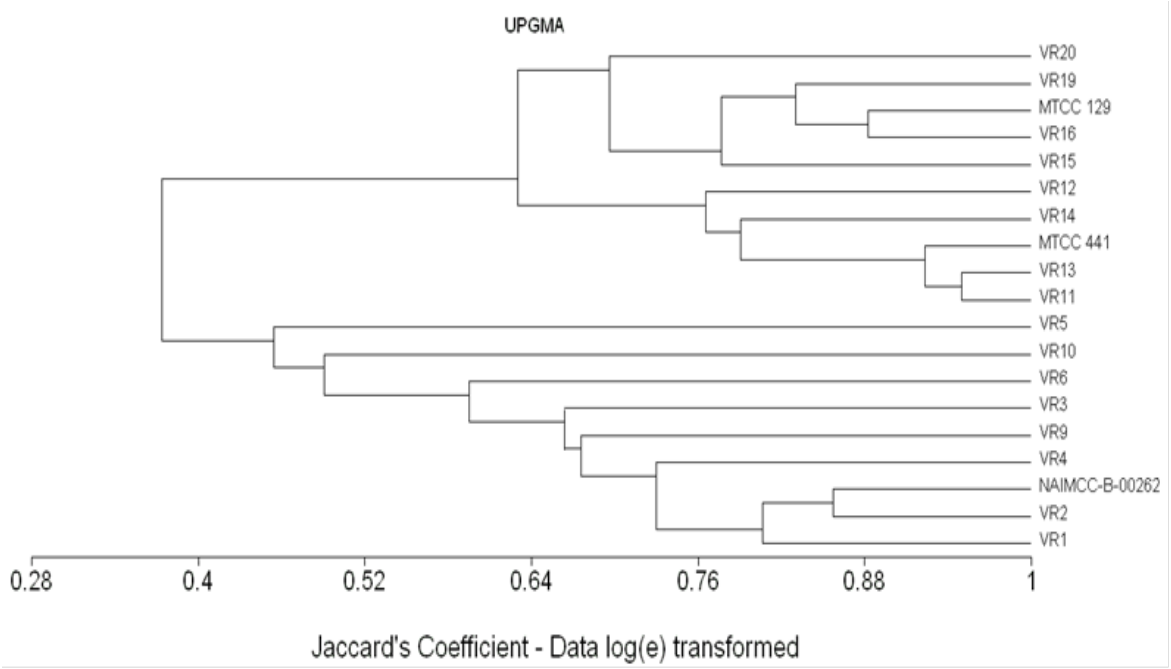

Fig. 2. Dendrogram was obtained from 19 bacterial isolates VR1 – VR20 with UPGMA (unweighted pair-group method with arithmetic mean) clustering method based on Jaccard's coefficient by using carbon utilization. Distance is based on genetic similarity coefficient.

Interactions among bacterial isolates *in vitro*: Inter-bacterium interaction was studied *in vitro* to evaluate their synergistic or antagonistic effect of bacterial isolates. The four strains VR1, VR2, VR11 and VR13 did not inhibit each other but displayed synergistic interaction between them after incubation at 28±1ºC for 72 h. Besides, the strain VR19 and VR20 also did not inhibit one another thus showed synergism, whereas the other isolates inhibited the growth of each other, showing the antagonistic interaction (Table 3).

Table 3. *In vitro* interaction between bacterial isolates.

Isolated strains	VR1	VR2	VR3	VR4	VR5	VR6	VR9	VR10	VR11	VR12	VR13	VR14	VR15	VR16	VR19	VR20
VR20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
VR19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
VR16	-	-	-	-	-	-	-	-	-	-	-	-		+		
VR15	-	-	-	-	-	-	-	-	-	-	-	-	+			
VR14	-	-	-	-	-	-	-	-	-	-	-	+				
VR13	++	++	-	-	-	-	-	-	++	-	+					
VR12	-	-	-	-	-	-	-	-	-	+						
VR11	++	++	-	-	-	-	-	-	+							
VR10	-	-	-	-	-	-		+								
VR9	-	-	-	-	-	-	+									
VR6	-	-	-	-	-	+										
VR5	-	-	-	-	+											
VR4	-	-	-	+												
VR3	-	-	+													
VR2	++	+														
VR1	+															

+, Synersism; -, Antagonism.

Effect of cell-free culture filtrate (CFCF) of *Bradyrhizobium* sp. strain VR2 on growth of *Bacillus* sp. VR11: Interestingly, the cell-free culture filtrate of *Bradyrhizobium* sp. VR2 positively affected the growth of *Bacillus* sp. VR11 when compared with control. An increased cell growth of *Bacillus* sp. VR11 was recorded in terms of absorbance (at 600 nm) at different intervals, which gradually increased with incubation time even up to 96 h; but this increase was greater in cell-free culture filtrate treatments than control (Fig. 3).

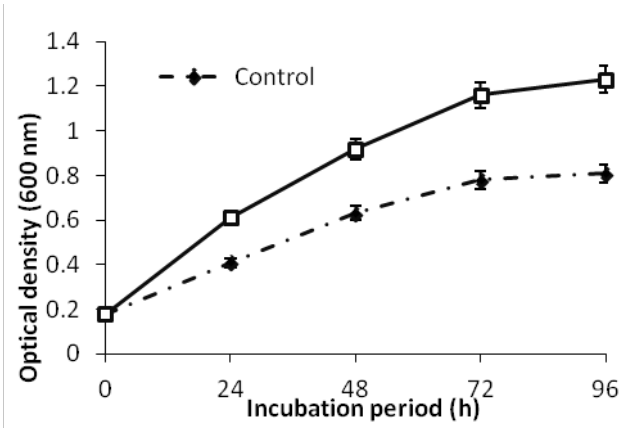

Fig. 3. Effect of cell-free filtrates of *Bradyrhizobium* sp. (*Vigna)* strain VR2 on growth of *Bacillus* sp. strain VR11 (values are mean of three replicates; ± standard error).

DISCUSSION

NTSYS-pc version 2.02e and MVSP version 3.21 software analysed the characteristics of all 20 bacterial isolates of *V. mungo* and put them into three different groups. Phenotyping is based on morphological, physiological or biochemical aspects and, in the case of the family *Rhizobiaceae*, also on symbiotic compatibility with legume host plants. The official classification of the genus *Bradyrhizobium*, *Bacillus* and *Pseudomonas* as presented in Bergey's Manual of Systematic Bacteriology (Jordan, 1984), considers only phenotypic features and mol% G+C. Later on, genotypic features were also described (Elkan and Bunn, 1992).

Bradyrhizobium sp. strains VR1 and VR2, and *Bacillus* sp. strains VR11 and VR13 displayed synergistic interaction. *Pseudomonas* strains VR19 and VR20 also showed synergism between them. Some sorts of similarities such as common physiochemical properties, carbon utilization, etc. were the reasons of synergistic interaction. However, they been share some of the identical features of PGP properties, such as IAA production, absence of HCN production, type of siderophore, chitinase production, etc. Positive interaction between *Rhizobium* and *Pseudomonas* sp. LG or *Bacillus* sp. has been reported by Stajkovic *et al*. (2011). Kumar (2012) have also reported the positive interaction among *R. leguminosarum* RPN5, *B. subtilis* BPR7 and *Pseudomonas* sp. PPR8 by successfully growing as mixed cultures.

In the present work growth of *Bacillus* sp. VR11 was increased after amending the CFCF of *Bradyrhizobium* sp. (*Vigna*) strain VR2 as compared to control. This shows that the culture filtrate of *Bradyrhizobium* sp. strain VR2 synergistically enhanced the growth of *Bacillus* sp. VR11. Limited studies have been carried out on the effects of extracellular metabolites of other competitor bacteria, especially on the enhancement of biocontrol efficiency of antagonists. Samavat *et al.* (2011) found that certain rhizobia had capacity to interact synergistically with *P. fluorescens* isolates UTPF68 and UTPF109 having potential biocontrol activity. They found that the cultural filtrates of tested rhizobial isolates were able to effectively increase the growth of both *P. fluorescens* isolates (UTPF68 and UTPF109). The

probable synergetic mechanism of rhizobia intensifies the biocontrol potential of the other bacteria. Moreover, El-Batanony *et al.* (2007) found that the cultural filtrates of *Rhizobium leguminosarum* showed potential synergetic activity with arbuscular mycorrhizal (AM) fungi in the biocontrol of *R. solani*, *Fusarium solani*, and *F. oxysporum* of faba bean. Enhanced soybean plant growth resulting from co-inoculation of *Bacillus* strains with *Bradyrhizobium japonicum* has been reported by Bai *et al.* (2003). Synergistic effects of plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and nitrogen fixation by pigeon-pea (*Cajanus cajan*) has been reported by Tilak *et al*. (2006).

Antagonistic properties shown by the other isolates of *V. mungo* suggest the operation of antagonism among them, which may be due to production of antibiotic(s), bacteriocins, HCN, and several other extracellular metabolites, resulting in bacteriostatic or bactericidal effects individually or in combination. Garbeva *et al*. (2009) reported the competitive interactions between two taxonomically non-related bacterial strains, *Pseudomonas* sp. A21 and *Pedobacter* sp. V48 isolated from a dune soil. The strains showed strong effects on each other's behavior and gene expression patterns when growing together under carbon-limited conditions on agar. The gene expression patterns suggest an interference competition strategy by the *Pseudomonas* strain and an escape/explorative strategy by the *Pedobacter* strain during confrontation with each other. They found that the bacterial strains can distinguish between intra- and inter-specific carbon competitions.

CONCLUSION

It may be concluded that *Bradyrhizobium* sp. strains VR1 and VR2 and *Bacillus* sp. strain VR11 that act synergistically can be used as co-inoculants for plant growth promotion and/or biocontrol of soil-born phytopathogens.

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Plant Growth Promoting Rhizobacteria for Biocontrol of *In-Vitro* Studies on Anti-*Salmonella enteritidis*-IgY for Passive-Immunotherapy in Broiler Chicks

T. Diraviyam, M. Menaka and A. Michael*
Department of Microbiology, PSG College of Arts and Science, Coimbatore -641 014, India

**Corresponding Author: A. Michael, Department of Microbiology, PSG College of Arts and Science, Coimbatore -641 014, India. E-mail: amichela2000@gmail.com*

ABSTRACT

In India, poultry is one of the fastest growing segments of the agricultural sector. But during the last few years there is also an increase in pathogen loads in poultry farms due to excessive stress on the birds. At early days of age, Salmonellosis is one of the main diseases with economic concern to all phases of poultry. In general, the diseases are being controlled by administering antibiotics, but now it is under inspection due to the emergence of antibiotic resistance. Therefore, the present study was focused to characterize Anti- *S.enteritidis*-IgY by *in-vitro* methods. White leghorn chickens (21 weeks old) were immunized with the *S.enteritidis* whole cell antigen and eggs were collected and stored at 4^{o}C. IgY antibodies were purified by PEG extraction method (Polson *et al.,* 1980). The purity of IgY antibodies was determined by SDS-PAGE and the specificity was evaluated by slide agglutination and ELISA. *In-vitro* efficacy was evaluated by Growth Inhibition Assay. The generated antibodies were specific to *S.enteritidis* whole cell antigen and the titre of more than 1:100000 were observed from 35^{th} day onwards by ELISA. *In-vitro* study result showed that the binding activity of Anti-*S.enteritidis*-IgY with bacterial cells has resulted in inhibiting the growth of *S.enteritidis* in liquid medium after 4 hours of incubation. The present study could form a platform for further research on yolk antibodies and its commercial application in India.

Key words: Chicken IgY, *Salmonella enteritidis*, ELISA and Growth inhibition assay

INTRODUCTION

World poultry industry is expanding, as the population is increasing. In India, poultry is one of the fastest growing segments of the agricultural sector. The growth rate of Indian poultry sector has been raising at 8 to 10% per annum. As a result the country is now the world's 5^{th} largest egg and broiler producer. India also leads in producing specialized products like Specific Pathogen Free (SPF) eggs in Asia, which perform as key contribution for manufacturing poultry vaccines and health products (ICAR Rating Feature, 2011). But during the last few years there is also an increase in pathogen loads in poultry farms due to excessive stress on the birds. This stress could be production stress or climatic stress which affects directly or indirectly on immune system of the bird and once immunity goes down birds easily acquire infection. At early days of age, Salmonellosis is one of the main diseases with economic concern to all phases of poultry industry (Bharti, 2008) and also a source of food borne transmission of disease to humans (Chalghoumi, 2009). Generally, the diseases are being controlled by administering antibiotics. But, the antibiotic usage is under inspection due to the emergence of antibiotic resistance. Therefore, it is important to find a viable alternative to antibiotics (Karlsson *et al.,* 2004). With this view, the present study was focused to generate chicken egg yolk antibodies (IgY) against *Salmonella enteritidis* and its in-vitro characterization for passive-immuno therapy in broiler chicks.

MATERIAL AND METHODS

Experimental chicken and bacterial strains

White leghorn chickens (19 weeks old) were obtained from the LK Poultry farm, Ayyampalayam, Coimbatore with good health conditions and known history of vaccinations. They were maintained in thoroughly cleaned and disinfected houses under complete hygienic measures; feed and water were given *adlibitum*. They were used for the generation of polyclonal antibodies against *Salmonella enteritidis* (SE) whole cell antigens.

Salmonella enterica subsp. *enteritidis* ATCC13076 (American Type of Culture Collection) standard strain was procured through Fisher Scientific, Ernakulam (Plate 1c). Standard strains were revived and maintained in the laboratory condition as per the standard protocols.

Preparation of whole cell antigens

Salmonella enteritidis was inoculated into brain heart infusion (BHI) broth and incubated at 37°C for 24 hours. Then, the cells were harvested by centrifugation and washed thrice with 0.1 N PBS buffer (pH 7.4); the pellet was re-suspended in PBS to achieve $1.5x10^8$cells/ml (McFarland's No.1). The colony forming units (CFU) of antigen cultures were determined by plate counts on Trypticase soy agar and then, the cells were treated with 3.7% formalin over night. The residual formaldehyde was removed by centrifugation in PBS buffer. Finally, the formalin inactivated cells were re-suspended in PBS and the concentration was adjusted to an optical density equivalent to McFarland's standard No.1. The cell suspension was aliquoted and stored under refrigeration for study (Lee *et al.,* 2002).

Immunization of chickens

21 weeks old white leghorn chickens were intramuscularly immunized with $1x10^9$ cells/kg of body weight in the pectoral muscle. The prepared whole cell bacterial antigens were used for immunization and the hens received booster doses ($1x10^9$cells/kg) at 14 days interval. The preimmune sera and postimmune sera were collected at before and after the various immunizations in chicken. They were tested for the presence of agglutinating antibodies by Slide Agglutination. Then the eggs were collected and stored at 4^oC for further investigation.

Purification of IgY from immune eggs

The yolk was separated and the egg yolk volume was measured. Twice the egg yolk volume of PBS was mixed with the yolk, thereafter 3.5% PEG 6000 of the total volume was added, vortexed and stirred for 30 minutes. Then the suspension was centrifuged at 4^0C for 20 minutes at 10,000 rpm. The supernatant was poured through a folded filter paper and transferred to a new tube. 8.5% PEG 6000 in gram was added, vortexed and stirred for 30 minutes. Then the tube was centrifuged at $4^{\circ}C$ for 20 minutes at 10,000 rpm. Then the supernatant was discarded and the pellet was carefully dissolved in 1ml of PBS and made up to final volume of 10ml. Then the 12% PEG 6000 was added and vortexed. Finally, the solution was centrifuged at 4^oC for 20 minutes at 10,000rpm and the final pellet was dissolved in 800µl PBS (Pauly *et al.,* 2011). Then, the IgY extract was dialysis. Thereafter the IgY-extract was pulled from the dialysis capsule by a pipette and transferred to storage vials and used for further studies. The purity of IgY-extracts was determined by SDS-PAGE technique and the total protein concentration was estimated by Lowry *et al.,* (1951) method. The total IgY concentration in the IgY-extract was estimated photometrically at 280nm (1:50 diluted with PBS) with the extinction coefficient of 1.33 for IgY (Pauly *et al.,* 2011).

Specificity of IgY

The specificity of Anti-*Salmonella* antibodies of the chicken serum and Egg yolk was determined by Rapid Slide Agglutination Test (RSA). Test was done on a plastic strip; 20µl of Coloured antigen and 20µl of IgY were placed and mixed thoroughly by stirring with the help of applicator stick. Then the slide was observed for the appearance of Agglutination with in 2minutes. The presence of

clumping in the test sample was similar to that of positive control (Rabbit serum against *Salmonella* antigen) indicated the agglutination reaction, which confirms the presence of specific IgY antibodies against specific antigen.

Estimation of Anti-*S.enteritidis*-IgY titre by ELISA

Micro titre wells were coated with 150µl/well *Salmonella* whole cell antigen solution at a concentration of 10µg/ml (Reference value - 500µg is corresponding to $1x10^8$ CFU/ml[9]) using 0.05M Carbonate bicarbonate buffer pH 9.6 and incubated at 4°C over night for binding. After coating, unbound antigens in the wells were removed by washing with PBS containing 0.05% tween 20 (PBST) for 3 times. The empty sites were blocked by adding 200µl per well of 1% bovine serum albumin in PBS and the Plate was incubated at 37°C for 1 hour. Plate was subsequently washed with PBST and incubated with 100µl IgY-extract at appropriate dilutions. Control wells contained PBST and pre-immune sera served as respective controls. Plate was incubated for one hour at 37°C and subsequently washed with PBST. For the chicken antibodies 100µl of diluted (1:1000) rabbit anti-chicken immunoglobulin coupled to horseradish peroxidase (Genei Pvt. Ltd, Bangalore) was added and the plate was incubated for 1 hour at 37°C. After incubation the plate was washed with PBST and enzyme activity determined by adding 100µl of freshly prepared substrate solution (1ml of TMB added with 19ml of sterile $D.H_20$). The plate was allowed to stand at room temperature in dark for 20 minutes. The reaction was stopped by adding 50µl of 4N H_2SO_4 and plates was read at 450nm in an ELISA reader.

