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Biodegradation of the Organophosphorus insecticide Dichlorvas by *Bacillus* species isolated from Grape wine yard Soils from Sangli District, M.S., India

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Abstract

In countries like India insecticides play an important role in horticulture sector. Their importance in crop yield improvement, especially in Grape production, is understood and well accepted. But still they pose a threat to the environment due to their toxic nature and persistence in the food chain. Therefore, the study focuses on the development of a method to reduce the environmental burden of the insecticides by way of biodegradation. During this study, 7 different Bacillus strains isolated from grape rhizosphere soils were found to show significant tolerance ability towards an organophosphorus insecticide, Dichlorvas. Results indicated that out of 7 strains, only one strain could degrade the insecticide at the level of 10mg/L and 15mg/L. Also, the degradation of Dichlorvas was enhanced when medium was provided with glucose as an inducer for growth of bacteria.

Keywords: Grape, *bacillus*, dichlorvas, glucose, organophosphorus.

Introduction

Grape is an important commercial fruit crop in India, which receives frequent application of large number of agrochemicals that is pesticides, throughout the cropping season for management of various pests and diseases (CIB of Ministry of Agriculture, Government of India, 2008). Grape is mostly consumed as fresh fruit in intact form without any processing. Therefore, pesticide residues left on the grapes as well as in soil and during harvest can be carried through into the wine which causes many harmful effects on the plants, animals and human being[1].

Unlike most pesticides, biodegradable insecticide polymers when disposed in the environment (e.g. compost, soil or waste water) are acted upon and utilized by the indigenous microorganisms as sources of carbon and energy, thus insecticides in the soil are degraded[2]. In India, most insecticides as organophosphates e.g. Dichlorvas is used on grapes against various insects. These organophosphorus products are widely used on various fruits in the world and thus their residues are constantly found in a variety of fruits including grapes[3]. So, survival of bacteria under insecticide stress can provide an efficient, cheaper and eco-friendly solution for bioremediation of these xenobiotic contaminated soils. These bacterial strains isolated from various agricultural fields i.e. grape wine yards can be employed in the microbe based bioremediation of insecticide contaminated soils. Soil typically has high microbial species diversity[4]. Because of species diversity many pesticides get degraded.

Therefore, common practices are monitored for microbiological degradation of xenobiotic compounds by determining the residual concentration. However a decrease in the concentration of the toxic compound means that the toxic xenobiotic compounds such as insecticides are converted into nontoxic compounds[5]. There is number of possible routes of removal of toxic substrates such as insecticides from soil, including mineralization (i.e. complete biodegradation), biotransformation, and assimilation as nutrient into microbial biomass, polymerization, leaching and sorption[6].

Therefore, proposed research work is aimed at the study of organophosphorous insecticide, Dichlorvas, biodegradation by *Bacillus* strains isolated from insecticide contaminated soils that is grape wine yards. The study will be useful for the bioremediation of soil pollution and pesticide free agricultural practices.

Material and Methods

Collection of Soil Samples: Twenty soil samples (5-6g) were collected from grape wine yards of seven different locations of Sangli district (M.S.), to isolate the Dichlorvas degrading bacteria. These grape wine yards have been sprayed with Dichlorvas organophosphorus insecticides for last 5-6 years. These samples were collected at different sites of each location by using spatula and at a depth of about 20cm (rhizosphere region) and were taken in sterilized polyethylene bags and kept at 4^0C until analysis.

Chemicals and Media: Standard analytical grade preparation of organophosphorus insecticide as Dichlorvas (2, 2-dichlorovinyl dimethyl phosphate) (76% E.C.) was brought from the local market of Sangli, Maharashtra, India. A liquid

preparation of Mineral Salts Medium (MSM) containing 0.05% yeast extract, 1.0% dextrose, 0.3% sodium nitrate, 0.001% ferous sulphate, 0.05% potassium chloride, 0.05% magnesium sulphate, 0.1% K_2HPO_4 and 0.05% KH_2PO_4 was used for enrichment of soil samples. For the screening and maintenance of cultures Nutrient Agar was used.

