Isolation and characterization of melamine degrading Bacterial strains from soil

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Abstract The use of agrochemicals has been critically important in increasing the yield of agricultural crops. On the other hand, there is evidence that significant environmental pollution and impacts on human health arise from the inappropriate use of agro chemicals. All-win wonder and Allwin top are the two agrochemicals produced by Sree Ramcides Chemicals Pvt. Ltd. It contains melamine as a major constituent. It contains melamine as a major compound. Melamine is a nitrogen-rich heterocyclic triazine. It contains 66 % of nitrogen which can be vitally used by plants as nitrogen source. Now-a-day's melamine is adulterated in milk products because of its protein content and it also found as a contaminant in food and food products. Hence, a laboratory enrichment study was conducted at Department of Environmental Sciences, Tamilnadu Agricultural University for the isolation of melamine degrading microorganisms.

Index Terms— Melamine degradation, Bacterial strains, Isolation, Characterization

1 INTRODUCTION

Environmental pressures are increasing as existing land and water resources come under threat from rapid urbanization. Furthermore, the need to use large amounts of agrochemicals to control pests and weeds has raised environmental and human health concerns. Agrochemicals pose health and environmental risks, and they can pollute rivers and lakes through runoff and groundwater through leaching. There are proven alternatives to this expensive agriculture system: farmers are already fertilizing soils and protecting crops with organic and sustainable techniques that work with nature, not against it, and can provide food for all (Pretty *et al.,* 2003, Badgley *et al.,* 2007).

M/s Sree Ramcides Chemicals Pvt. Ltd., is a Chennai- based company, which manufactures the agro inputs such as herbicides, insecticides, fungicides and plant nutrients for various crops grown all over India and in more than 10 countries. The company focuses on plant health products which facilitate absorption of nutrients and improve the plant health by increasing the resistance to diseases and pests in plants, thereby leading to increased crop yields. It contains melamine as a major component.

During the year 2008, more than 2 crore children in China were clinically evaluated for urolithiasis due to the fear of consuming melamine adulterated powdered infant formula. At least 53,000 children were hospitalized (Xin and Stone, 2008) and 6 deaths were reported. The severity of melamine adulteration and the resultant renal toxicity has placed the urgent need to ensure public health in the spotlight (Brown *et al.*, 2007; Buur *et al.*, 2008; Langman, 2009). Since, melamine is now treated as a chemical hazard threatening food safety, several international agencies are working together to monitor and control melamine contamination in food and food products (Bhalla *et al.*, 2009). The chances of water resources and soils being contaminated by these man-made pollutants has become more and more likely and even micro-quantities of some chemicals may cause catastrophic tragedies. Hence a systematic research was carried out to isolate the melamine degrading microorganisms in soil. Such findings highlight the need for long term monitoring of fate of agro chemicals to ensure environmental safety.

2 Materials and Methods

Enrichment culturing with Allwin wonder and Allwin top

Five hundred gram of Allwin wonder and Allwin top applied soil samples collected from wetlands were taken in a cleaned and dried incubation cups. Three replicated incubation cups were maintained for each soil

sample. The soil samples were treated with Allwin wonder and Allwin top at different concentrations at an interval of seven days to maximize the survival of the potential degrading species alone.

Treatments details

T_1 : Control
T_2 : Allwin wonder @1 mg kg^{-1} of soil
T_3 : Allwin wonder @1.25 mg kg^{-1} of soil
T_4 : Allwin wonder @1.5 mg kg^{-1} of soil
T_5 : Allwin wonder @ 2.5 mg g^{-1} of soil
T_6 : Allwin wonder @ 5 mg $kg^{-1}$of soil
T_7 : Allwin top @ 0.125 mg kg^{-1} of soil
T_8 : Allwin top @ 0.187 mg kg^{-1} of soil
T_9 : Allwin top @ 0.25 mg kg^{-1} of soil
T_{10}: Allwin top @ 0.50 mg kg^{-1} of soil

Isolation of the microorganisms

Pseudomonas and *Bacillus* were isolated from the soil using serial dilution and plating technique (Waksman and Fred, 1922). One gram of the soil was taken in 10 ml sterile water blank in a test tube, which gave 10^{-1} dilution. The contents were serially diluted by taking 1ml from 10^{-1} dilution and adding it to another 9 ml sterile blank which gave 10^{-2} dilution. This was repeated till 10^{-6} dilution was obtained. 1ml each of the solution from suitable dilution was pipetted out into sterile petriplates. The selective media, Nutrient agar medium for *Pseudomonas and Bacillus* was prepared and added to the respective petriplates and rotated for uniform mixing. The plates were then incubated in an incubator at 30°C for 24 h. The colonies developed were preserved for purification and identification.