In-vitro efficacy of Anti-*S.enteritidis*-IgY

Growth inhibition assay was performed to investigate whether the Anti-*S.enteritidis*-IgY could inhibit the growth of SE in liquid medium by the method prescribed by Lee *et al.,* (2002) and Guimaraes *et al.,* (2009) with some modifications. *S. enteritidis* was sub-cultured on blood agar plates. Then the colonies were scraped and suspended in Trypticase soy broth (TSB) and allowed to reach the optical density of 0.05 at 600nm, corresponding to a cell density of approximately $2.7x10^7$ CFU/ml. And then a 50µl of each bacterial culture was mixed with 5ml of TSB separately and incubated at 37ºC with shaking (blank control). The purified IgY from eggs of hens immunized with *Salmonella* antigen was dissolved in TSB at a concentration of 360mg/ml. The same concentration of Non-specific IgY in TSB was used as control. The reconstituted IgY solutions were filter sterilized with a 0.2µm filter. Then 5ml of filter sterilized TSB with desired concentration of specific IgY and Non-specific IgY were mixed with 50µl of prepared bacterial culture separately and incubated at 37ºC with shaking. The suspension were transferred to a micro-titer plate (100µl per well) and the turbidity of the each culture samples was measured at 600nm in 2 hours intervals until the culture sample without IgY (blank control) reached the stationary phase. The optical density was measured using a micro-titre reader. The growth inhibition assay was conducted for 12 hours.

RESULTS AND DISCUSSION

Concentration of total protein and IgY

The concentration of total protein in the IgY-extract was found to be 40.07 ± 0.57 mg/ml and the total IgY concentration was 30.24mg/ml after 24^{th} weeks of chicken age and then it was found to be relatively constant during the study period.

Purity and specificity of IgY-extract

The purity of IgY-extracts was determined by SDS-PAGE technique (Laemmli, 1970). A clear 180 kDa protein bands were observed. It was compared with the protein band obtained in the lane loaded with commercial standard IgY (Genei, Bangalore). Bands observed in test lanes and standard IgY lane were similar, which indicated the purity and also confirmed the molecular weight of the IgY extracted from the egg yolk. Some other minor impurities protein bands were also observed, but it was negotiable.

In Rapid slide agglutination test, the agglutination reaction was observed within 2minutes after mixing the serum and antibody solutions with respective antigens separately. The observation was compared with the agglutination reaction of standard anti-serum (IVRI, Izatnagar) with the *Salmonella* antigens, which was considered as positive control for interpretation of test results. The result indicated the presence of Anti-*S. enteritidis*-IgY in the serum samples and egg yolks. With this qualitative determination further titration of specific IgY was carried out by ELISA.

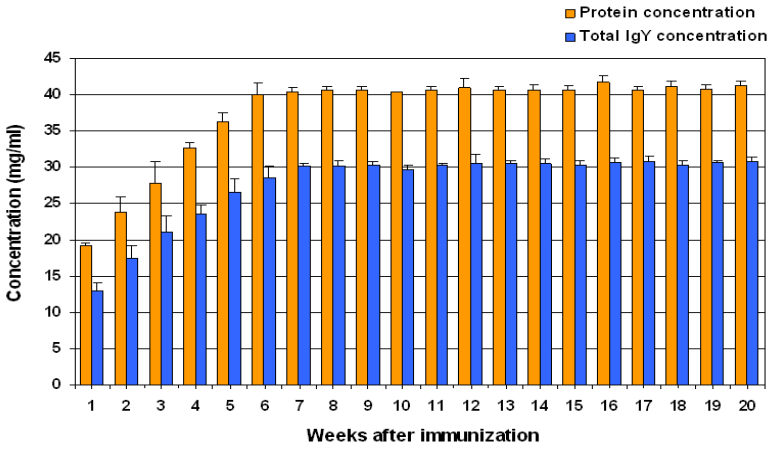

Fig.1 : Estimation of total protein and total IgY concentration in IgY-extract of immune eggs laid by the hens immunized with *S. enteritidis* whole cell antigen. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation.

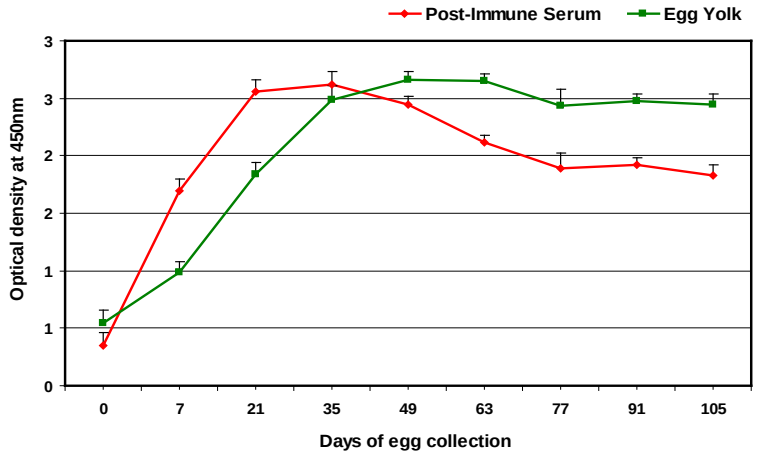

Fig.2: Level of specific-IgY in serum and egg yolk of chicken immunized with *S. enteritidis* antigen during the study. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation.

Titre of Anti-*S. enteritidis*-IgY in chicken serum and egg yolk

The specific antibody level in chicken serum and egg yolk was estimated by ELISA. The level of specific antibodies against the respective antigen in serum was increased after 1 week and slowly it reached the maximum titer on day 21st from the date of initial immunization.

However, the specific antibody level in the egg yolk was very weak on 21st day and gradually increased and reached the peak on 35th and 49th day. The titer of specific antibody was found to be

1:100000 on 35th Day and the titer were maintained with booster doses. Results indicated that there was a delay in the appearance of Anti-*Salmonella*-IgY in yolk when compared to serum after the first immunization. It was possibly due to the gradual accumulation of IgY during the yolk formation period by selective active transport (Kitaguchi *et al.*, 2008) similar findings was reported by Mahdavi, *et al.*, (2010).

Growth inhibition assay

The growth of *S. enteritidis* was similar in patterns that showed a lag phase of 0 to 2 hours, exponential phase of 2 to 6 hours and then stationary phase after 6hours of incubation at standard conditions. With the similar conditions, the growth of *S. enteritidis* with their respective specific IgY and non-specific IgY was plotted for growth inhibitory assay. In this assay the IgY concentration i.e., 360mg/ml was used on the basis of previous study done by Lee *et al.*, (2002). The growth of *S. enteritidis* with specific IgY showed significant reduction after 4 hours of incubation when compared to the growth with non-specific IgY.

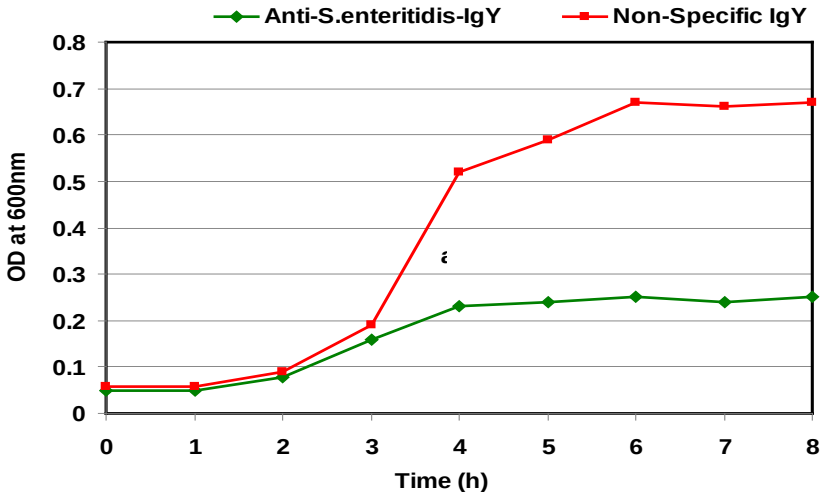

Fig.3: In-vitro efficacy of Anti-*S.enteritidis*-IgY against *S. enteritidis* growth in the liquid medium by Growth inhibition assay. Values are the mean of duplicate samples.

The results were comparable to the report of Lee, *et al.*, (2002). As a result it was observed that the *S.enteritidis*-specific IgY was found to be inhibited the growth of homologous cells in a liquid medium. The mechanism of action by which the antibodies suppress the growth was not clearly understood, but there are some proposed reasons discussed by Lee *et al.*, (2002) and Mahdavi *et al.*, (2010). The binding of specific IgY to bacterial surface components could cause some structural alterations of the bacterial surface, which may block the opportunity to take nutrients and proliferate. This could be because antibodies were generated against whole bacterial cell, which could possess binding activities against various epitopes of the bacterial surface as is the characteristics of a polyclonal antibody. Therefore, binding activities of IgY against bacterial surface components, including fimbriae and outer membrane protein may cause the growth inhibition.

The study indicated that the chicken are suitable alternative to generate polyclonal antibodies because of the cost-effectiveness and high yield than generating in mammals. The growth inhibition assay results revealed that the specific binding activity of IgY is an important factor for the major antibacterial property. However, it is essential to conduct more intensive studies to explore the exact mechanism of inhibition. The present study may help to some extent for the justification of applications of IgY antibodies in passive immuno therapy but how the chicken are normally responding to a treatment with

IgY to be critically studied by in-vivo efficacy in broiler chicks. This report could form a platform for further research on egg yolk antibodies and its commercial application in India.

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Biosorption of Heavy Metals from Magnesite Soil by Actinobacteria and Synergistic Effect of Biosorbed Actinobacteria on Plant Growth

V. Suganya, T. Shanmugasundaram, S. Ramya and R. Balagurunathan*
Department of Microbiology, Periyar University, Salem - 636 011, Tamil Nadu, India.

**Corresponding author: R. Balagurunathan, Department of Microbiology, Periyar University, Salem - 636 011, Tamil Nadu, India. E-Mail: rbalaguru@yahoo.com*

ABSTRACT

Heavy metal affects the all group of organisms and eco system, including microbial activities. Hence, the present study reports the biosorption of heavy metals from magnesite soil by actinobacteria and synergistic effect of biosorbed actinobacteria on plant growth. The soil samples were collected from magnesite mine, Salem and 20 different actinobacterial strains were isolated. These isolates were screened for their heavy metal resistance activity in the plate and liquid culture method with increasing metal concentration (50 and 650 µg/ml). The dominant metal reduction isolates were identified based on the OD value and the maximum metal absorption was showed by isolate AT9 against Mg – 90.3%, Mn – 92%, Pb – 59.3% and Zn – 93.2% respectively. Finally the treated soil was further used for plantation study. The plant root length, shoot length, total length, Wet weight, dry weight were analyzed after treatment and the results were compared with the fertile soil, and untreated mine soil. This is the first study to report the actinobacteria to reclamate the magnesite soil for crop cultivation purpose.

Keywords: Actinobacteria, magnesite mine, metal resistance, heavy metals

INTRODUCTION

The massive growth of industrialization and population has aggressively infected the human health care and environment either directly nor indirectly (Eisazadeh, 2008). Pollutants like dyes, heavy metals, effluents from different industries like cement, leather and household waste causes health threats to human beings (Saifuddin, 2007). The mining industry is playing a vital role in the economy of every country, mean time the wastage produced from the industry causing the environmental pollution due to improper disposal of waste. It has been estimated that over $2x10^9$ tons of environmentally hazardous mined and processed wastes could be generated per year due to mining activity in India (Natarajan, 2009). Among these pollutants, heavy metals are the most hazardous, infects human and cattle through a lot of diseases and disorders (Volesky and Strandberg, 2009). The effluents processed soil of mining industry contains high heavy metals such as Mn, Cu, Cr, Hg, Zn, Fe, Cd, Pb, As, Co etc. These are highly toxic to the environment by their increasing quantity in the soil (Giller *et al.,* 2009). Heavy metals affect all groups of organisms and ecosystem processes, including microbial activities. These metals which are not biodegradable are accumulated in living organisms when released into the environment from the accumulated sites. Though some of the metals like Cu, Fe, Mn, Ni and Zn are essential as micronutrients for life processes in plants and microorganisms, while many other metals like Cd, Cr and Pb have not known for their physiological activity, but they are proving detrimental beyond a certain limit (Bruins *et al.,* 2006). Changes in the trace elements profile of the soil cause physiological genetic changes in various forms of life, such as plants, aquatic and benthic fauna, insect, fish, birds and mammals as evidenced by recent research workers (Tyagi *et al.,* 1986; Samarth *et al.,* 2012).

Various methods are available for the removal and management of heavy metals, which involve technical inputs. Current methods are used for the treating soils contaminated with toxic metal are classified into three types such as, Physical, Chemical and Biological treatment. Among these physical and

chemical methods are having many drawbacks. So, the biological methods are withstood with comfortable position. Hence the current study is aimed to find the metal resistance capacity of actinobacteria from mine soil and to evaluate the use of microbially treated magnesite soil for the plant growth.

MATERIALS AND METHODS

Soil sample collection

In the present study, soil samples were taken from magnesite deposit (*Lat 11° 40' N; Long 78º 5' - 78º 11' E*) of Salem. Samples of different in colour and texture were collected in sterile polythene bags. The samples were immediately taken to laboratory for isolation of actinobacteria and physico-chemical analysis (Radhakrishnan *et al*., 2007).

Isolation and enumeration of actinobacteria

One gram of magnesite soil sample was dissolved in 10 ml of sterile distilled water and mixed thoroughly. Then the soil suspension was serially diluted upto 10^{-6} and inoculated 0.1 ml of each diluted suspension on Starch casein agar (SCA) medium at the pH of 8.5. The plates were incubated at 28°C for one month. The suspected colonies were observed, selected and inoculated in ISP2 agar medium and incubated at 28°C for 96-120 hrs (Shriling and Gottileb, 1966).

Screening of metal tolerance actinobacteria

All the isolated actinobacterial strains were taken for metal tolerance test using the modified method of Alvarez - Iglesias *et al.,* (2003). One loopful of the selected actinobacterial cultures were streaked aseptically into the sterilized ISP2 agar medium. Each individual plate was supplemented with different metals such as magnesium, manganese, zinc, lead, copper and mercury, in concentration of 50 µl. Each heavy metal containing plates were divided into six equal sectors and inoculated with the cultures. The streaked plates were incubated at 28ºC for five to seven days. After the appropriate incubation period the actinobacterial growth was observed on the plate.

Determination of metal sorption capacity of actinobacteria

The metal tolerant actinobacteria were further screened for metal sorption capacity based on the method described by Lee *et al.,* (1983) and Colomiris *et al.,* (1984). ISP2 agar plates were prepared and supplemented with different concentrations (50 to 650 µg/ml) of various heavy metals ($ZnSO_4$, $MnCl_2$, $MgSO_4$, $CuSO_4$, $HgCl_2$ and $PbNO_4$). All the culture was inoculated aseptically in the exponential growth phase and the plates were incubated for 3 - 5 days at 28 ºC along with the control plates. The metal sorption capacity of actinobacteria was determined based on the indications such as, no growth (plates was considered as the metal sensitive organism) and good growth (considered as metal tolerant).

Determination of metal sorption using liquid state fermentation method

The ability of the selected five actinobacterial strains for metal sorption was determined based on the OD value in UV - vis spectrophotometer at 542 nm (modified method of Hemambika, 2011). All the five actinobacterial cultures were inoculated in the freshly prepared, sterilized ISP2 broth individually and incubated in the rotator shaker at 120 rpm for 5 days. Five millilitre of sample was taken at the end of 5th day and centrifuged at 4500 rpm for 40 min. Then the supernatant was taken for the measurement of OD value. Based on the OD value dominant culture for metal sorption was determined.

The dominant metal sorption actinobacteria were determined by using following formula;

$$PAM = \frac{Co - Ce}{Co} \times 100$$

Co - Initial absorbance value, Ce - Final absorbance value, PAM – Percentage of metal sorption value

Metal sorption of the mine soil using adequate treatment

Four different magnesite soils were collected, processed and remediated by isolated actinobacterial strains. An adequate treatment was followed for the metal sorption of mine soil such as, Sterilized magnesite soil + actinobacterial consortium. In this method, negative control (only magnesite soil) and Positive control (Fertile land soil) also were used. All the reactions were carried out at 28°C in the duration of 30 days.

Metal analysis and nutritional characteristics of the soil

After the completion of 30 days the concentration of metal and nutritional characteristics of the soil before and after was analyzed.

Plantation study

The treated Magnesite mine soil color was changed due to the treatment. Further the efficiency of the soil in the growth of the plants was evaluated by the plantation study taken for a period of eight weeks against two different types of plants such as, *Vigna aconitifolia* (moth bean) and *Vigna radiata* (mung bean). The known amount of air-dried soil samples (500 g) were taken from the treated magnesite mine soil in a polyethylene bags. The seeds *Vigna aconitifolia* (moth bean) and *Vigna radiate* (mung bean) seeds were collected from the Department of Agriculture, Danispet, Salem district were sown in polyethylene bags in triplicates. The fertile land soil was used as a control. The plants were checked for the germination and watered continuously for the growth of the plants. The whole experiment was carried out under greenhouse condition. Moisture content of each bag was maintained at 75%. After germination, each plant group was transferred into three triplicates in a plastic pack and maintained for eight weeks. The maintenance of these crops were followed by accepted agricultural practices (Topalov and Hendri, 2009). The cup of each experiment was arranged according to completely randomized design with 3 triplicates. The experiment was started with crops study. The seeds were sown in the treated soil and regularly irrigated water daily. At the end of the eight week the plant root, shoot length, number of the leaves present and its fresh and dry weight were measured.

RESULTS AND DISCUSSION

Ultra-basic igneous rocks or dolomitic limestones is always called as magnesite ($MgCO_3$). The macro- and micronutrients, pH, temperature, humidity and climate of the magnesite mines were different from the other normal ecosystem. Actinobacterial diversity in the magnesite soil was better when compared to bacterial diversity, because the actinobacteria having the ability to produce various enzymes to convert the bicarbonate and to utilize as a carbon source from the magnesite soil (Das *et al.*, 1997).

In the present study five different soil samples were collected from Magnesite Ore, Salem and the physico - chemical parameters of the samples were analysed **(Table 1**). Totally 20 actinobacterial strains were isolated from the 5 different magnesite soils. The feature of magnesite soil and their fertility status created an ecosystem which considered as rich source for isolation of different extremophilic actinobacteria. The actinobacteria having the capacity to survive with these ecological niches due to the sun heated alkaline soil rich in magnesite which was not well studied upto date.

Among the isolates, morphologically different 10 actinobacterial strains were subjected to check heavy metal tolerance activity. For screening process, five actinobacterial species (AT2, AT4, AT6, AT9 and AT10) were showed resistance against four different types of heavy metals (Mg, Mn, Pb and Zn) at 50 µg/ ml concentration (**Table 2)**. The growth of the actinobacteria in the plates indicates the resistance of the strain towards the respective metals. In the case of Cu and Hg supplemented plates were showed no growth, which indicates that the strains were sensitive to copper and mercury, respectively. Similarly, Desala Prithviraj, (2012) stated that the actinobacterial species are effective in bioleaching, bioaccumulation of metal, ores beneficiation and desulfurization of fossil fuel and metal contaminated

soils. In addition to this, Ahmed, (1999) reported that the isolation of mercury and chromate tolerant diastrophic bacteria from the long term contaminated soil and industrial effluents.