Enrichment and isolation of Dichlorvas degrading bacterial strains: To remove large pieces of vegetation and debris the soil sample was passed through a sieve (4.0mm mesh) For isolation of Dichlorvas insecticide degrading bacteria from collected soil samples, enrichment culture technique was used with varying concentrations of Dichlorvas (5- 20mg/L) in Mineral Salts medium. Soil sample (1gm) was inoculated in to 100ml of mineral salt medium supplemented with above various concentrations Dichlorvas of in 500ml Erlenmeyer flasks. The enrichment medium containing flasks were kept for incubation on a rotary shaker at 150 cycles per minute for 7 days at room temperature (25-30oC). At 24hrs of incubation intervals, 0.1ml from above enriched culture was streaked on sterile Nutrient Agar plates containing Dichlorvas (5- 20mg/L) and incubated at room temperature for 24-48hrs. Single colonies of bacteria which differ in shape and color were picked up and were subcultured onto sterile Nutrient agar plates consisting of above same concentration of Dichlorvas till pure culture was obtained. The isolated strain was subcultured every three months and maintained at 4^0C. The selected bacterial isolate was identified by considering Bergey s Manual of Systematic Bacteriology[7].

Degradation of Dichlorvas by WL Dump D10: To study the degradation of the Dichlorvas, the isolated strain WL DumpD10 was inoculated in the Mineral Salts Medium as above with dextrose and 10mg/L Dichlorvas as the source of carbon and energy. The flasks containing medium were kept for incubation on rotary shaker at 150 cycles per minute at room temperature. After incubation, contents were analyzed by taking 5ml of degradation broth and centrifuged at 10000 rpm for 15 min. The pellet was discarded and supernatant was analyzed by UV-Visible spectrophotometer to detect the Dichlorvas degradation by isolate WL DumpD10. Degradation was determined at intervals of 2 days up to 8 days. The rate of Dichlorvas break down by isolate WL DumpD10 was also determined in the form of percentage.

Optimal Temperature and pH for degrading Dichlorvas by WL DumpD10: To determine the optimum conditions as temperature and pH for efficient degradation of Dichlorvas by WL DumpD10, for determination of optimum temperature, degradation broth was placed for incubation at various temperatures (10^0C, 20^0C, 25^0C, 30^0C, 35^0C and 40^0C). For determination of optimum pH for breakdown of insecticide, MSM medium was formulated with different pH buffers (4.0 to 11.0), fortified with 10mg/L Dichlorvas.

Figure-1
Study Area- Sangli District Map

Extraction of the metabolites and GCMS analysis: After incubation for about 8 days, the degradation broth was centrifuged at 10000 rpm for 15 min. Then after centrifugation, pellet was discarded and supernatant obtained was used to extract metabolites with ethyl acetate (1:1). The extract was dried and kept for evaporation to dryness in an evaporator. For GCMS analysis obtained residues were dissolved in small volume of methanol.

Gas chromatography was performed in temperature assisted mode with a DB 530-m fused silica capillary column (0.25mm, i.d. x 0.25 µm film thick) joined to a mass spectrophotometer. Samples were applied in to a split mode temperature program of 180^{0}C for 1.5 min, 260^{0}C for 20 min, at the rate of 10^{0}C/min, application temperature was 260^{0}C, detector temperature was 280^{0}C and Nitrogen was used as carrier gas. The components were detected and identified on the basis of mass spectra and were compared using National Institute of Standards and Technology (NIST) library[8].

Results and Discussion

The use of chemical insecticides in grape wine yards is a common practice in India and also throughout the world. The environmental contamination caused by intensive and indiscriminate use of these types of insecticides in these sites and their elimination by physical and chemical techniques compared to the magnitude in which they are used is impossible over a short time. Therefore, it is necessary to study alternatives in order to reduce the major effects of these pesticides on the environment[8]. Biological removal of insecticide is the cheapest way, as soil microorganisms may utilize such toxic compound and converts them into non-toxic metabolites.

Isolation and Identification of Dichlorvas Degrading Bacteria: About 7 different strains of bacteria with different colony morphologies were obtained from collected grape wine yard soils after repeated purification processes. The isolate WL DumpD10 possessed comparatively higher degradation ability of degrading Dichlorvas (10mg/L) by 87.0% after incubation for 8 days at pH 7.2 and at room temperature about 30^{0}C. WL DumpD10 utilized Dichlorvas as its carbon and energy source in Mineral salt Medium. Then, for further detail study strain WL DumpD10 was selected.