Purification of Microorganism

The purification of isolated microorganism was done by Streak plate technique. The selective media for *Pseudomonas and Bacillus* was prepared and poured into petriplates in sterile conditions and kept for solidification. A loopful of the isolated microorganism was streaked in a zigzag manner on the respective media. The plates were then incubated for 24 h in an incubator for colony formation. The colonies were subcultured in agar slants and preserved in the refrigerator for future use.

Morphological, biochemical and Molecular characterization of bacterial isolates

Morphological examination of isolates was performed by scanning electron microscopy (Fig. 1). Morphological and biochemical characterization of the bacterial isolates was done as per the methods specified in the Table 1. Molecular characterization was carried out for the isolated microbial cultures at Chromous Biotech Pvt. Ltd., Bangalore and sequenced through single pass analysis from forward and reverse direction. Sequence data was compared with already available sequence data by BLAST analysis in NCBI sequence data bank (Fig.2).

Table 1. Standard methods followed for morphological and biochemical characterization of bacterial isolates

S. No	Characteristics	Reference
1	Colony morphology	Gerhardt *et al.* (1994)
2	Gram staining	
3	Acid production from glucose, sucrose and lactose	
4	Hydrogen sulphide production	Cappuccino and Sherman (2002)
5	Nitrate reduction	
6	Indole test	Gerhardt *et al.* (1994)
7	Methyl Red- Vogues Proskauer (MR-VP)	
8	Citrate utilization	Simmons (1976)
9	Urease Activity	Gerhardt *et al.* (1981)
10	Catalase Activity	Smibert and Koeigi (1981)
11	Oxidase Activity	Cappuccino and Sherman (2002)
12	Gelatin hydrolysis	
13	Starch Hydrolysis	
14	Mackonkey Agar	

Results and Discussion

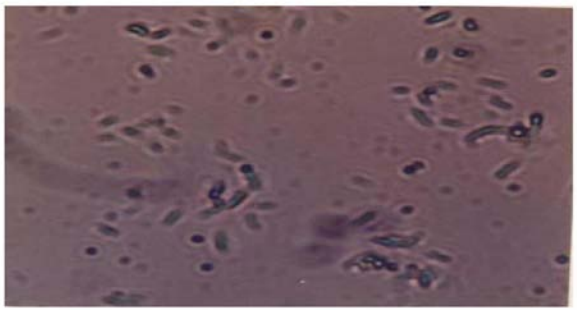
Two predominant bacterial (BS1 and BS2) were isolated from soil enriched with Allwin wonder and Allwin top. Morphological and biochemical characterization of the isolates showed that the isolate BS1 was opaque, yellow coloured, wrinkled colonies, gram negative, oxidase and catalase positive and rod shaped on nutrient agar media. According to these properties it was iden-

tified as the genus *Pseudomonas* sp. Culture of BS2 showed gram positive, catalase positive, rod shaped, opaque, white, smooth and raised colonies on nutrient agar media. From the morphological and biochemical characterization of bacterial isolates, they were tentatively identified as *Pseudomonas* sp. and *Bacillus* sp. Further the molecular characterization of above isolates confirmed the above results given by Chromous Biotech Pvt. Ltd., Bangalore. They could get an amplification size of approximately 546 bp and 560 bp, respectively for *Pseudomonas* sp. and *Bacillus* sp. Morphological and biochemical characteristics of the melamine degrading isolates are listed in Table 2.