Table 1: Physicochemical and metal characteristics of waste dumps of magnesite mine

S. No	Physicoche-mical/Metals	Magnesite soil			Average of three sites	Control soil	Permissible limit
		Site I	Site II	Site III			
1	pH	8.12	8.32	8.14	8.19	7.07	6-8
2	Temperature	30 ^{o}C	30 ^{o}C	30 ^{o}C	30 ^{o}C	30 ^{o}C	-
3	EC (mS/m^{-1})	0.1	0.1	0.1	0.1	1	0.1-1
4	$Cacl_2$	Nil	Nil	Nil	-	Nil	-
5	Texture	SCL	SCL	SCL	-	RLL	-
6	WHC (%)	58	53	54	55	81	-
7	N (Kg/acre)	*28	*35	*35	32.66	*89	114-180
8	P (Kg/acre)	**10	**13	**10	11	9	4.6-9
9	K (Kg/acre)	*18	*20	*20	19.33	75	49-113
10	Ca (mg/kg^{-1})	4307±2.17	4907±2.57	4430±2.09	4548	3129	52000
11	Mg	5286±2.96	5330±3.32	5400± 3.75	5338.66	4476.32	9000
12	Fe	1592±1.35	2070± 2.43	1773± 1.40	1811.66	6.01	2-6
13	Cu	57.09±0.03	65.96±0.12	53.97±0.08	59	87.98	100
14	Zn	2200±1.98	2222±1.07	2200±1.01	2207.33	2980.8	129000
15	Pb	1081±1.65	1141±1.79	1070±0.86	1097.33	489.56	300-600
16	Hg	51.75±0.21	69.96±0.27	43.97±0.17	55.22	24.54	1000
17	Mn	3025±0.35	3173±2.81	3221±1.07	3139.66	3489.53	1000
18	Cd	432±0.75	443±1.96	410±0.70	428.33	117.45	200

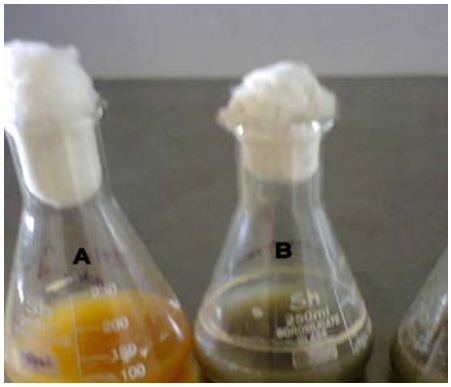
SCL: Sand-Clay-Loamy, **RLL**: Red-Loamy-Lateritic. *****lower than permissible limits, ******higher than the permissible limits. **WHC:** Water Holding capacity. The values are average of mean and the data present in the parenthesis are standard deviation of triplicates. The permissible limit for serial number 1-8 adopted from Tamil Nadu soil testing laboratory and 9-17 (in ppm) data's were adopted from Ramamurthy and Kannan, (2009); Akpoveta *et al.,* (2010)

Table 2: Heavy metal tolerant potentiality of actinobacterial strains (50 µg/ml)

S. No	Concentration of metals (µg/ml)					
	Mg	Mn	Pb	Zn	Cu	Hg
AT1	-	-	-	-	-	-
AT2	+++	++	++	+	-	-
AT3	-	-	-	-	-	-
AT4	+++	++	+++	+	-	-
AT5	-	-	-	-	-	-
AT6	+++	+++	+++	+	-	-
AT7	-	-	+	+	-	-
AT8	-	-	-	-	-	-
AT9	+++	+++	++	+	+/-	-
AT10	+++	+++	+++	++	-	-

+++: Effective growth, **++:** Good growth, **+:** Normal growth, **+/- :** Trace growth, **-:** No growth

The microorganisms (bacteria, fungi, algae, and actinobacteria) are highly effective in sequestering heavy metals and these have been used to remove metals from polluted industrial and domestic effluents on a



large scale (Tom and Wong, 1993). The metal sorption capacity of the selected actinobacterial strains were checked by using different metals like Mg, Mn, Pb and Zn, but it exhibits different levels of resistance for each metal (**Figure 1**).

Figure 1: Biosorption of Metals by actinobacterial culture (A – Control, B - 50 µg/ml metal concentration, C – 650 µg/ml metal concentration)

The results showed that the metal sorption limit of AT2, AT4, AT6, AT9 and AT10 against Cu and Hg was about 100 µg/ ml^{-1} and Mg, Mn, Zn and Pb was about 650 µg/ml^{-1} respectively. The percentage of tolerance level of the test actinobacterial strains were presented in Table 3 and Figure 2. The tolerance level suggests that the resistance level against individual metal was dependent on the isolates. The five isolates (AT2, AT4, AT6, AT9 and AT10) showed relatively no tolerance activity to all metals. The tolerance among the isolated actinobacteria were observed in order of Mn>Mg >Zn >Pb. The metal sorption efficiency was effectively occurred at 38 ± 2 ^{o}C and in the alkaline environment and the activity was enhanced by the water soluble organic compounds (Table 4). Effects of toxic metals on fungal growth have shown intra and inter specific variability and dependence on type of metal contaminants and speciation (Plaza *et al.*, 1998).The variation in the metal tolerance might be due to the presence of one or more types of tolerance strategies or resistance mechanisms exhibited by different actinobacteria (Gadd, 1993).

Table 3: Metal tolerance capacity of the actinobacterial strains

Strain No.	Heavy metal concentration 650µg/ml			
	Mg	**Mn**	**Pb**	**Zn**
AT2	82.12%	79.7%	57.4%	91.3%
AT4	72.21%	70.25%	38.6%	90.4%
AT6	84.7%	81.3%	57.9%	89.8%
AT9	90.3%	92%	59.3%	93.2%
AT10	80.2%	78.2%	56%	92.9%

Table 4: Metal adsorption studies by adequate treatment

S. No	Experiments	Cu	Zn	Pt	Cd	Ni	Mn
1	Control	335.5	270	855	682.5	495	870.5
2	Soil + actinobacteria	235.75	203	272.5	147.5	**80**	510.5

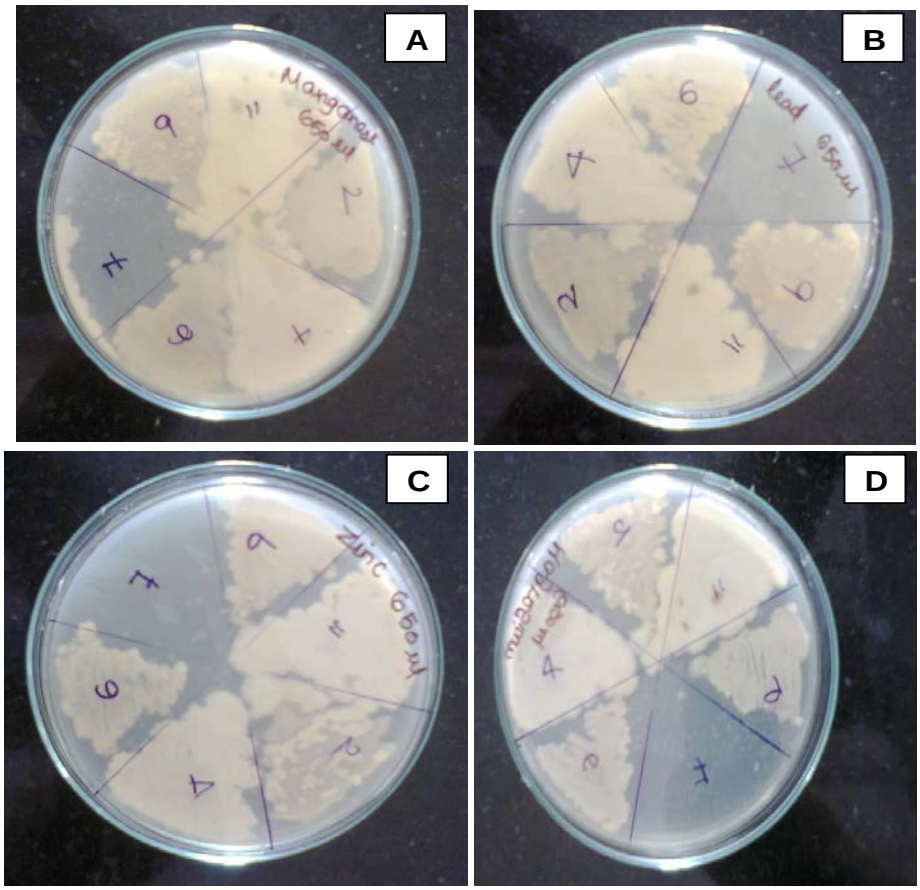


Figure 2: Metal tolerance capacity of actinobacteria in 650µg/ml concentration (A - Lead acetate, B - Mangenese chloride, C - Zinc sulphate and D - Magnesium chloride)

Based on the treatment, the metal concentration was reduced due to the activity of actinobacteria in the magnesite soil samples (Figure 3). The treated soil was analyzed by the atomic adsorption spectra photometer. The results are given in the (Table 5)**.** Earlier reports also suggested that the capability of several bacteria involved in removal of uranium, Cd, Pb and other toxic metals from polluted soil (Strandberg and Gohi, 1981). The temperature and pH of the soil was an essential parameters that affecting the microbial growth, metal utilization and activity on soil of mining industries (Ehrlich and Brierley, 1990).

Figure 3: Stages of magnesite soil treated with actinobacterial consortium (A – Magnesite soil, B – Soil + Actinobacteria Consortium and C- Treated soil)

Table 5: Metal sorption of magnesite mine soil by using actinobacteria

S. No	Study	Metal	Untreated soil (ppm)	Treated soil (ppm)
1	Actinobacteria+	Mg	2312	1899
	Magnesite soil	Mn	7745	5433
		Pb	867	689
		Zn	723	561
2	Control -Magnesite soil	Mg	2312	2319
		Mn	7745	7749
		Pb	867	854
		Zn	723	743

In the magnesite mine soil, the nitrogen content is very low and also micronutrients and trace elements concentration were high. So it may be toxic to the plant growth. The results of nutritional characteristics of the soil after the treatment and before the treatment were given below (**Table 6**).

Table 6: Nutritional characteristics of the soil

S. No	Study	Untreated soil (kg/acre)			Treated soil (kg/acre)		
		N	P	K	N	P	K
1	Actinobacterially treated	55	13	28	68	24	63
2	No treatment	55	13	28	55	13	28

In the plant growth study, both plants *Vigna aconitifolia* and *Vigna radiata* were germinated well in all the treated soil within the time period of two days. The seed germination was occurred in the untreated mine soil was very less. At the end of the eight weeks period, the plant root, shoot length, number of leaves, fresh and dry weight were observed and the results were represented in the Table 7 and 8. Comparing with the fertile soil, the plants grow in the treatment 2 i.e. magnesite soil containing the actinobacteria showed good level of the shoot, root length number of leaves and fresh and dry weight. All the results in the triplicates were represented by the mean and standard deviation**.**

Table 7: Plantation experiment using *Vigna aconitifolia*

TREATMENT	Root length (cm)	Shoot length (cm)	Total length (cm)	No of leaves	wet weight (g)	Dry weight (g)
1 (Fertile soil)	3.1±0.4	11.8±2.48	15.7±3.4	2.6±1.1	1.28±0.13	0.37±0.06
2 (Magnesite soil + Actinabacteria)	2±0.3	9.8±0.55	11.8±0.5	2±0	1.13±0.15	0.25±0.04
3 (Magnesite soil)	1.8±0.25	8.8±0.60	10.7±0.35	2±0	0.95±0.16	0.18±0.06

Table 8: Result of plantation experiment of *Vigna radiata*

Treatments	Root length (cm)	shoot length (cm)	Total length (cm)	Number of leaves	Wet weight (g)	Dry weight (g)
1 (Fertile soil)	3.06±0.5	12.4+3.6	15.5±4.1	2±0	1.05±0.17	0.23±0.06
2 (Magnesite soil + Actinabacteria)	2.2±0.4	11+2.3	13.2±2.4	1.6±0.5	0.85±0.15	0.21±0.05
3 (Magnesite soil)	1.8±0.5	7.9+1.5	10±1.7	1.5±0.8	0.82±0.17	0.14±0.05

The results obtained from the study revealed that the bioremediation mainly possible only by the actinobacterial metabolism. In situ method of reclamation can be very efficient for the treatment of soils contaminated with toxic heavy metals. The target actinobacterial flora was isolated from the metal containing mine soil, they possess metal tolerant potential for several heavy metals. These metal resistant potential were made by the metal containing environment (waste dumps of mining industry) and they could posses effective bioremediation or metal sorption potentiality. Due to heavy metal tolerant and metal absorption potentiality of these bacterial isolates from magnesite soil is a suitable choice for bioleaching and bioremediation processes. Hence, this study finally reveals that the treatment with actinobacteria is a good way to treat and to reclamate the mine soil into the fertile land. This is the first pilot scale study carried out in this aspect and this results proves that the potential ability of the microbes and to improve the nutritional status of the soil.

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Tannin Degrading Ability of Microorganisms Isolated from Soils Differing in Tannin Contents

N. Hemalatha[1*], O.K. Remadevi[2] and Chandrasekaran Ramanigopal[3]
[1]Department of Microbiology, Periyar University, Salem - 636011, India
[2] Institute of Wood Science and Technology, Malleswaram, Bangalore -3, India
[3]Department of Management Studies, VMKV Engineering College, Salem-636 308, India.

**Corresponding Author : N. Hemalatha, [1]Department of Microbiology, Periyar University, Salem - 636011, India, Email : aarthihema2004@yahoo.com*

ABSTRACT

Tannins are plant secondary metabolites which has antimicrobial activity. Though absent in some places they are rich in other places which enter the soil mainly by plant leachates, litter, tannery effluents, etc. In the present study the type and counts of microbes in the tannin containing soil from under Acacia tree and tannin free soil from pasture land was investigated. A preliminary test was done to find the tannin content of the test soils using Folin – ciocalteau total phenolics method. Though undetected in pasture soil, the tannin content of the tree soil was found to be 4.5%, The microbial counts were significantly lower in tannin-containing soil. Organisms, belonging to the same species were selected from both soil types for tannin degradation study. While 80 % of the tested organisms collected from tannin rich soil showed tannase producing ability in the tannin agar medium, only 20% of the tested organisms from non tannin site exhibited tannin degrading ability.

Key words: Tannin, tannin degraders, tannase, bacteria, microbial count, allelopathy

INTRODUCTION

Tannin is a complex recalcitrant phenolic compound that influences the chemical nature of the soil. The growth and viability of soil microorganisms are influenced by the physical and chemical nature of the soil. Microorganisms are responsible for the biological properties of the soil. Nutrient cycling, organic matter formation and decomposition, soil structure formation, and plant growth promotion are among the beneficial functions that bacteria perform. Tannins are the plant secondary metabolites. Tannins make up a significant portion of forest carbon pools. Tannins are being added to soil directly as plant litter, foliar leachates, root exudates or after passage through herbivore guts. A large fraction of tannins and simpler phenolics appear to enter the soil as foliar leachate (Stevens *et al.,* 2002). The active fraction of root exudate was found to be composed of gallic acid (Rudrappa *et al.,* 2007). Tanneries and other industrial effluents also add tannin to soil or water sources near the tannin and leather manufacturing industries. Reduction of vegetation and failure of cultivable new vegetation growth are being experienced in such tannin polluted areas due to allelopathic effect of tannins. Carbon and nitrogen mineralization were also inhibited by tannin-addition (Ushio *et al.,* 2013). Allelopathy, may deleteriously affect interactions between rhizospheric microbial communities and native plant species (Wardle *et al.*, 2004; Callaway *et al.,* 2008).

Soil quality can be evaluated by its biological, chemical and physical properties (Ashton and Macintosh, 2002). Tannins have profound influence on the chemical and biological properties of the soil that in turn affect the physical nature of the soil. Tannins may play an important role in plant–plant, plant-microbe and plant–litter–soil interactions. Tannnis are known to affect the microbial biodegradation process in soil and the microbes are the main source for the release of nutrients for plant growth and yield

(Spaccini, 2002). Tannins have a range of effects on various organisms – from toxic effects on animals to growth inhibition of microorganisms.

A number of studies demonstrated that tannins inhibit microbial activity (Fierer *et al*., 2001). Tannin concentrations required to inhibit microbial populations vary. Yeasts appear to be rather tolerant of tannins, but inhibitory effects are reported at high concentrations of 10 % (Scalbert, 1991). At a concentration higher than 10 mM tannins are literally toxic to their environment (Arunachalam 2001).Some microbial groups are more sensitive to tannins than others. Concentrations of 1 – 2 % tannin reduce the overall decomposition of organic materials applied to soil (Field & Lettinga 1992). Carbon and nitrogen mineralization were also inhibited by tannin-addition. Smith *et al.* (2003) studied with green tea catechins, which are monomeric polyphenolics and reported that gram-positive bacteria are more sensitive to their bactericidal effect than gram-negative bacteria. Ushio *et al.*, 2013 found that the fungi-to-bacteria ratio was higher after tannin-addition. Tannins may affect the types and distribution of microorganisms present in a soil. Altered soil microbial community may negatively impact the ability of native species to survive in the same soils (Batten *et al.*, 2008).

Some organisms have the capacity to degrade tannins. Tannin degraders can produce extracellular tannase to breakdown tannins. Tannase production has been reported in fungi and bacteria (Scalbert, 1991). Recently many investigations are done to isolate tannin-degrading microbes from different ecosystems and to study the process of degradation. But how the tannin presence influences the tannin degrading ability of the indigenous microbial flora had not been yet studied. Also most of the organisms capable of degrading tannins isolated till date are either anaerobic ruminant bacteria or fungal strains associated with the degradation of wood and forest litter. A major problem with the above organisms is their slow degradation process. Hence the present study was carried out to find the type of bacterial load existing in a tannin rich soil site. Their tannin degrading ability was checked and compared with those of their counterparts isolated from soils where tannin was absent.