The isolated 7 bacterial strains were confirmed as belong to the Genus *Bacillus,* on the basis of their morphological, cultural and biochemical characteristics, according to Bergey's Manual of Systematic Bacteriology. The growth profile of the selected isolate showed maximum growth after 24-30 hrs of incubation in insecticide supplemented medium. *Bacillus* strain used (WL DumpD10) in this study had shown a range of degradation capability as glucose enhanced the degradation of Dichlorvas than mineral salts with insecticide alone where glucose acts as a growth inducer.

Optimum Temperature and pH for Dichlorvas Degradation in MSM by WL DumpD10: The insecticide was degraded by *Bacillus* (WL DumpD10) during incubation temperatures ranging from 30^{0}C to 40^{0}C. Dichlorvas residues were detected after 8 days experiment. Figure 2 shows medium incubated at higher temperatures 30^{0}C and 35^{0}C, the degradation rate reached 87.0% and 85.0% within 8 days, but the rate was low at any other temperatures. Eight different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) were used in the optimization experiment. Optimum pH value for degradation of Dichlorvas by WL DumpD10 determined was 7.0 (figure 3)

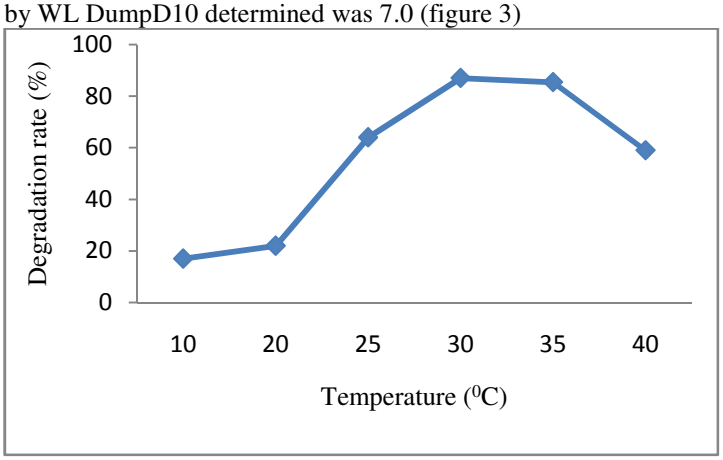

Figure-2
Optimum temperature for the degradation of Dichlorvas by WL DumpD10

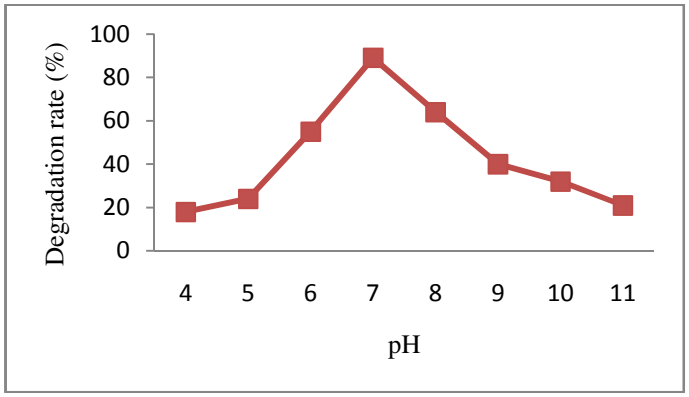

Figure-3
Optimum pH for degradation of Dichlorvas by WL DumpD10

Detection and Identification of Dichlorvas degradation metabolites: Metabolites of Dichlorvas degradation by WL DumpD10 was analyzed by GC/MS using NIST library. Upon GC/MS analysis Dichlorvas shows retention time of 7.208 min. This detection of metabolites showed the presence of three components. This result observed after degradation of Dichlorvas by isolated *Bacillus* strain obtained metabolites as compared to the reports previously observed. Reports obtained from GC/MS analysis show the presence of (Pyridine, 3-ethenyl-), (5-Phenyl-1H-tetrazole) and (2-Chlorohistidine) (figure 4)