According to these properties it was identified as *Bacillus* sp. Similar observation was made by Sahin and Tamer (2000) who isolated the culture of *Bacillus* from soil contaminated by Thiram, with the characteristics of gram positive and aerobic rods. Mirgain *et al. (*1993) and Struthers *et al. (*1998) isolated *Pseudomonas, Rhizobium, Acenetobacter* and *Agrobacterium* from atrazine contaminated soils which are capable to initiate atrazine degradation by a hydrolytic dechlorination reaction. Nafeesa (2009) also isolated chlorpyriphos and carbofuran degrading bacterial isolates *viz., Pseudomonas* sp., *Bacillus* sp., *Klebsilla* sp., *Acenitobacter* sp. *and Serratia* sp. from soil through enrichment. Bacteria metabolize melamine by sequential deamination to ammeline, ammelide and cyanuric acid (Seffernick *et al.,* 2002). *Pseudomonas* spp. was isolated from soil, which are capable of degrading the compound of s-Triazine (Hernandez *et al.,* 2008).

Fig.1. Morphology of bacterial strains isolated from soil

***Pseudomonas* sp.**



***Bacillus* sp.**

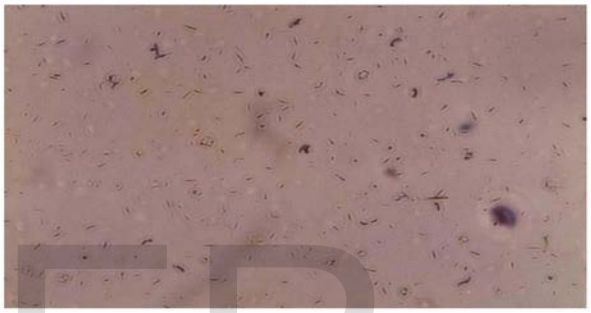


Fig.2. Confirmation of genomic DNA of *P. fluorescens and B. subtilis* using specific primer

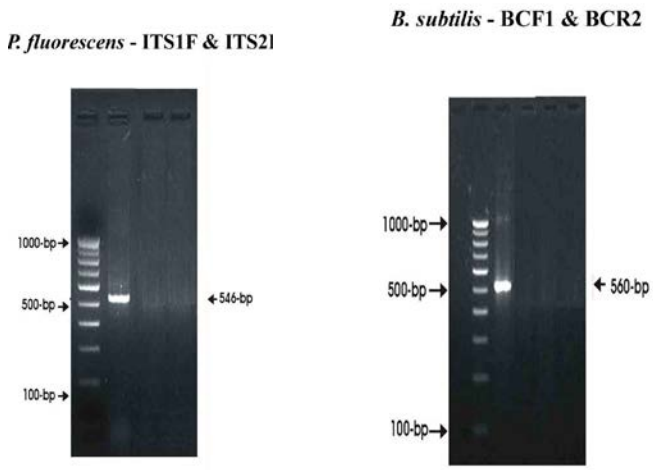


Table 2. Morphological and biochemical characteristics of melamine degrading bacterial isolates

Isolate	Grams Stain reaction and Shape of the cell	Nutrient Agar Plate characteristics	Fermentation			H_2S Production	NO_3 Reduction	Indole Production	MR Reaction	VP Reaction	Citrate Use	Urease Activity	Catalase Activity	Oxidase Activity	Gelatin Liquefaction	Starch Hydrolysis	Growth on Mackonkey Agar	Tentative identification
			Glucose	Sucrose	Lactose													
BS-1	Gram (-)ve, rods	Thin, opaque, yellow coloured wrinkled colonies	-	-	-	-	+	-	-	-	+	-	+	+	-	-	+	*Pseudomonas* sp.
BS-2	Gram (+)ve, rods	Opaque, white raised and smooth growth	A	A	-	-	+	-	+	-	-	-	+	-	-	+	-	*Bacillus* sp.

A – Acid production; G – Gas production; +ve – positive; -ve - negative

Conclusion

Agro chemicals have largely played a part in India's growth into a self-sufficient nation in food production and also play an important role in intensive agriculture. There was lack or absence of corrective and preventive measures, presence of persistent, bioaccumulative and toxic agrochemicals and its transformation in water, fish, vegetables and human fluids. Keeping in view of the present scenario, it is strongly recommended that extensive awareness creation for safe use of agrochemicals be introduced, epidemiological studies and impact of agrochemical usage in the country be instituted.

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