MATERIALS AND METHODS

Collection of soil samples

Two categories of soil samples viz. tannin-soil (**TS**- soil from under *Acacia* tree) and non-tannin soil (**NTS**- soil from pasture land with no *Acacia* growths) were collected from a depth of 10-30 cm from top surface soil. The surface soil was preferred because the microbial population is most dense due to higher organic matter which is the dominant reservoir of microbial food. A preliminary test was done to find the tannin content of the test soils using Folin – ciocalteau total phenolics method (Makkar *et al*, 1993). Tannins were expressed as tannic acid equivalent in the dry matter as percentage. Though undetected in pasture soil, the tannin content of the tree soil was found to be 4.5%.

Sampling of non-tannin soil (NTS)

A sampling field of 3000 square feet in Periyar Palkalai Nagar region pasture land with an even topography was chosen for the collection of soil samples. Five representative areas (replications) of 1 m^2 each equidistantly apart (5 m), were chosen randomly from the field. Five intact soil cores (≈150cm^3) from each representative area were carefully removed at 0-25 cm depths using a 5 cm diameter La Motte soil sampling corer with a removable inner tube. Clean soil sample bags were used for collection of samples**.** The sub samples of soil were composited for each site and used for analysis.

Sampling of tannin soil (TS)

The soil samples under five *A. nilotica* trees in Periyar Palkalai Nagar region were taken. Samples were collected from five equally spaced (1 m) spots around each *A. nilotica* tree trunk. The five sub-samples with similar core size were bulked to produce one composite sample for each tree site**s** (replicate).At the time of sampling, the soil temperature was 20-25^o C and soil moisture content was 50-

60 % as determined on gravimetric basis. The soil samples were subjected to microbial assay within 24 hrs of collection to minimize the effects of storage on microbial activity.

Soil microbiological assays

There are thousands or millions of organisms in a small quantity of soil and indeed, the estimated number of organisms in a soil depends on which technique is used. In this study serial dilution method described by Dubey and Maheshwari (2002) was followed for the enumeration of active bacterial population using nutrient agar medium. Before each transfer the tubes were mixed gently to get a uniform suspension. Five replication plates were prepared from each dilution. Incubation period was 24 - 72 hrs at 35^{o}C. Plates which contained 30-300 colonies were selected for counting. Within this range the count can be accurate or else the numbers will become too low to count or too numerous to count. In plates with more than 300 colony numbers, the possibility of interference of the growth of one organism with that of another may occur. If discrete colonies are not growing in the medium their exact number could not be found out. The result was expressed as number of colony forming units per ml.

Colony selection

Plates of higher dilutions were used to identify morphologically distinct colonies. Similar colonies were identified based on colony appearance and subjected to pure culture technique using quadrant streaking. Isolated colonies can be maintained in subcultures until further tests. Biochemical characterization of prominent isolated microbes was done for their genus and species level identification confining to Bergey's Manual of determinative bacteriology. The identified microbes were tested for their tannin degrading ability.

Screening for tannin degraders

Screening experiment for tannase enzyme production (Bradoo *et al.,* 1996**)** was done on the isolated and identified colonies. Microbial tannin degrading ability was performed in plates of selective medium which contained (gm/litre): tannic acid (Sigma Chemical Co.), 10.0, $NaN0_3$, 3.0; KH_2P0_4, 1.0; $MgS0_4$. $7H_20$, 0.5; KCl 0.5; $FeS0_4$. $7H_2O$, 0.01; agar, 30.0, pH 4.5. Point inoculations were carried out and plates were incubated at 32ºC for 72 hours. Two experimental batches were set up. Ten bacterial species isolated from Tannin soil were selected to constitute the experimental batch one. Similarly ten bacterial species isolated from Non tannin soil were selected to constitute second experimental batch. Five replications were maintained for all the 20 colonies selected to form a total of 100 experimental plates. The tannin degrading ability was found by the zone of clearance around the colonies.

Statistical analysis

Statistical analysis of total bacterial counts in non-tannin and tannin soil was analyzed using students "t" test. Central tendency measure like average and percentages were used for result interpretations.

RESULTS

The tannin content of the test soils were found using the spectrophotometric results of Folin - ciocalteau total phenolics method. Tannins were expressed as tannic acid equivalent in the dry matter as percentage. Though undetected in pasture soil, the tannin content of the tree soil was found to be 4.5%.

Total microbial counts

The nutrient agar plates inoculated with prepared dilutions of the test soil samples were observed carefully for the total number of bacterial colonies after 24 hours of incubation. Each colony represented one bacterium. The difference in the bacterial counts of two soils was highly significant being high in NTS ($108 \pm 22 \times 10^4$ vs. $3.8 \pm 1.1 \times 10^4$, SED - 9.85) ($p<0.001$).

Bacterial colonies

Ten morphologically different colonies based on colony appearance were identified in Tannin soil. These colonies were identified to belong to the genus Bacillus, Mycobacterium, Micrococcus, Lactobacillus, Enterobacter, Pseudomonas, Citrobacter, Serratia, Escherichia and Klebsiella, after biochemical characterization based on Bergey's manual of determinative bacteriology. Apart from those mentioned in Table-2, NTS contained additional bacterial genus, namely Staphylococcus, Streptococcus, Acinetobacter and Aeromonas. Biochemical characterization of isolated colonies to their species level was carried out until 10 similar bacterial species were identified common to both types of test soils (with and without tannin). The 10 bacterial species common to both the soil types (Table 2) were taken for screening the tannin degrading ability separately.

Tannin degraders

Tannic acid was the sole carbon source in the medium. After three days of incubation, the colonies were checked in both the experimental batches (the organism isolation sites being different) for the clear zones of tannin hydrolysis. In NTS experimental batch, among the ten tested organisms only two of them namely *Pseudomonas fluorescens* and *Bacillus megaterium* were able to form tannin hydrolysis zone. In TS experimental batch, eight of the tested organisms inclusive of the above two, exhibited tannin degrading ability. Thus two of the tested organisms namely *Pseudomonas fluorescens* and *Bacillus megaterium* were able to form tannin hydrolysis zone in both the experimental batches though the zone of clearance was more around colonies isolated from tannin site (Table 2) with the maximum zone of clearance of 4.5 and 3.6 cm respectively. The other six of them exhibited tannin degrading ability only in the experimental batch II, the organism's source of isolation being tannin site. In all the other test plates though there was growth of inoculum, there was no zone of tannin clearance (Table 2). Gram negative organisms were more among the isolates from tannin soil.

Table 2. Tannin degrading ability of microbes isolated from the two types of soil

Bacterial species	Grams Reaction	Tannin degradation	
		From Tannin soil Exp. Batch I	From Non tannin soil Exp. batch II
Bacillus megaterium	Gram positive	+ (3.6)	+ (2.2)
Citrobacter freundii	Gram negative	+ (1.2)	-
Mycobacterium neoaurum	Gram positive	+ (2.1)	-
Micrococcus luteus	Gram positive	-	-
Lactobacillus plantarum	Gram positive	+ (2.8)	-
Serratia marcescens	Gram negative	+ (1.5)	-
Enterobacter agglomerans	Gram negative	+ (1.7)	-
Pseudomonas fluorescens	Gram negative	+ (4.5)	+ (2.5)
Escherichia coli	Gram negative	+ (2.9)	-
Klebsiella pneumoniae	Gram negative	-	-

+Occurrence of tannin degradation; - Absence of tannin degradation; Values in parenthesis – Tannin hydrolysis zone diameter in mm (Average of 5 replicas).

DISCUSSION

The falling litter increased the tannin content of the soil under the *A. nilotica* tree. The total microbial counts were low in tannin soil, which may be due to the allelopathic and microbicidal effect of the tannins. Tannins had found to limit the growth of Gram positive organisms. This is similar to the study made by Smith *et al.,* 2003 wherein the green catechin tannins are found to suppress Gram positive bacteria. The forms flourishing in any particular microenvironment are the ones best adapted to the environmental conditions (McLean and Parkinson, 1997). Soil microbial communities may adapt to specific tannin chemistries during forest succession (Cowley and Whittingham, 1961). In the present study more microbial species were isolated from non tannin containing pasture soil whereas fewer microbial species were identified from the soil with Acacia tree tannin. New conditions may be physiologically tolerable, allowing acclimatization (an adjustment of physiology within individuals) or adaptation (increased abundance and reproduction of tolerant genotypes over generations), or may be intolerable and get locally extinct if adaptation is not possible (Parmesan, 2006).

Organisms possessing tannin degrading ability were more in TS. After the incubation period the clear zones appeared around the tannase producing bacterial colonies. The clearing zone formation is because of the ability of producing extracellular tannase in the medium containing only tannic acid (Mondal and Pati, 2000). Zones were formed due to hydrolysis of tannic acid to gallic acid and glucose leading to a decrease in opacity of the medium. Tannase enzyme, catalyses the hydrolysis of ester bonds and depside bonds between phenolic acid and an alcohol, to release gallic acid and glucose (Barthomeuf *et al.,* 1994). Gallic acid is often complexed as gallotannins (Niemetz and Gross, 2005). It is now known that free gallic acid is released from complexed gallotannins by simple hydrolysis reactions, wherein a tannase activity breaks gallate ester to form free gallic acid, ellagic acid, and Glucose (Mahoney and Molyneux, 2004). Plant- or microbial-derived tannase may facilitate free gallic acid release. Tannase enzyme production might be one of the possible adaptive mechanisms to overcome the microbial toxic and allelopathic chemical, namely tannin. In the present study, 80% of the tested organisms from tannin site exhibited tannin degrading ability and only 20 % of the tested organisms from non tannin site exhibited tannin degrading ability.

It was found that the two organisms namely *Pseudomonas fluorescens* and *Bacillus megaterium* had tannin degrading ability irrespective of their isolation site, but bigger zones of tannin clearance around the colonies from tannin site indicated improved tannin degrading ability. From the studies conducted by Chowdry *et al.,* 2004, two strains showing relatively high efficiency in degrading tannic acid and gallic acid were identified to belong to the Pseudomonas species. Ijory *et al.,* 2007 isolated a tannin-degrading strain of Bacillus sp. AB1 able to utilize 1% (w/v) of tannic acid. Mingshu *et al*., 2006 in his studies had found the ability of *Citrobacter freundii* to degrade polyphenolics. Franco *et al.,* (2005) isolated Serratia bacterial strains from sediments collected at a tannery discharge place characterized by tannin degradation property. Zeida *et al.,* 1998, identified *Enterobacter agglomerans*, to be exhibiting a high level of gallic acid decarboxylase activity. Bacterial species known to use tannin as a sole carbon source include species of *Achromobacter*, *Azotobacter*, *Bacillus*, *Corynebacterium*, *Escherichia*, *Klebsiella*, *Pseudomonas* and *Staphylococcus* (Bhat *et al.*, 1998; Scalbert, 1991).

There is less evidence demonstrating bacterial degradation of tannins, although bacteria can tolerate high tannin concentrations (Gamble *et al*., 1996). In the present study, two organisms namely *Klebsiella pneumoniae* and *Micrococcus luteus* had lacked tannin degrading ability, but were able to grow in tannin containing media and soil that suggest their tannin tolerance ability. Also Klebsiella colonies on tannic acid medium appeared excessively mucoidy which might be a mechanism adapted by the organism to avoid direct contact with toxic tannin in the medium. Such physiological alterations may

increase the organism's tolerance ability. Physiological performance is the principal determinant of a species' tolerance to environmental variability and change (Somero, 2012).

Forty percent of tannin degraders from TS site were able to produce tannin degradation zone, but the same organisms from NTS site failed to produce tannin degradation. Thus some microbes can adapt to the environmental conditions where they were growing in the long run, which can even decide their gene expression patterns. Tests for tannin degrading ability of microbes showed that though the organisms belonged to the same species, they differ physiologically based on the soil condition in which they are growing. It was suggested that microbial species exposed to stress condition tends to overcome it by developing adaptive mechanisms that is lacking when grown in stress free environment. Bacterial cells continuously adjust gene expression in response to challenges from their environment (Berthoumieux *et al.*, 2013). In response to the availability of carbon sources in the environment, the transcription factors regulate, a large number of genes encoding enzymes in central metabolism (Gutierrez-Ríos *et al.*, 2007; Baldazzi *et al.*, 2010). All the living individuals of a species contain a distinct combination of genes, and the intrinsic interaction among the gene pool influences evolution, survival, and phenotypic/genotypic changes of the part of the biodiversity, that is, community (Dastager *et al.*, 2011). Such species level difference may form the base for strain level differentiation based on tannin degrading ability.

CONCLUSION

The organisms that lacked tannin degrading ability originally, had developed the ability to degrade tannins while growing in tannin rich environment. Thus some microbes can adapt to the environmental conditions where they were growing during the long run by developing resisting mechanisms. The present study can be further extended to find out the tannin degrading ability of other predominant soil microbial flora namely fungi and actinomycetes. Also tannin degradation can be checked under higher tannin concentrations. Efficient tannic acid degraders can be used for tannin bioremediation, and in the study of genes involved in the production of tannase, an industrially important enzyme. The research on biodegradation of tannins is in an incipient stage and further studies have to be carried out. Such studies apart from helping us to understand the phenomenon of nature, ecology of microbial succession and allelopathic effects, will also aid to exploit the potential of various tannins for large scale applications in food, fodder, and medicine and tannery effluent treatment.

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Bioremediation of Ferric Iron in Synthetic Metal Oxide Using *Bacillus* Sp. (SO-10)

G. Sivakami[1], R. Priyadarshini[1], V. Baby[1], S. Rajakumar[2] and P.M. Ayyasamy[1]*
[1] Department of Microbiology, Periyar University, Salem - 636 011, India.
[2] Department of Marine Biotechnology, Bharathidasan University,
Tiruchirappalli - 620 024, India

**Corresponding Author: P.M. Ayyasamy, Department of Microbiology, Periyar University, Salem - 636 011, India, Email : pmayyasamy@gmail.com*

ABSTRACT

Now a day heavy metal Fe in soil is considered a major environmental problem facing many countries around the world. Contamination of Fe occurs in soil due to both anthropogenic and natural causes. During the last two decades, extensive attention has been paid to the management and control of soil contamination. Decontamination of heavy metal especially Fe in the soil has been a challenge for a long time. Bioremediation is one of hopeful process for removal of Fe from contaminated sites. In the present study, an attempt was made to detoxify Fe in synthetic metal using a bacterium *Bacillus* sp. (SO-10). The effect of various carbon sources on the dissolution and transformation of Fe was investigated using a mineral salts medium containing 1 g of Fe oxide to select the most effective carbon source. Among three carbon sources, namely glucose, starch and cellulose, glucose at 1% was found to be the most effective. Therefore, glucose was used as a representative carbon source for the second part of the biological treatment in the mineral salts medium at various pH.

Keywords: Bioremediation, *Bacillus* sp., Iron, Metal Oxide, Mineral Salts Medium

INTRODUCTION

Heavy metals are a serious threat to soil quality due to their persistence after entering the soil. Heavy metals in soils may adversely affect environmental quality and ecosystem health. The soil can become contaminated with metals from a variety of various anthropogenic and natural sources. However, agriculture, manufacturing, mining and the land application of sewage have made significant contributions to soil contamination in a relatively short period of time (Gadd and White, 1993). The commonly found heavy metals in the subsurface environment include iron, lead, mercury, arsenic, chromium, cadmium, nickel, zinc and copper etc (Han *et al*., 2002). Among the heavy metals in the water and soil, the concentration of iron higher than the others. In oxic environments, only the ferric state was energetically stable, common forms are poorly crystalline goethite and hematite (Schwertmann and Cornell, 1991). Iron is the fourth most abundant element in earth (Greenwood and Earnshaw, 1984). Unlike most organic pollutants, heavy metals con not be chemically or biologically degraded; however, microorganisms can interact with these heavy metals and change their oxidation state through the addition (reduction) or removal of (oxidation) electrons. Hence, the decontamination of heavy metals in the soils has been a challenge for a long time.

Heavy metals are sorbed and retained by soil leading to their accumulation. Unfortunately, the capacity of the soil to store this toxic form such as ferric state of iron is not infintite (Bridges and Baren, 1997). Iron oxyhydroxides are ubiquitous reactive constituents of soils, sediments and aquifers they exihibit large surface area, which bind trace metals, nutrients and organic compounds. Most of the Fe(III) in the soils and sediments occurs as solid phase minerals that range in their degree of crystallinity, particle size surface area and reactivity (Murray, 1979). If a change occurs through natural and other processess, the toxic form of solid phase minerals can directly contaminate the ground water as they percolate through the medium. There is a need to develop inexpensive extraction processes to remove the ferric oxy hydroxides from contaminated soils.

Within the past few years, a number of reports have appeared on the isolation and properties of iron reducing microorganisms. Numerous bacteria which reduce Fe(III) during growth on organic substrates have been described (Lovley, 1987). Although the ability of some microorganisms to bring about the reduction of Fe(III) has been known since the 19th century (Adeny, 1984). Until recently, even

microbiologists considered that much of Fe(III) reduction in natural environments was result of nonenzymatic processes.

Microbial transformation of ferric ions with oxidation of organic matter in soil as a minor pathway. However, the only ferrous reducing microorganisms known to metabolize fermentable substrates, such as sugars and amino acids or fermentative microorganisms which only reduce Fe(III) as a minor side reaction in their metabolism. Iron reducing and iron oxidizing microorganisms gain energy through reduction or oxidation of iron since the organisms are significant in the biogeochemical cycling (Zhang *et al.*, 2010). In anoxic soil, ferric iron reduction is one of the most important terminal electrons accepting processess (Hori et al., 2010). Microbial Fe(III) reduction also contributes to many phenomena of environmental and/or practical importance. The coupled reaction of Fe reduction and organic matter oxidation is an important mechanism in a broad diversity of environments (Lovely *et al.*, 1991). The Fe reducing microorganisms which can conserve energy to support growth for the oxidation of organic compounds coupled to the reduction of Fe.

The substrates such as carbon sources sand organic acids play a vital role in the dissolution of both natural and anthropogenic components in the oil environment through a variety of biogeochemical process. The effects of carbon sources on the remediation of heavy metals from water and soil systems for biological processes have captivated the attention of many researches (Webb *et al.*, 1998). Accordingly, several authors have examined the effects of carbon sources on the dissolution of heavy metals from water and soil systems for biological process examined the potential use of three different carbon sources lactate, acetate and succinate, in the reduction of Ferric irons from sediment samples (Lovley and Klug 1986). Webb *et al.* (1998) used four different carbon sources, lactate acetate, propionate and glycerol. They reported that the rate of bacterial growth and Fe removal were higher using acetate and propionate. Ayyasamy and Lee (2009) tested the dissolution rate of ferric ions using three carbon sources glucose, lactic acid and acetic acid at various concentrations. The results revealed that glucose support maximum bacterial growth to enhance highest dissolution efficiency. The complete oxidation of glucose to carbon-di-oxide with Fe(III) serving as the sole electron acceptor was energetically favourable and thus could potentially support the growth of Fe(III) reducers (Lovley and Phillips, 1986).