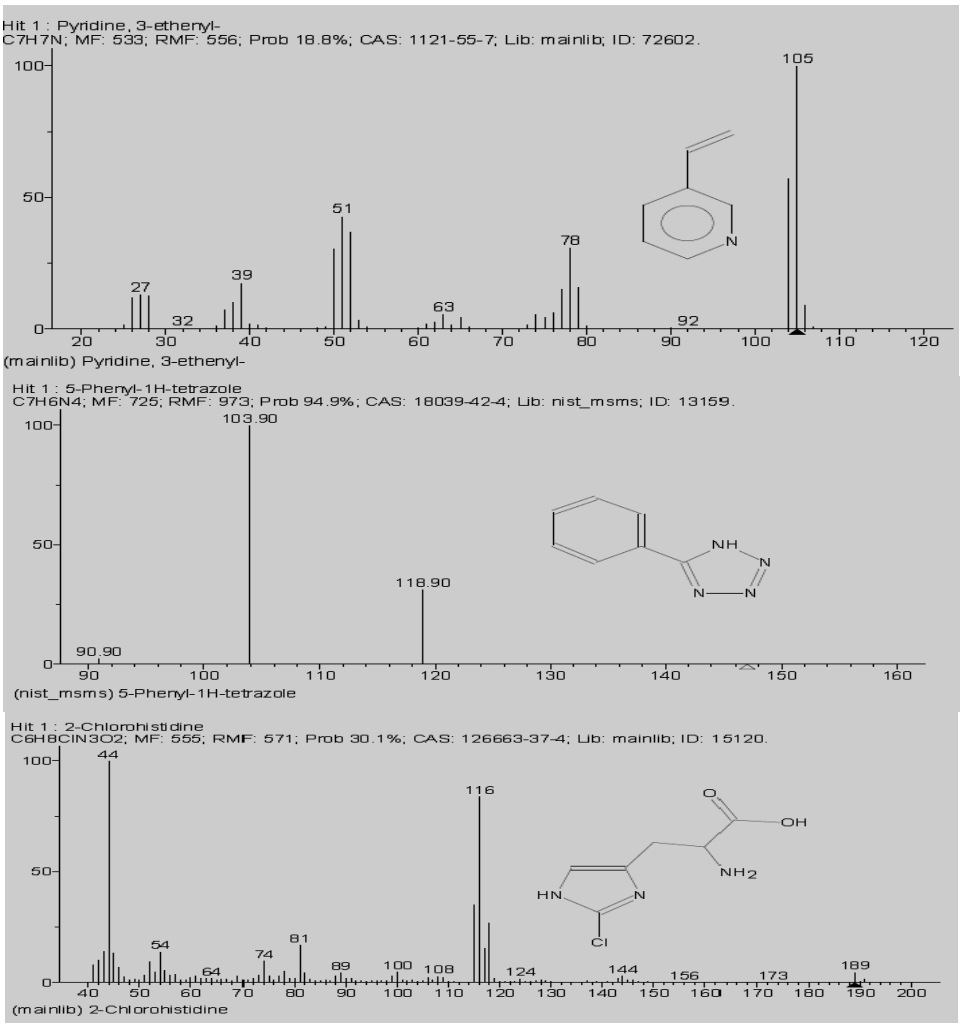

Figure-4
GCMS analysis of Dichlorvas degradation metabolites

Discussion: The treatment of chemical or pesticide contaminated soil with microorganisms involves the conversion of complex and toxic compounds into non-toxic forms[9]. Previous studies indicated that soil bacteria such as *Acinetobacte*r species have been used successively in both environmental microbiology and biotechnology applications[10]. As glucose accelerated the degradation of Dichlorvas, previous studies found that the glucose as growth inducer had increased the rate of degradation[11]. Also earlier studies found that, many soil contaminated pesticides are degraded more rapidly by soil bacteria because respective pesticide resistance was developed in these bacteria following repeated application at the same site[12]. It was concluded that *Xanthomonas* degrade Dichlorvas but unable to degrade Chlorpyrifos[13]. Similar observation was found in which research conducted on the effect of OP pesticides on salt affected alkaline soil bacteria[14]. The variation in *Xanthomonas* population gave an indication that OP pesticides had either stimulatory or inhibitory effects on different soil microbial groups and had some role to play in the degradation of selected organophosphates.

Conclusion

In conclusion our reports indicated that the isolated soil *Bacillus* strain may be a good choice for the bioremediation of organophosphorus contaminated soil and water. However, further investigations such as degradation enzymes and other biochemical aspects are still needed before the application.

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