Influence of pH on the ferric iron reduction is also a important factor. If a change in pH occurs in soil through natural or anthropogenic processes the ferrous ion may also contaminate the ground water. The effects of pH on the remediation of ferric oxides from soil systems were examined by many authors. Halverson and Starkey (1927) predicted that pH would favor Fe reduction. The soil pH at neutral was strongly correlated with Fe(III) concentrations. In pH neutral, aquifers and aquatic and marine sediments, the reduction of Fe(III) was regarded as an important process for the degradation of naturally occurring organic compounds and anthropogenic matter. Therefore, in this study examined to find out efficiency of carbon sources at various pH on the transformation of ferric iron from soil.

MATERIALS AND METHODS

Collection of samples

The soil samples were collected at the depth about 10 cm from metal contaminated areas, in pre cleaned, acid washed plastic containers for the analysis of heavy metals. The soil samples were carefully transported to the laboratory and stored at 4^{o}C in the laboratory for the analysis of microbial quality. Pour plate technique was employed to enumerate the bacterial count using nutrient agar. Well-defined bacterial isolates were selected on the basis of colony and morphological characteristics and transferred to nutrient agar, and they were identified up to the genus level after purification.

Adaptation of bacterial genera at high Fe concentration

In this study, *Bacillus sp.* (SO-10) was selected because of its resistance, metal degrading abilities and physical versatility. The selected isolate was grown in 500 ml of nutrient broth and incubated on a rotary shaker at 30^{o}C and 120 rpm for 15 hrs. Cells were then centrifuged at 10,000 rpm and 30^{o}C for 20 min and washed three times in phosphate buffer solution.

Prior to use this organism the multi-tolerance was developed using nutrient agar included with various concentration of Fe solution. Nutrient agar was prepared and sterilized for 15 minutes at 15 lb pressure. About 100 to 1000 mg.l^{-1} iron (filter sterilized - 0.2µm) was added and the medium was aseptically transferred into sterile petridishes. The plates were kept over night for surface drying.

The *Bacillus sp.* (SO-10) was tested by spot inoculation on agar surface against various concentration of iron. The plates were incubated at 30°C for 72 hrs. After 72 hrs, the growth of the isolates was observed. The resistant isolates were stored on the same medium at 4°C for further studies.

Biosolubilization of Fe from synthetic medium amended with various carbon sources

Amorphous ferric (FeIII) oxide was systemized by neutralizing 0.4 M solution of $FeCl_3.6H_2O$ with 1M of NaOH and subequently dialyzing it to remove Na^+ and Cl^- ions or by sun drying to remove the Na^+, $C1^-$ (Bonneville, 2004). Prior to us the bacterial strain, inoculam development process was employed. A loopful of culture was inoculated in presterilized 100ml nutrient broth. The flask was kept in a shaker at 120 rpm for 12 hrs at 30°C. The culture broth was centrifuged at 10000 rpm for 20 min. Cell suspension was prepared using sterile distilled water and adjusted to 0.5 OD (10^7 CFU.ml^{-1}) using colorimeter**.** One ml (10^7 CFU.ml^{-1}) of the above suspension was used as inoculum for the solubilization of Fe.

About 200 ml of mineral salts medium (MSM) was prepared in 500 ml bottle and supplemented with various concentrations (0.5, 1.0 and 1.5%) of glucose, starch and cellulose. The pH of the medium was adjusted to 7 and sterilized at 121°C. To this 1 g of synthetic amorphous ferric oxide and 1% of bacterial inoculum (10^7 CFU.ml^{-1} of the cells) were aseptically added and closed with cork. Strict anaerobic condition was maintained by flushing N_2 gas (99.99%) and kept in a shaker (120 rpm) at 30°C for 10 days. The control case was also maintained with the same medium with ferric oxide but without carbon sources and bacterial inoculum. Every 24 hrs the samples were drawn aseptically using 5 ml micro-syringe and analyzed the bacterial growth by pour plate technique, the amount of solubilized iron using 1, 10 Phenanthrolines method. The end of 10^{th} day, oxides were separated by centrifugation (4000rpm) and dried at 60°C for over night and analyzed remaining concentration of Fe.

Effect of pH on solublisation of Fe(III) in MSM containing 1% glucose and ferric oxide

The biotransformation of iron was carried out in 500 ml of serum bottles containing 200 ml mineral salts medium supplemented with glucose at various pH. Glucose (1%) was found to be ideal in releasing metals from ferric oxide compared with the other concentrations. Therefore, 1% glucose was used as the carbon source in this study. The pH of the medium was adjusted to the required range (5, 6, 7, 8 and 9) using 5 N HCl and 10 N NaOH. In addition, 1g of metal oxide and 1% of the bacterial inoculum (10^7 CFU ml^{-1}) were added to the medium and kept in a shaker (120 rpm) at 30°C for 10 d. Every 24 h, the samples were drawn aseptically using a 5 ml micro-syringe and the level of bacterial growth was analyzed using a pour plate technique. The 1,10 Phenanthrolines method was used to analyze the concentration of ferrous iron.

RESULTS AND DISCUSSION

Bacterial genera isolated from iron contaminated samples

Totally about 24 types of morphologically different colonies were obtained from various iron contaminated soil by pour plate technique. The organisms were identified based on the morphology and various biochemical characteristics. From the isolates, the genera of *Bacillus, Aeromonas, Clostridium, Cornybacterium, Lactococcus* and *Lactobacillus, Micrococcus, Shigella* and members of *Enterobacteriaceae* were identified. Those genus are Fe (III) reducers according to earlier study reported by Runov, (1926). These organisms shown to use Fe (III) as an electron acceptor while growing under anaerobic conditions were microorganisms.

Iron resistant bacterial species

The primary and secondary screening was performed to select potent iron degrading bacteria. For the primary screening, all the test isolates were inoculated on nutrient agar plate amended with various concentration of Fe. Among the 24 isolates, 5 organisms *Bacillus*, *Clostridium, Enterobacteriaceae* family organisms, *Klebsiella* and *Lactobacilli* were grown efficiently and considered as iron resistant. These efficient strains were grown up to the maximum concentration (500 ppm). For secondary screening, those 5 efficient strains were allowed to grow on synthetic mineral salts medium amended with 500 ppm of Fe. Periodically growth was measured at 600nm using colorimetry. Among the organisms, *Bacillus* sp. (SO-10) was grown well till 500 ppm of iron. Hence, the organism SO-10 was selected as potent strain for biotransformation of iron from iron oxide. Roberts (1947) reported that *Bacillus* sp. has high solubilization ability of Fe (III).

Effect of carbon sources on the bacterial growth and solubilization of iron from Fe oxide

The growth of the organisms and the solubilization of iron in the synthetic medium containing iron oxide having a significant association with the carbon sources. The effects of various carbon sources (glucose, starch and cellulose) at various concentrations (0.5, 1 and 1.5%) on the growth of bacterial species and solubilization of ferrous iron was studied and the results are given in Figure 1 to 3. The bacterial growth was found to be maximum (48 x 10^7 CFU/ml) in the medium supplemented with 1% glucose, followed by 1.5% glucose (46 x 10^7 CFU/ml) on 10^{th} day (Figure 1). For all carbon sources the rate of solubilization increased rapidly up to 8 days and then maintained stable thereafter. The solubilization of ferric iron in defined medium containing carbon sources denoted as following glucose > starch > cellulose. The highest rate of solubilization occurs on glucose supplemented medium. The solubilization rate rapidly increased up to 7 days and then decreased. Rapid solubilization of iron was noticed in the medium supplemented with glucose at a concentration of 1%. The solubilization of iron increased up to the level of 6374 mg/kg. This might be due to the utilization of oxygen as an electron acceptor by bacteria and to the dissimilatory enzymatic action from the input concentrated Fe Oxide. Roberts (1947) reported that *Bacillus* sp. under anaerobic conditions is capable of respiratory-chain linked, high rate dissimilatory iron reduction, via both a constitutive and an inducible Fe(III) reducing system. Other evidence indicates that Fe(III) reduction may involve extracellular cytochromes which shuttle electrons to insoluble ferric iron (Seeliger et al., 1998). Turick et al. (2002) reported that iron reducing microbes produced an extra cellular melanin that contained quinoid compounds that could act as the sole terminal electron acceptor for growth. Bacterially reduced melanin can also reduce Fe(III) in the absence of cells, suggesting that the melanin can act as an electron shuttle between the cells and Fe(III) oxides. In the case glucose at 0.5 and 1.5% glucose, the rate of iron solubilization showed 4253 mg/kg and 5912 mg/kg respectively. The solubilization rates increased only up to 5^{th} day. After 5^{th} day incubation the sulubilization rate starts declined.

In the medium amended with starch, the rate of bacterial growth and solubilization of ferrous iron was less when compared to glucose. Among the three concentrations, starch at 0.5% showed high solubilization rate which was 3793 mg/kg (Figure 2). Cellulose at 1% showed the solubilization level only about 2011mg/kg (Figure 3). Among the carbon sources, the rapid and high solubilization of ferrous iron was noticed in the medium supplemented with glucose at a concentration of 1%. Therefore glucose at 1% was selected as a potential carbon source to find out solubilization and removal of ferric irons from soil environment

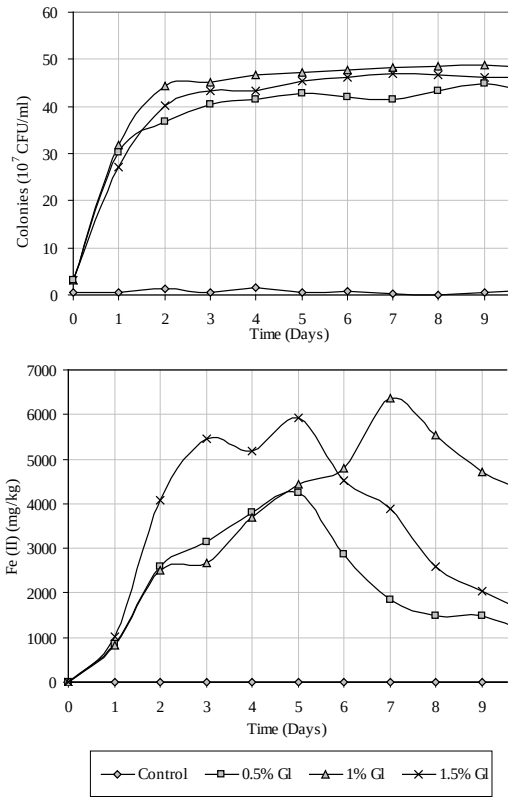

Fig.1. Effect of various concentration of glucose on the growth of *Bacillus* sp (SO-10) and solubilization of ferrous iron

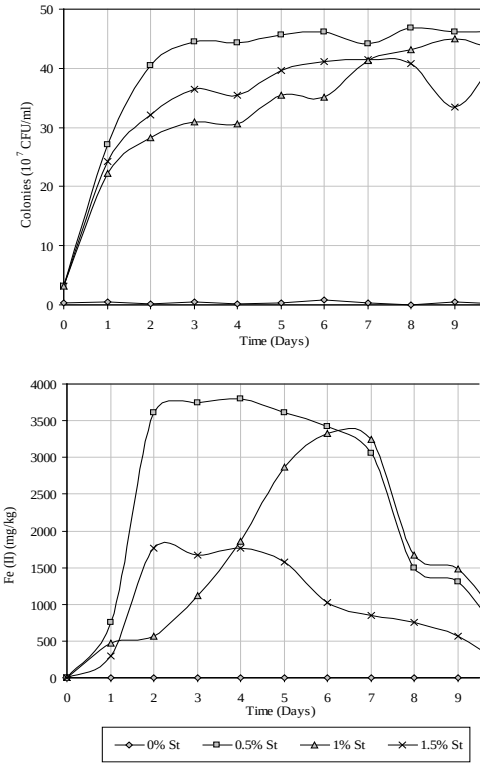

Fig.2. Effect of various concentration of starch on the growth of *Bacillus* sp. (SO-10) and solubilization of ferrous iron

The reason why glucose exhibited the highest solubilization rate can be explained by the fact that our bacterial culture is an heterotroph and is capable of utilizing glucose as a carbon source. Most of the *Bacillus* sp. utilizes glucose as a nutrient substrate (Roberts, 1947). Our result clearly indicates that *Bacillus* sp. (SO-10) is capable of gaining energy from glucose as an electron donor and Fe(III) as an electron acceptor. Under oxygen-limiting conditions, ferric iron can be reduced to ferrous iron. The dissimilatory Fe(III) reduction under anoxic state runs usually according to the following reaction stated by Coates et al. (1998):

Glucose Bacteria Acetic acid

$$C_6H_{12}O_6 + 2H_2O + 8Fe^{3+} \xrightarrow{\text{Bacteria}} 2CH_3COOH + 2CO_2 + 8Fe^{2+} + 8H^+$$

Bacterial growth and solubilization of iron in the synthetic medium at various pH: Figure 4 shows the growth of *Bacillus* sp. (SO-10) during solubilization of iron in the synthetic medium supplemented with1% glucose at various pH. The maximum growth of 49.5 x 10^7 CFU ml^{-1} was recorded in the medium at pH 7 on the 6^{th} day followed by 46, 43 and 41 x 10^7 CFU ml^{-1} at pH 8, 9 and 6 respectively. The growth was much lower at pH 5. Nevertheless, the growth was relatively constant at all pH after the 6 days incubation. *Bacillus* sp. (SO-10) solubilized the maximum level of ferrous iron from the Fe oxide in the synthetic medium at pH 7 after incubation for 4 day. The rate of solubilization increased to 9047.2 mg/kg at pH 7 followed by 8955 mg/kg at pH 8. On the other hand, the solubilization was much lower at pH 5, 8 and 9. The synthetic medium with the initially alkaline and acidic pH did not solubilize as much ferrous iron.

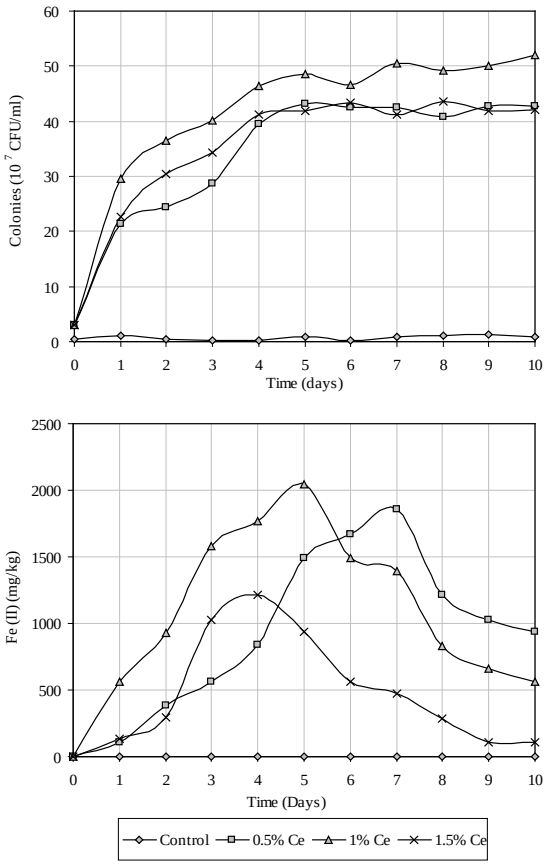

Figure 3. Effect of various concentration of cellulose on the growth of *Bacillus* sp. (SO-10) and solubilization of ferrous iron

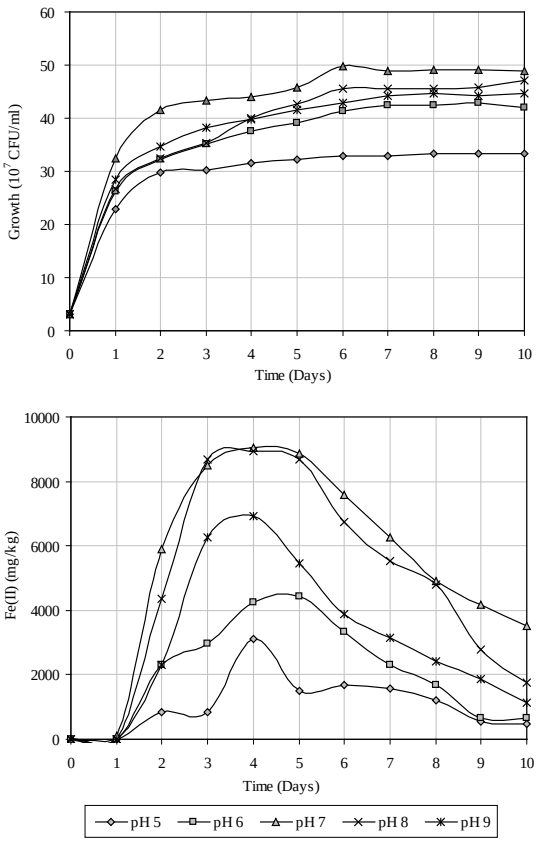

Figure 4. Effect of various pH on the growth of *Bacillus* sp. (SO-10) and solubilization of ferrous iron

Bacillus sp. (SO-10) solubilized the maximum level of Fe in the synthetic medium at pH 7. This was attributed to the neutral pH of the medium. Where as Karaka (2004) reported an acidic pH is generally favorable for the extraction and solubilization of heavy metals in solid/liquid systems. Deng (1997) examined the effect of pH on the reductive solubilization rates of Fe(III) hydroxide by ascorbate as a reductant. They reported that the rate of solubilization is relatively constant at pH ranging from 4.3 to 6.0, and decreases apparently at pH 6.0 to 7.6. Apart from pH the presence of inorganic molecules makes a powerful binding with Fe(III) oxides and assists extreme leach out from the soil (Matthiesen et al., 2001). Chuan et al. (1996) also examined the effects of pH on the solubilization of heavy metals from soil. They reported that the solubilization rate reached a maximum under slightly acidic conditions (pH 5). However, the releasing of metals was negligible at pH 8 and 9. The above results clearly showed that an alkaline pH (>7) is unsuitable for mobility and biotransformation of heavy metals.

Iron is the most abundant heavy metal contaminates the surface of soils and sediments. The availability and mobility of the iron contaminate the ground water and ecosystem health. So decontamination of iron is very important. Most of the iron present in soil as in the form of Fe (III) oxide. Unlike most other organic pollutants, heavy metals especially iron cannot be degraded naturally. Biological method is more applicable and versatile method to decontaminate iron from soil systems. The

biological reduction is a coupled reaction with oxidation of organic matter a carbon sources by terminal electron accepting process. The Fe (II) oxides act as electron acceptor for biogeochemical cycles. There are 24 metal resistant's were isolated and in that *Bacillus* sp. (SO-10) showed a iron transformation potentiality. Then, glucose was selected as electron donor for the bacterial growth and reduction of iron from the iron oxides. The releasing and the solubilization of iron from the soil by *Bacillus* sp. (SO-10) were influenced by various pH. Normally, pH plays an important role in the solubilization of heavy metals from soil. An alkaline pH (>8) did not have any specific influence on the solubilization and transformation of Fe. From the study it was concluded that, glucose at 1% could be suggested as the best carbon source to support the bacterial growth and also removal of Fe from metal oxide.

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Biodiversity of the Endophytic Fungi Isolated From *Euphorbia Hirta* of Yercaud Hills, Salem

R. Dhanalakshmi, S. Umamaheswari and D. Arvind Prasanth*
Department of Microbiology, Periyar University, Salem-636011, Tamil Nadu, India.

**Corresponding Author: D.Arvind Prasanth, Department of Microbiology, Periyar University, Salem - 636011, Tamil Nadu, India, E-mail: prasanthviji@gmail.com*

ABSTRACT

Endophytes are viewed as an outstanding source of bioactive natural products as there are many of them occupying unique biological niches. Endophytic fungi residing inside the medicinal plants are of gaining importance for their bioactive compounds. This study involves the isolation and identification of endophytic fungi from *Euphorbia hirta*, a medicinal tree which is used for the treatment of coughs, chronic bronchitis, diarrhea, dysentery and other skin disorders. A total of 24 segments each 12 from leaf and stem were collected and inoculated on SDA plate. The plates were incubated at 25^{o} C for 72 hrs. The plates were observed for colony morphology. The macroscopic features and wet mount preparation was done to identify the fungi. The fungal isolates identified were *Alternaria spp., Aspergillus flaviceps., Colletotrichum falcatum, Exophiala spp., Nigrospora spp.* and *Mycelia sterilia*. The Colonization Frequency (CF) and Endophytic Infection Rate (EIR) were 66.66% and 33.33% respectively. The endophytic plants could be exploited for the discovery of novel bioactive pharmaceutical compounds considering the fact that these plants harbor a diverse group of endophytic fungi which possess pharmacological properties similar to the host plant. Hence there is a need for preserving the medicinal plants which constitute a larger part of the global environment.

Key words: Biodiversity, Bioactive compounds, Endophytic fungi, Medicinal plants, Symbionts.

INTRODUCTION

Endophytes are the repository of chemical constituents that reside inside plants as symbionts, living within the tissue of their host medicinal plants and are not affected by surface sterilization techniques (Selvanathan *et al.,* 2011). A variety of fungal relationships exits between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonist (Rajagopal *et al.,* 2010). The diversity of fungal endophytes are of gaining importance, nowadays as they produce a plethora of substances of potential use to modern medicine, agriculture, industry, such as novel antibiotics, antimycotics, immunosuppressants and anti-cancer compounds (Huang *et al.,* 2008). Only a few plants have been extensively screened for their endophytic fungi and their potential bioactive secondary metabolites (Rebecca *et al.,* 2011). Medicinal plants are reported to harbor endophytes which in turn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions (Kumar and Hyde, 2004). It is therefore important to determine the endophyte diversity of medicinal plants. *Euphorbia hirta* which belongs to the family Euphorbiaceae, is a small annual herb common to tropical countries. The plants are used in treatment of asthma, respiratory tract inflammations, coughs, chronic bronchitis and other pulmonary disorders. The plant is also widely used against diarrhea and dysentery, especially amoebic dysentery. In some countries the leaf extracts or exudates of the plant are used as ear drops and in the treatment of boils, sore and promoting wound healing (Ogueke *et al.,* 2007).

MATERIALS AND METHODS

Collection of samples

Healthy and mature plants were carefully chosen for sampling. Fresh mature leaves and stem of *Euphorbia hirta* (L.) (Euphorbiaceae) without symptoms of ripening were collected from a healthy plant from different locations at Yercaud forest, Salem. The plant materials were brought to the laboratory in the sterile zip-lock bags and transported to the medical microbiology laboratory. The samples were then processed immediately to reduce the chance of contamination.

Isolation of Endophytic Fungi

The plants were rinsed in running tap water to remove soil particles and unwanted debris. After washing, the leaves and the stem were selected for further processing under aseptic conditions. Highly sterile conditions were maintained for the isolation of endophytes and the entire process was carried out inside the laminar air flow. Sterile glassware (Conical Flask) and mechanical things such as scissor, forceps, scalpel, and blades were used in sterile conditions for this experiment. The stem and leaves were cut into segments (0.5-1cm) by the use of sterile lancet blades.

The segments of stem and leaves were immersed in 70% ethanol for 5s for surface sterilization. The branch portions were further sterilized sequentially in 4% sodium hypochlorite solution (Merck Laboratories) for 90s, and then rinsed in sterile distilled water for 10s. The excess moisture was blotted in a sterile filter paper. The surface sterilized segments were placed in petridishes containing Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol (5 mg / ml). Six segments were placed for one plate. The petri dishes were incubated at 25to 27°C for 72 hrs in dark conditions and they were monitored every day to check the growth of endophytic fungal colonies from the segments. Most of the fungal growth was initiated within two weeks of inoculation. The pure cultures were maintained on PDA slants. The endophytic fungi were identified according to their macroscopic (front and reverse side of fungal colonies) and microscopic characteristics such as the morphology of fruiting structures and spore morphology under a bright-field microscope (10X and 40X).

Statistical analysis

The colonization frequency (CF) and Endophytic Infection Rate (EIR) were calculated as described by (8). Samples were incubated and growth was examined daily during 6 weeks and Colonization Frequency was calculated.

Colonization frequency (CF %)

$$CF = \frac{\text{No. of individual fungi recorded}}{\text{Total no. of segments screened}} \times 100$$

Endophytic Infection Rate (EIR %)

$$EIR = \frac{\text{Total no. of endophytic fungi recorded}}{\text{Total no. of segments screened}} \times 100$$

RESULTS

About 24 segments (12 segments of leaves and stem respectively) of *Euphorbia hirta* were screened for the isolation of endophytic fungi. The leaf segments showed a maximum repository for endophytic fungi than the stem segments. A total of 8 endophytic fungal genera were isolated and identified from different parts of stem and leaves. The endophytic fungal genera isolated included *Alternaria spp* (Fig.1), *Aspergillus flaviceps.* (Fig.2), *Colletotrichum falcatum* (Fig.3), *Exophiala* spp.,

Nigrospora spp. (Fig.4) and *Mycelia sterilia.* The Colonization Frequency (CF) was 58.33% and 8.33% for stem and leaves respectively. The Endophytic Infection Rate (EIR) was found to be 33.33% (Table 1).

Table 1: Endophytic fungi isolated from *Euphorbia hirta*

S. No	Plant Part	No. of samples	Isolated Endophytes	Fungal group	Total number of endophytes	Statistical Analysis	
						CF	EIR
1.	Leaf	12	*Colletotrichum falcatum* *Nigrospora spp.* *Alternaria spp.* *Aspergillus flaviceps*	Coelomycetes Dematiaceous Hyphomycetes Hyaline	7	58.33 %	33.33%
2.	Stem	12	*Mycelia sterilia* (3) *Exophiala spp.*	hyphomycetes Hyaline Hyphomycetes	1	8.33%	
Total no. of segments		24	Total no. of isolates		8	66.66%	33.33%

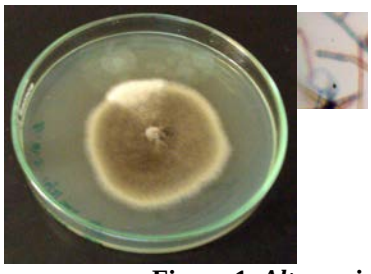

Figure 1. *Alternaria spp.*

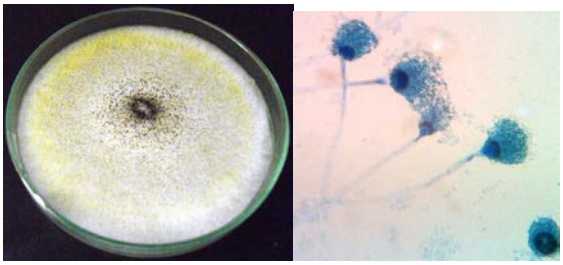

Figure 2. *Aspergillus flaviceps*

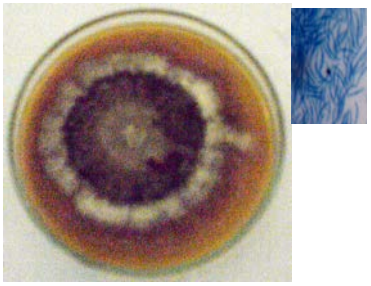

Figure 3. *Colletotrichum falcatum*

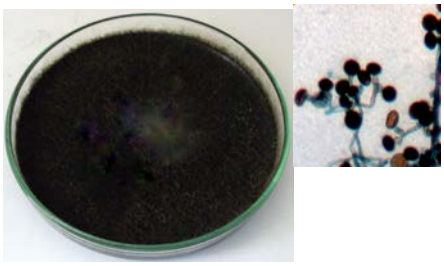

Figure 4. *Nigrospora spp.*

DISCUSSION

Traditional medicines from plants are one of the oldest forms of the health care known, they continue to be a rich source of therapeutic substances (Selvanathan *et al.,* 2011). Endophytic fungi are one the most unexplored and diverse group of organisms having symbiotic association with higher life forms and may produce beneficial substances for host (Khan *et al.,* 2007). The diversity of fungal endophytes in few medicinal herbs of South India is already reported.

Carl Linnaeus, the great Botanist and taxonomist assigned the name Euphorbia to the entire genus in the physician's honor. *Euphorbia hirta* is a very popular herb amongst practitioners of traditional herb medicine, widely used as a decoction or infusion to treat various ailments including intestinal parasites, diarrhoea, peptic ulcers, heartburn, vomiting, amoebic dysentery, asthma, bronchitis, hay fever, laryngeal spasms, emphysema, coughs, colds, kidney stones, menstrual problems, sterility and venereal diseases. Moreover, the plant is also used to treat affections of the skin (Shih *et al.,* 2012).

The studies reported so far in this endophytic species have reported presence of distinct endophytic community in each of these host plants studied suggesting host preference of these endophytes with regard to the plant community in which they reside (Rajagopal *et al.,* 2010). This study has also showed such a trend of host preference which was apparent with the leaves, and stem parts of *E. hirta*. The Colonization Frequency (CF) was found to be high in leaves (58.33 %) when compared to leaf segments (8.33 %). The various endophytic genera reported in this study were mainly belonging to the class of Hypomycetes and was the first report to isolate endophytic fungi from the small herb along diversity of Yercaud region when compared to the study of (Banu *et al.,* 2009) who reported the absence of endophytic fungi from E. hirta along with some medicinal plants.

The screening of these Endophytic fungi for the presence of bioactive compounds constitutes an important area of future research. The discovery of such novel bioactive compound from these diverse endophytic fungi arise the need to preserve these medicinal plants which is an important constituent of the global environment.

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The Effect of *Azadirachta Indica* Leaves on *Monocystis* of Earthworm

N. Sundaramoorthy[1], Priya Roy[2] and R. Dhandapani[2*]

[1] Department of Microbiology, Adhiparasakthi College of Arts and Science, Kalavai, India.

[2] Department of Microbiology, Periyar University, Salem – 636 011, India.

*Corresponding Author: R. Dhandapani, Department of Microbiology., Periyar University, Salem – 636 011, India, Email : danpani@gmail.com

ABSTRACT

Monocystis species is an apicomplexan parasite which lives in the seminal vesicle of terrestrial earthworms, and affects their reproduction. Neem is proven to have anti-helmintic, anti-fungal, anti-diabetic, anti-bacterial and antiviral properties. This paper focuses on the effect of neem on various stages of *Monocystis*. An earthworm was dissected and the seminal vesicle was examined using Haematoxylin Eosin stain and Lugol's iodine wet mount. Neem powder was applied to the seminal fluid containing cystic stage of *Monocystis*. The degradation of the oocytes after treatment with neem powder was observed through staining. Thus neem was found to knock out parasite infestation and thereby improve the reproduction rate of the earthworms.

Keywords: *Azadirachta indica*, *Monocystis*, coelomic fluid, oocyst

INTRODUCTION

Earthworms are efficient environmental monitoring tool. They can accumulate certain heavy metals, industrial effluent, various biocides, pesticides and their residues. Earthworms are involved in one of the most critical job of recycling or reusing the basic materials that plants and animals need to survive. By eating waste, earthworms help to decompose the materials into smaller, simpler parts that can be reused by other organisms. Without this process of decomposition, the basic chemicals of life would stay locked up and unavailable for use by other organisms. Earthworms increase the plant growth by modifying the soil chemically and biologically by mineralizing the soil organic matter and activating, dispersing or controlling some plant parasites or symbionts.

Earthworms play a major role in converting large pieces of organic matter (e.g. dead leaves) into rich humus, and thus improving soil fertility. This is achieved by the worm's actions of pulling down below any organic matter deposited on the dried dirt, such as leaf fall or manure, either for food or when it needs to plug its burrow. Once in the burrow, the worm will shred the leaf and partially digest it, then mingle it with the earth by saturating it with intestinal secretions. Worm casts contain 40% more humus than the top 9" of soil in which the worm is living as well as dead organic matter, the earthworm also ingests any other soil particles that are small enough-including stones up to 1/20 of an inch (1.25mm) across-into its gizzard wherein minute fragments of grit grind everything into a fine paste which is then digested in the stomach. When the worm excretes this in the form of casts which are deposited on the surface or deeper in the soil, minerals and plant nutrients are made available in an accessible form.

Majority of earthworms are infected by the parasite. *Monocystis* species is an apicomplexan parasite that lives in the seminal vesicle of terrestrial earthworms. The earthworm always shows the presence of the parasite in the seminal vesicle. The earthworm becomes infected when it ingests a spore containing several sporozoites. These hatches in a gizzard, where the released sporozoites penetrate the intestinal wall, enter the dorsal blood vessel, and then make their way to one of the host's 5 or so 'hearts'. From there they penetrate the seminal vesicle, where they enter the sperm – forming cells in the wall and destroy the developing spermocytes. The parasite feeds on the sperm morula by extruding enzymes and

absorbing the digested products through the pellicle. It frequently moves to another morula and consume the cytoplasm before it is fully grown. This process of consuming the sperm cytoplasm affects the reproduction of earthworm. Then they move into the lumen of the vesicle where they become mature trophozoites. After a period of feeding, two of these will come together, flatten against each other, and secrete a common cyst around each other. This is the gametocyst, usually containing 2 gamonts. Each now undergoes extensive division of their nuclei, pinches of a small portion of cell cytoplasm, which together then bud off to become the gametes. The fusion of a pair of gametes forms a zygote, each ultimately becoming a spore. Three cell divisions later form 8 sporozoites. Thus, each gametocyte now contains many oocysts. New hosts become infected by ingesting gametocyte or more commonly, by ingesting individual oocysts. Thus, meiosis is zygotic. Only the zygote is diploid, and reductional division in sporogony returns the sporozoites to the haploid condition. To overcome the problem regarding parasites in earthworms study was focused on *Azadirachta indica* leaves so that the reproduction rate of the earthworms could be improved.

Neem (*Azadirachta indica*, syn. *Melia azadirachta* L., *Antelaea azadirachta* (L. Adelb.) is a tree in the mahogany family Meliaceae. It is one of two species in the genus *Azadirachta*, and is native to India, Myanmar, Bangladesh, and Pakistan growing in tropical and semi-tropical regions. Products made from neem have proven medicinal properties, being anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, anti-fertility, and sedative. Neem is deemed very effective in the treatment of scabies although only preliminary scientific proof exists which still has to be corroborated, and is recommended for those who are sensitive to permethrin, a known insecticide which might be an irritant. Also, the scabies mite has yet to become resistant to neem, so in persistent cases neem has been shown to be very effective.

There is also anecdotal evidence of its effectiveness in treating infestations of head lice in humans. A tea made of boiled neem leaves, sometimes combined with other herbs such as ginger, can be ingested to fight intestinal worms (Ganguli, 2002). Significance of neem is the following, Neem products are non toxic, environmental friendly and bio degradable, cheaper than the synthetic products, one product can be used for various purposes, increases soil fertility, thus crop yield can be used for both cash as well as food crops, pests and insects don't develop resistance to neem products.

Coelomic fluid-Innate immune Respose

The coelomocytes of *Lumbricus terrestris* have been classified and described based on Wright's stained preparations and on living cells. The five major categories consist of basophils, acidophils, neutrophils, granulocytes and chloragogen cells. Both the acidophil and chloragogen cell groups contain two subgroups. Granulocytes also exhibit heterogeneity with respect to staining properties of granules. Some possess acidophilic granules, some basophilic granules, and others contain both types. Granules of acidophils have been observed to be occasionally excreted from the cells. All cell types, with the exception of chloragogen cells, produce pseudopodia and are capable of phagocytosis, a vital component of the earthworm's immune response (Stein *et.al.*, 1977).

MATERIALS & METHODS

Earthworm and examination of coelomic fluid

Eisenia foetida were collected from the field and maintained throughout the study. The earthworms were washed with clean water, rinsed and dried. The earthworms were then excited with 5V electrical stimulations to produce coelomic fluid through their epidermal dorsal spores. The earthworms were then placed on UV-transilluminator to observe the production of coelomic fluid. The healthy ones will produce coelomic fluid through the epiderma dorsal pores whereas the infected earth worm will produce less coelomic fluid.

Seminal vesicle harvesting

Dissection of the earth worm: Dissection of the earth worm showed prominent digestive system and reproductive system (Figure 1).

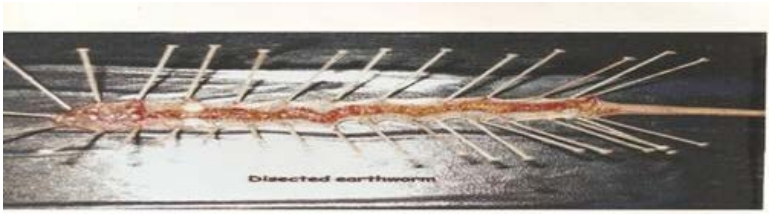

Fig.1: Dissected earthworm

Removal of seminal vesicle

Cream coloured seminal vesicles located in between 10^{th} - 15^{th} segments were clipped out using sharp dissecting scissors and transferred on to the watch glass containing distilled water. With the help of teasing needle the contents of the seminal vesicles were released out into the clean glass slide and smear was made on the slide and air dried. The harvested seminal fluid was examined using haematoxylin Eosin staining and Lugol's Iodine Wet mount.

Preparation of the leaf Extract

The leaves of *Azadirachta indica* were collected and dried under shadow for 2-3 days and further dried at 60◦c for 6-8 hrs in hot air oven. The leaves were then made into fine powder using powder extractors. In every step, the leaves were dried without moisture to overcome the fungal contamination. The air dried powder of plant material was stored in air tight container for further use.

Effect of neem on seminal fluid of *Monocystis*

The prepared neem powder (0.1g) was added to the eppendroff containing the diluted seminal fluid (1ml) containing the cystic stage of *Monocystis.* The suspension was kept for two days at room temperature. The contents of the suspension were examined by Lugol's iodine wet mount technique.

RESULTS & DISCUSSION

Examination of Ceolomic fluid of Earth worm under UV-Transilluminator

Luminescence was observed on healthy earthworms whereas poor luminescence was observed on infected earthworms (Figure 2). The coelomic fluid of earthworms contains cells and many molecular components involved in innate immune system of these species. Among those are the glycoproteins of lectin which contribute to the recognition of foreign material in coelomic cavity by binding to carbohydrates. Moreover they cause its immobilization by agglutination and destruction by membrane lysis. Humoral components phenoloxidase, protease and other enzymes are involved in the elimination of foreign materials.

Examination of seminal vesicle: Microscopic examination of milky white seminal fluid identified by Lugol's iodine wet mount and Haematoxylin - Eosin stain showed the presence of various stages of *Monocystis* (Fig-3).

The small, spindle shaped, thick-walled (20 μ m) spores containing sporozoites were identified. Mature trophozoites which are large (200 μ m), fusiform unicellular with a smooth exterior, with no spermatozoa or flagella on the surface but are mobile. Their movements are reminiscent of euglenoid motion but the mechanism by which it is accomplished is not understood.

Figure 2: Coelomic fluid of healthy earthworms showing luminescence

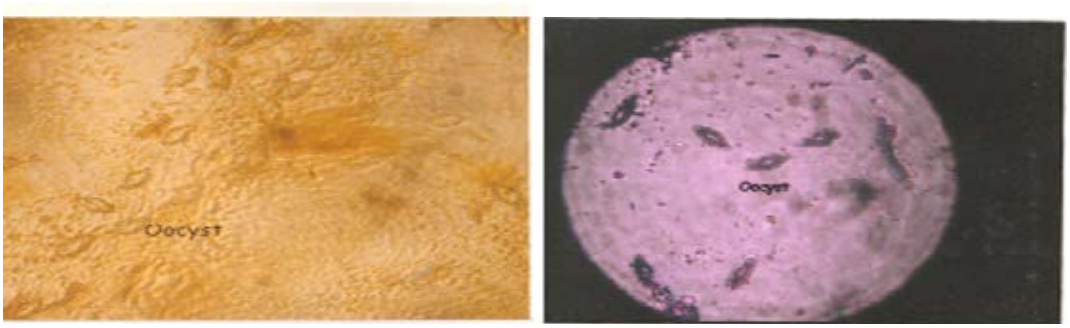

Fig. 3: Various stages of monocycstis observed in Lugol's iodine and Haematoxylin- Eosin stain. a) Lugol's iodine wet mount b) Haematoxylin - Eosin stain

Effect of Neem on *Monocystis*

The effect of Neem on *Monocystis* was studied by incubating the seminal fluid in the presence of Neem extract (Fig:4). Considerable reduction in the Gametocytes of Monocystis was observed in the presence of Neem (Fig: 5). The sporozoites which fed on the developing spermatocytes in the wall of the seminal vesicle effectively destroyed by the extract of *Azardirachta indica*, thus improving the reproduction rate of earthworms.

Fig. 4: Incubation of seminal fluid with neem extract

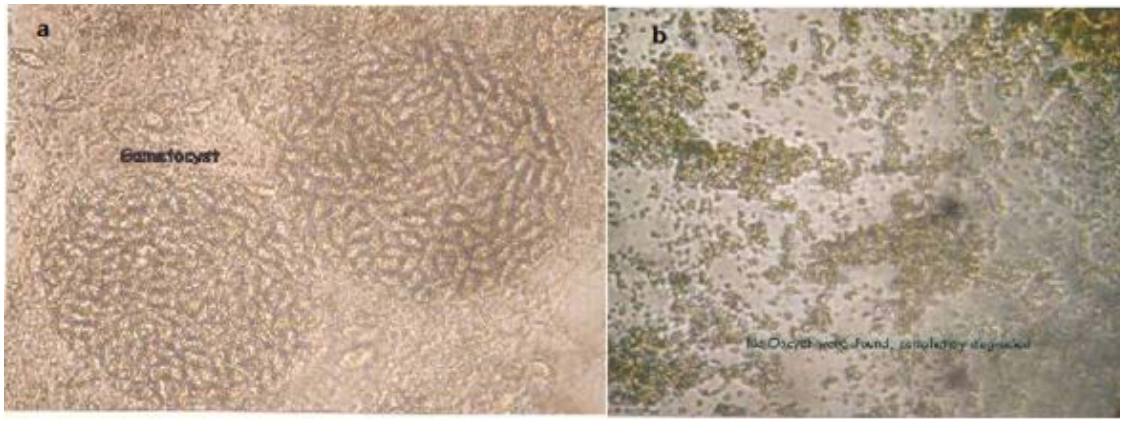

Fig. 5: Effect of Neem extract on the Gametocytes of *Monocystis,* a)Gametocyte before treatment with extract of *Azardirachta indica* b) Degraded gametocyte after treatment with extract of *Azardirachta indica*

The present study of coelomic fluid of earthworm reveals that infestation by the parasite showed poor luminescence property. Gamatocyst of *Monocystis* was isolated from the seminal vesicle of *Eisenia foetida*. The sporozoites which fed on the developing spermatocytes in the wall of the seminal vesicle were effectively destroyed by the extract of *Azardirachta indica*, thus improving the reproduction rate of earthworms. When these leaves were used for organic farming it helps to improve the reproductive rate of the earthworm, which in turn can improve the soil fertility directly or indirectly through casts. *Azadirachta indica* thus helps to improve organic matter source, nutrient recycling, free nitrogen, weed control and also helps in pest and disease management.

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Incidence, Survival and Antibiotic Resistance of *Aeromonas Hydrophila* Isolated From Lamb and Chicken Meat Retail Outlets

S. Rajakumar[1*], P.M. Ayyasamy[2], K. Shanthi[3], Y. C. Song[4] and P. Lakshmanaperumalsamy[5]

[1] Department of Marine Biotechnology, Bharathidasan University, Tiruchirappalli - 620 024, India
[2] Department of Microbiology, Periyar University, Salem - 636 011, India
[3] Department of Environmental Science, P.S.G. College of Arts and Science, Coimbatore - 641 014, India
[4] Division of Civil and Environmental Engineering, Korea Maritime University, Busan - 606 792, South Korea
[5] Registrar, Karpagam University, Coimbatore – 641021, India.

**Corresponding Author : S. Rajakumar, Department of Marine Biotechnology, Bharathidasan University, Tiruchirappalli - 620 024, India, Email : kodairaj@gmail.com*

ABSTRACT

This study was carried out in lamb and chicken meat retail outlets located in Coimbatore city, Tamilnadu, India during September 2005 to February 2006. Lamb and chicken meat and the accessories like chopping boards, knifes, balance trays, washing water and hands of the butchers were examined. A total of 63 *A. hydrophila* strains were isolated from lamb and chicken meat retail outlets. The highest incidence was found in butchers hand and washing water. Survival study was carried out using selected strains of *A. hydrophila* (SLM-4) on lamb, chicken meat and egg samples at various temperatures (30, 10 and 4 ^{o}C). The temperature at 30 ^{o}C stimulates the survivability of *A. hydrophila* in all the samples. However, the organism survives even at low temperature (4 ^{o}C). Similarly, the bacterium survived significantly in the stainless steel, wood and iron pieces showing the adhering ability of the organism. Traditional cooking process (100 ^{o}C) proved complete elimination of *A. hydrophila*. In the study of antibiotic sensitivity, all the strains isolated from lamb and chicken meat outlets were resistant to ampicillin and bacitracin. The resistance showed < 20% by using the antibiotics, chloramphenicol, gentamycin, nalidixic acid, streptomycin and sulphamethoxazole.

Keywords: *Aeromonas hydrophila*, Lamb meat, Chicken meat, Retail outlets, Antibiotics.

INTRODUCTION

Aeromonas hydrophila is a widespread representative species of *Aeromonas* found in water, water habitants, domestic animals and foods (Rashid *et al.*, 1992; Dascalov, 2006; Djuikom *et al.*, 2008). It is mainly considered to be an emergent food-related bacterium (Velazquez *et al*,. 2001; Motta *et al.*,. 2007). Food and water-borne illnesses are the major important problems in the world, because of improperly treated water and unhygienic animal foodstuffs. The mesophilic *Aeromonas* has been likely to cause many human infections (Condon *et al.*,. 1991). The organism has been isolated from a range of foods in almost every country in which it has been investigated. Individuals affected by *A. hydrophila* show nausea, vomiting, diarrhea and stomach cramps, which develops within 24 to 48 h. In addition, accounts of *Aeromonas* associated food-borne outbreaks and considerable numbers of sporadic cases have been reported (Kobayashi and Ohnaka 1989). Actual sourced food-borne outbreaks are few, however epidemiological evidence suggests that the bacterium can cause self-limiting diarrhea, with children being the most susceptible population (Isonhood and Drake, 2002). *Aeromonas* species are present in a wide range of foods, which rely on low temperature for preservation; some strains can grow and produce exotoxins in bacterial media at refrigeration temperatures (Eley *et al*,. 1993).

Prevalence of organism on the surface of the foodstuff enhances cross contamination. A major concern with cutting boards in home is that bacteria of animal origin may cause cross contamination (Kirov, 1993). Fluid from raw meat remaining on the work surface might transfer pathogenic agent to other foods to be cooked before eating. Earlier reports states that the bacteria of greatest concern as cross contaminants on kitchen cutting boards were of animal origin, which were being responsible for human infections and diseases transmitted via foods (Abeyta and Wekell, 1988).

During the processing of raw chicken in retail shops, the cutting board, knife, hands of the butcher, washing water and weighing balance tray are the principle surfaces to which chicken meat comes in to contact and any remaining food might enhance the growth of microorganisms and cause more frequent cross contamination. To keep the contamination minimum and obtain good quality meat, all equipments coming into contact with the food should be adequately cleaned, sanitized and tested. There are well recognized reports regarding the association of *A. hydrophila* with animal foodstuffs from other countries having hygienic mode of handling and no systematic study has been conducted on the prevalence of this organism in the animal meat from retail markets of India. Hence, this study was aimed to investigate the sources of cross contamination in retail outlets and to characterize their survival capacity on different types of wood, metal surfaces and meat samples. Further, antibacterial activity of the *A. hydrophila* strains were also carried out and the resistant pattern has been investigated.

MATERIALS AND METHODS

Sample collection

The study was carried out in the lamb and chicken meat retail markets of Coimbatore city, Tamil Nadu, India. In retail outlets, lamb meat, chicken meat, chopping board, knife, balance, butcher's hand, washing water and intestine were examined. The samples were collected between 7 to 9 am for the period of six months during September 2005 to February 2006. Small pieces of lamb and chicken meat and intestine were placed in Alkaline Peptone Water (APW) (per liter contains: 10 g of peptone and 5 g of sodium chloride) for pre-enrichment and incubated at 37 ^{o}C for 18 h. Sterilized cotton swabs were used to sample the surface of chopping board, knife, balance and butcher's hands. The swabs were directly inoculated in to 10 ml APW in screw capped bottles and incubated at 37 ^{o}C for 24 h. During the same period, a total of 75 eggs were collected in sterile polyethylene bags from different retail outlets of Coimbatore city. For egg samples, sterile cotton swabs dipped in sterile APW were used to swab the entire surface area of the egg shell. Further procedures were followed as mentioned in earlier.

Isolation and Identification

The enriched cultures were streaked on Starch Ampicillin Agar (SAA) plates and incubated at 37°C for 24 h. This medium contains (g/l): pancreatic digest of casein, 10; sodium chloride, 5; soluble starch, 1; ambicillin, 0.01; phenol red, 0.018; agar, 15 (Palumbo *et al.*, 1985). The yellowish honey coloured and oxidase positive colonies were isolated and presumptively considered as amylolytic *Aeromonas* species (Popoff, 1984). Further the isolates were identified as *A. hydrophila* using Kaper's multitest medium containing (g/l): proteose peptone, 5; yeast extract, 3; tryptone, 10; L-ornithine hydrochloride, 5; mannitol, 1; inositol, 10; sodium thiosulfate, 0.4; ferric ammonium citrate, 0.5; bromocresol purple, 0.02; agar, 3 (Kaper, 1979) and confirmed on the basis of biochemical characteristics (Esteve, 1995). A reference strain, *A. hydrophila* MTCC646 collected from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India was used for comparison.

Survival of *A. hydrophila* on lamb and chick meat and eggs at various temperatures

In this study, five gram of lamb and chicken meat were taken and sterilized at 121 ^{o}C at 15 lb pressure. The bacterium, *A. hydrophila* (SLM-4) isolated from lamb meat during September, 2005 were

selected for the entire survival study. The culture was enriched in nutrient broth at 37 ^{o}C for 18 h and centrifuged at 15000 rpm for 15 min. The pellets were collected and dilution at 10^3 was made to get 2.16 log CFU/ml. From that dilution, 1 ml was drawn out and injected into entire area of lamb and chicken meat and incubated at 30, 10 and 4 ^{o}C respectively for 24 h. Every 4 h intervals, 1 g of meat samples were taken and homogenized under aseptic condition, pour plate technique was employed using SAA. The plates were incubated at 37 ^{o}C for 24 h and yellow to honey coloured, amylase and oxidase positive colonies were observed.

For egg samples, eggs were surface sterilized by immersion in 70% alcohol for 2 min and air dried in a sterile chamber for 10 min. A hole was made in each egg under aseptic condition using egg driller. Further, the eggs were injected with 2.16 log CFU/ml of enriched cells (*A. hydrophila*), closed with paraffin wax and kept at 30, 10 and 4 ^{o}C respectively for 24 h. Every 4 h, 1 g of each egg contents were taken and pour plate technique was made to enumerate *A. hydrophila* using SAA.

Survival of *A. hydrophila* (SLM-4) on stainless steel, wood and iron pieces

In this study, stainless steel, wood and iron pieces of 4 × 4 cm size were taken and sterilized at 121 ^{o}C at 15 lb pressure. The total area of the materials are expressed as 16 cm^2 (area = length x width). These materials were selected based on the accessories such as balance, chopping board and knife used in the meat shops. The strain, *A. hydrophila* (SLM-4) was enriched in nutrient broth at 37 ^{o}C for 18 h. The enriched culture was centrifuged (15000 rpm for 15 min) and diluted to get 2.16 log CFU/ml. One ml inoculum was taken and added drop wise on stainless steel, wood and iron pieces respectively. Further, the inoculum was spreaded by an inoculation loop to coat entire area of the materials and incubated at 30 ^{o}C for 24 h. Periodically the materials were taken and washed thoroughly with sterile distilled water until to get entire biofilms. The washed solutions were made into 100 ml with sterile distilled water and pour plate technique was employed to enumerate *A. hydrophila* using Starch Ampicillin Agar. The bacterial populations were calculated according to the area (cm^2) of materials.

Survival of *A. hydrophila* (SLM-4) in lamb and chicken meat and egg samples under traditional cooking process

Five grams of lamb and chicken meat samples were taken and sterilized at 121 ^{o}C for 15 lb pressure. Readily available mixture of cooking ingredient (the Indian name is called masala) marketed by Sakthi Masala Pvt Ltd, Erode, Tamil Nadu were taken and mixed with sterile distilled water until to get stickiness. Further, the ingredients were applied under aseptic condition in each meat samples and air dried in a sterile chamber for 1 h. About 2.16 log CFU/ml of *A. hydrophila* (SLM-4) was injected into entire area of each samples and incubated at 30 °C for 24 h. Two sets were prepared to check survival of bacterial cells before and after cooking. At a regular interval of 4 h, 1 g of each samples were cooked at 100 ^{o}C for 10 min. Further, the samples were homogenized and pour plate technique was employed on SAA. After incubation for 24 h, yellow to honey coloured, amylase and oxidase positive colonies were counted. Similar procedure was followed in the samples without cooking. For egg samples, the ingredients were applied on the boiled eggs under aseptic condition. The cells of *A. hydrophila* 2.16 log CFU/ml were injected into two sets of eggs and kept at 30 ^{o}C and 4 ^{o}C for 24 h. One set of eggs was re-boiled at 100 ^{o}C for 10 min, homogenized and tested for bacterial growth.

Antibacterial sensitivity test

Antibacterial susceptibility tests were carried out by the disk diffusion method reported by Bauer *et al.*,. (1966). A total of 63 *A. hydrophila* strains isolated from both lamb and chicken meat outlets and were tested against various antibiotic discs (Himedia, Mumbai, India) are as follows: Ampicillin (10 mcg); Bacitracin (10 units); Chloramphenicol (30 mcg); Erythromycin (15 mg); Gentamycin (10 mcg); Kanamycin (30 mcg); Nalidixic adid (30 mcg); Novobiocin, (30 mg); Neomycin (30 mcg); Polymixin B

(300 units); Streptomycin, (10 mg); Sulphamethoxazole (25 mcg); Tetracycline (30 mcg) and Vancomycin, (30 mg).

All the strains were enriched in brain-heart infusion broth (Himedia, Mumbai, India) at 37 ^{o}C for 6 to 8 h. The enriched cultures were then streaked over Mueller Hinton agar plates (Himedia, Bombay, India) using a sterile cotton swab. The antibiotic discs were dispensed using a disc dispenser and sufficiently separated from each other so as to avoid overlapping of inhibition zones. After 30 min the plates were inverted and incubated at 37 ^{o}C for 16 to 18 h. Results were recorded by measuring the diameter of the inhibition zones and compared with the interpretive chart of performance standards for antimicrobial disk susceptibility tests, supplied by the Himedia laboratories, Bombay and classified as resistant, intermediate and sensitive.

Statistical analysis

All experiments were conducted in triplicates and the mean values were calculated with error bars. Both mean and standard deviation were performed where appropriate, using the statistical package within Microsoft® Excel (Version, 2003).

RESULTS

Incidence of *A. hydrophila* in lamb and chicken meat and egg samples during the study period

The occurrence of *A. hydrophila* in the shop of lamb and chicken meat and eggs were analyzed and the results are given in Table 1. In lamb meat shop, out of 75 samples analyzed, 20 samples were found to *A. hydrophila* and the percentage of incidence was 26.6. In chicken meat shop, 43 of 75 samples were found to be contaminated with *A. hydrophila* and the percentage of incidence was 57.3. There was no incidence of *A. hydrophila* in egg samples out of 75 during the study period. Among the samples analyzed, the samples from chicken outlets showed maximum incidence of 57.3%.

The materials used in lamb and chicken meat shops were chopping board, balance, knife, butcher's hand and washing water. Among the above materials analyzed in the lamb meat shop, the maximum incidence (60.0%) was recorded in washing water, followed by butcher's hand (40.0%). There was no incidence of *A. hydrophila* in knife and chopping board during the study period in lamb meat shops. Apart from the above mentioned materials, intestines of lamb were also analyzed for the incidence of *A. hydrophila*. There was no incidence of *A. hydrophila* during this survey in intestine. In chicken meat shop, the samples collected from butcher's hand showed the maximum incidence (90.0%) followed by chicken (66.6%). Here all the samples i.e. balance, knife constituted about 50% of incidence. Washing water and intestine showed 40% of incidence respectively. Intestines of chick samples showed 40.0% of incidence. The prevalence of *A. hydrophila* in outlets of lamb and chicken meat and eggs were observed in month-wise during September 2005 to February 2006. The maximum incidence was observed both in lamb and chicken meat during December. There was no incidence in egg samples though out the study period (Table 2).

Table 1. Percentage incidence of *A. hydrophila* in lamb and chicken meat and egg samples collected in retail markets at Coimbatore during September 2005 to February 2006

Samples	Total number of samples analyzed	Number of samples showed positive	Percentage of incidence
Lamb meat shop			
Lamb meat	15	8	53.3
Chopping board	10	0	0
Balance	10	2	20
Knife	10	0	00
Butcher's hand	10	4	40
Washing water	10	6	60
Intestine	10	0	0
Total	75	20	26.6
Chicken meat shop			
Chicken meat	15	10	66.6
Chopping board	10	6	60
Balance	10	5	50
Knife	10	5	50
Butcher's hand	10	9	90
Washing water	10	4	40
Intestine	10	4	40
Total	75	43	57.3
Egg shop			
Eggs	75	0	0
Grand total	225	63	31.5

Table 2. Monthly incidence of *A. hydrophila* in lamb and chicken meat and egg samples collected from retail markets of Coimbatore during September 2005 to February 2006

Months	Lamb meat shops			Chicken meat shops			Egg samples		
	Samples analyzed	+Ve	% of incidence	Sample analyzed	+Ve	% of incidence	Samples analyzed	+Ve	% of incidence
Septem	12	03	25.0	12	07	58.3	12	0	0
October	13	03	23.0	13	05	38.6	13	0	0
November	12	03	25.0	12	06	50.0	12	0	0
December	13	06	46.1	13	10	76.9	14	0	0
January	12	03	25.0	12	07	58.3	12	0	0
February	13	02	15.0	13	08	61.5	12	0	0
Total	75	20	26.6	75	43	57.3	75	0	0

Survival of *A. hydrophila* (SLM-4) in lamb and chicken meat and egg samples at various temperatures

Survival of *A. hydrophila* in lamb and chicken meat and egg samples at various temperatures 30, 10 and 4 °C were studied and the results are given in Fig. 1. The initial bacterial cells in the each samples showed 0.432 log CFU/g. In lamb meat, the cells were increased significantly up to 1.96 log CFU/g at

30 ^{o}C after 24 h. The number of cells at 10 °C showed a level of 1.026; whereas at 4 ^{o}C, the cells found that only 0.385. Corresponding to the results noticed in the chicken meat samples, the maximum growth in the chicken meat were 1.929 log CFU/g at 30 ^{o}C.

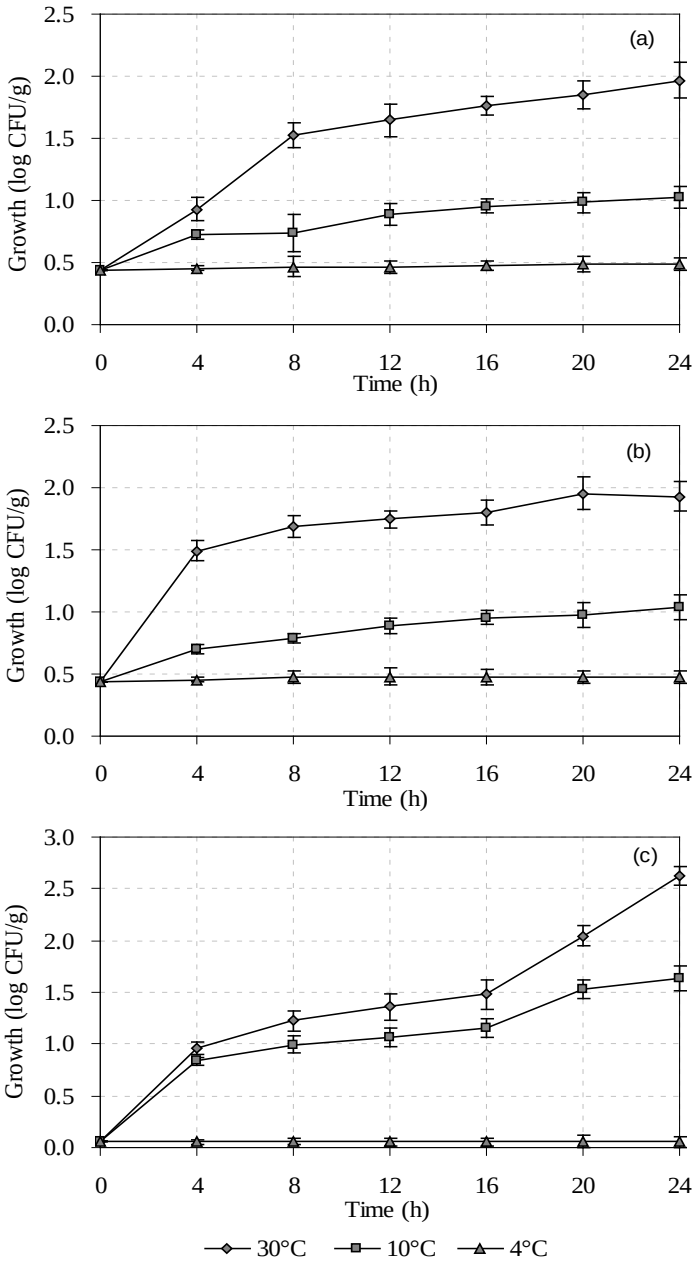

Fig. 1. Survival of *A. hydrophila* in lamb and chicken meat and egg samples at various temperatures. (a) Lamb meat (b) Chicken meat (c) Egg

Table 3. Percentage of antibacterial resistance among the *A. hydrophila* strains isolated

Antibiotics	Sampling Source	
	Lamb meat outlets (n = 20)	Chicken meat outlets (n = 43)
Ampicillin	100	100
Bacitracin	100	100
Chloramphenicol	10	11.6
Erythromycin	65	62.8
Gentamycin	5	4.7
Kanamycin	90	88.4
Nalidixic acid	10	16.3
Novobiocin	90	93
Neomycin	95	81.4
Polymyxin B	90	90.7
Streptomycin	10	11.6
Sulphamethoxazole	5	11.6
Tetracycline	25	23.2
Vancomycin	80	83.7

n = number of *A. hydrophila* strains

The survival of *A. hydrophila* in egg samples was carried out at various temperatures (30, 10 and 4 ^{o}C) for 24 h. The maximum growth of 2.63 log CFU/g was observed in the egg incubated at 30 ^{o}C followed by 1.64 log CFU/g at 10 ^{o}C. In the incubation at 4 ^{o}C, the growth was found to be very negligible. Among the samples (lamb meat, chicken meat and egg sample) used in this study, the *A. hydrophila* survive significantly in the egg at 30 ^{o}C.

Survival of *A. hydrophila* (SLM-4) on stainless steel, wood and iron pieces at various temperatures

The results from the survival of *A. hydrophila* on stainless steel, wood and iron pieces indicated clearly that the organism, *A. hydrophila* could be able to adhere on the surfaces of the knife, chopping board and balance. This study was carried out on various materials based on the accessories used in the meat shops at various time intervals (4, 8, 12, 16 and 24 h) with the inoculum concentration of 2.160 log CFU/ml. In the initial stage the bacterial cells were low (0.43 log CFU/cm^2). It was increased drastically up to 1.176 log CFU/cm^2 after 24 h in the material of iron piece (Fig. 2). The bacterial growth in the other materials such as wood and stainless steel were 0.863 and 0.756 log CFU/cm^2 respectively.

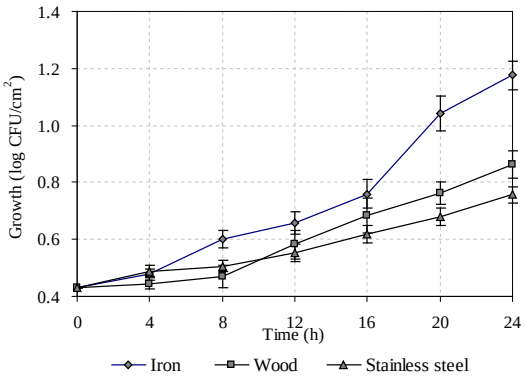

Fig. 2. Survival of *A. hydrophila* on iron pieces, wood and stainless steel at room

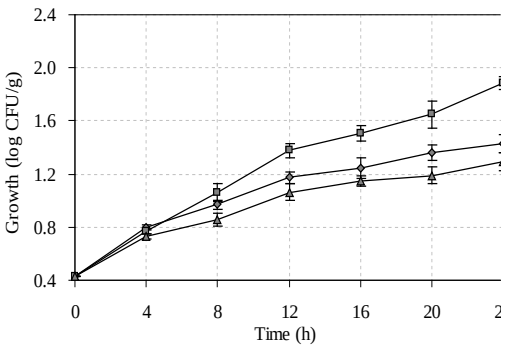

Fig. 3. Survival of *A. hydrophila* in lamb and chicken meat and egg samples before traditional Indian cooking process.

Survival *A. hydrophila* (SLM-4) in lamb and chick meat and egg samples under traditional Indian cooking process

The survival of *A. hydrophila* (SLM-4) in lamb and chicken meat and egg samples were carried out before and after Indian cooking process and the results are given in Fig. 3. This study also demonstrated that there was increasing bacterial growth in all the samples. The maximum growth was recorded in chicken meat which was about 1.883 log CFU/g after 24 h. However, it was little bit low when compared with untreated cooking ingredients. Similar observations were recorded both in lamb meat and egg samples. Complete elimination was recorded in the samples after cooking at 100 ^{o}C for 30 min (data not shown).

Antibacterial resistant patterns

The results of antibacterial resistance of *A. hydrophila* strains isolated from lamb and chicken meat outlets are given in Table 3. The results revealed that all the strains from lamb and chicken meat outlets were resistant to ampicillin and bacitracin. More than 90% of the strains were resistant to kanamycin, novobiocin and polymixin B. The slightest resistance (< 20%) was noticed for chloramphenicol, gentamycin, nalidixic acid, streptomycin and sulphamethoxazole. About 65% of strains from lamb meat and 62.8% from chicken meat were found resistant to erythromycin. The strains isolated form lamb meat and chicken meat resistant to tetracycline about 25 and 23.2% respectively. There was no significant variation in resistance levels among *A. hydrophila* from the samples of lamb and chicken meat outlets.

DISCUSSION

In the present investigation, the percentage incidence of *A. hydrophila* in lamb meat procured from local markets was found to be 53.3% and the incidence was slightly lower than the incidence reported by Ibrahim and Mac Rae (1991) as 58% in lamb meat procured from local retail supermarkets in Brisbane, Australia. Corresponding to lamb meat, percentage of incidence in chicken meat found that maximum level of 66.6% compared with lamb meat. About 36.4% incidence of *A. hydrophila* was reported from chicken by Kirov *et al.,*. (1990). They have explained routes of food borne transmission - one being food contaminated with infected water, with faeces of infected symptomatic or asymptomatic food handlers. Cross contamination of animal carcasses was likely for red meats as processing methods are different, but *Aeromonas* spp. was nevertheless consistently found on retail meat (Stern *et al.,*. 1987). One of the characteristics that strongly influence the potential importance of *A. hydrophila* with regard to food safety is their psychrotrophic nature.

The survival of *A. hydrophila* in meat and egg samples was severally influenced by various temperatures (30, 10 and 4^{o}C). However, the organisms resist even at low temperature (4 ^{o}C). In Bergey's manual lists, *A. hydrophila* as organism being capable of showing growth over a temperature range of 0 to 41°C, and at refrigerated temperatures (4°C to 7°C), the species grows at sufficiently rapid rate as to be competitive with other psychrotrophic species associated with foods (Popoff 1984). The survival of *A. hydrophila* in the present study was in good agreement with the earlier reports (Beuchat 1991). Similarly, Golden *et al.,* (1989) reported that sublethally heat injured cells of *A. hydrophila* were more sensitive than uninjured cells upon subsequent exposure to 5°C. In this present study, the growth of *A. hydrophila* was noticed all the temperature range. The presence of *A. hydrophila* in the lamb and chicken meat by contamination of the butcher's hand was about 40% and 90% respectively. In support to our result, there are many reports regarding the introduction of *Aeromonas* through food handlers (Wadstrom and Ljungh, 1991). In the survival study conducted on various accessory materials, the bacterial growth on the iron pieces showed higher. The reason why iron materials exhibited the highest adhering rate can be explained by the fact that *A. hydrophila* is an iron corrosive and is capable of utilizing Fe(III) as a electron acceptor (Woznica *et al.,* 2003). *A. hydrophila* cells became more hydrophobic and significantly increased their

ability to bind the iron-binding glycoprotein lactoferrin when the bacterium was stored under nutrient-poor conditions (Ascencio *et al.,* 1995).

An experiment was conducted to check the inhibitory effect of readily available Indian cooking ingredients (masala) on the growth of *A. hydrophila* in meat and chick samples. The results showed that *A. hydrophila* was able to survive on masala pasted meat blocks even after 24 h storage at 30°C and masala did not removed completely. However, the number was consistently reduced showing the antibacterial effect of the masala.

Water used in lamb and chick meat shops was found to contain *A. hydrophila* and its existence was about 60% and 40% respectively. The above results clearly support the fact that the contamination may be due to processing and other secondary factors, which paves way for the multiplication of *A. hydrophila*. From the overall observation, it is concluded that the meat samples, which does not originally contain any *A. hydrophila* initially, may be contaminated during processing. The present study clearly highlights the sources of cross contamination. There are no standard operational procedures to sanitize these utensils. This causes a health hazard to the consumers of lamb meat, chicken from retail outlets. Hence, legal measures should be implemented to install proper sanitation methods to control this type of cross contamination. Personal obligatory inspection of retail outlets by officials of the food safety department should be frequently monitored to prevent possible health hazard.

All the *A. hydrophila* strains were tested antibacterial resistance against various antibiotics. The results clearly exposed that all the strains from lamb and chicken meat outlets were resistant to ampicillin and bacitracin. Corresponding to our results, Palu *et al.,* 2006) observed 100% ampicillin resistance of *A. hydrophila* isolated from clinical samples. Recently, Penders and Stobberingh, (2008) observed all the *Aeromonas* spp. isolated from fish farms showed resistant to ampicillin. However, they have not reported any bacitracin resistant organisms. The *Aeromonas* spp. isolated from ice cream showed less (28%) resistance against ampicillin reported by El-Sharef *et al.,* (2006). Vivekanandhan *et al.,* (2002) who reported that all *A. hydrophila* strains from marketed fish were resistant to bacitracin. More than 90% of the strains resistant to kanamycin, novobiocin and polymyxin B. The investigation of Ansary *et al.,* (1992) supported the existence of kanamycin resistant strains, with a frequency of about 38.2%. The resistance showed little bit low when the organisms tested with other antibiotics such as chloramphenicol, gentamycin, nalidixic adid, streptomycin and sulphamethoxazole. Vivekanandhan *et al.,* (2002) recorded the similar patterns in *A. hydrophila* isolated from marketed fish and prawn. Though, they have not studied *A. hydrophila* against sulphamethoxazole. About 65 and 62.8% of strains from lamb meat and from chicken meat were showed resistant to erythromycin. This is partially supported by Ansary *et al.,* (1992) and Son *et al.,* (1997). About 25 and 23.2% of the strains isolated form lamb meat and chicken meat respectively resistant to tetracycline. Similar to our results, Palu *et al.,* (2006) reported that 22.2 and 7.7% of *A. hydrophila* strains isolated from clinical samples and vegetables respectively showed resistant to tetracycline. However, Jacobs and Chenia, (2007) observed most of the *Aeromonas* strains (78.3%) isolated from aquaculture systems resistant to tetracycline.

A. hydrophila is a widespread, emerging food pathogen and tend to cause illness in humans. Our study clearly indicates that the meat samples, which do not originally contain any *A. hydrophila* initially, may be contaminated during improper progress such as handling, washing and storage. This contaminant causes a health hazard to the consumers of lamb and chicken meat from the retail outlets. It can play a significant role in intestinal disorders in children under five years old, the elderly, and immunosupressed people. Most cases of illness are associated with marketed meats, aquaculture products or long-term refrigerated ready-to-eat foods. From the study conducted with various temperatures, the results point out that *A. hydrophila* is a psychrotrophic bacterium, as it grows at refrigeration (4 ^{o}C) temperatures. This ability of the pathogen may play an important role in food safety of foods for human consumption. The

organism was completely eliminated in each meat samples after cooking at 100 ^{o}C for 30 min. However, the produced toxins by *A. hydrophila* can cause illness even after cooking. This organism has a number of putative virulence properties, which are associated with enterotoxic, cytotoxic and hemolytic activities. Multiple resistances to some antibiotics have occurred in most of these organisms, and it may become a problem to cure intestinal disorders in human. *A. hydrophila* is quite resistance to many factors such as refrigeration, heating and application of traditional cooking ingredients etc. Hence, proper processing is needed to control the contamination of *A. hydrophila* before being used the meats.

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