



Soil Bacterial Isolate Showing A Potential Of Mancozeb Degradation And Plant Growth Enhancement

Priyanka Gajjar^{1*}, Bhavya Kiri², Hiral Shah¹

^{1*}Department of Microbiology and Biotechnology, School of science, Gujarat University, Navrangpura, Ahmedabad

²Department of Microbiology, Shri A. N. Patel PG Institute of Science and Research, Sardar Patel University, Anand.

¹Ananya Institute of Science, KIRC campus, Kalol, Gandhinagar

***Corresponding Author:** Priyanka Gajjar

*Department of Microbiology and Biotechnology, School of science, Gujarat University, Navrangpura, Ahmedabad

Abstract

Mancozeb, a dithiocarbamates pesticide, has been widely used for weed control. Despite mancozeb's endurance and toxicity towards biological forms, its removal or degradation from contaminated area has become a concern, leading to bioremediation processes. From agricultural fields contaminated with pesticides, two bacterial strains, *Pseudomonas aeruginosa* (PM3) and *Rhizobium pusense* (PM10) were identified that have the ability to degrade mancozeb. Within a 10-day timeframe, these isolates were able to break down between 97.49% and 98.99% of the mancozeb's original concentration (100mg/L). In both sterilised and non-sterilised soils, these strains showed the ability to degrade mancozeb by 95-100% of their initial concentration (200mg/kg). These strains also exhibited notable characteristics that supported plant development, including the ability to solubilize phosphate, produce both indole acetic acid and ammonia in absence and presence of mancozeb. Mancozeb has shown detrimental influence on plant through plant growth experiments and cause a drop-in growth indicator, such as percentage germination, plant biomass and plant height. Inoculation of bacterial isolates with mancozeb was shown to considerably improve plant growth in terms of plant weight and plant length. This study revealed that mancozeb degrading strains have the potential to become a strong contender for increasing agricultural productivity in pesticide contaminated soils.

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Keywords: Mancozeb, Dithiocarbamates, Bioremediation, *Pseudomonas*, *Rhizobium*.

1. INTRODUCTION

After rice, wheat and maize, potatoes are the world's fourth most widely produced crop in world. Potato tuber, the edible component of the potato, is a useful comestible due to its high fibre, carbohydrate and water-soluble vitamin concentration as well as its low-fat content. Figure 1 shows the chemical composition of potato tuber.

Potato cultivars need the use of several agrochemical products, including pesticides, because they are more susceptible to insects, weeds, fungus and viruses. Application of pesticides increases efficacy of crop; yet, because pesticides are not completely selective, they also impact nontarget species. Furthermore, agrochemicals may accumulate in crops, having a deleterious impact on food quality and consumer health (Kurek, M et al., 2017).



Figure 1 Chemical composition of potato

Mancozeb is well known fungicide that is composed of zinc ion and manganous ethylene bis dithiocarbamates and is widely employed in the treatment of vegetables, fruits and vines. Mancozeb degradation in plants is much slower than that of other dithiocarbamates, according to literature (Saeedi Saravi, S. S., & Shokrzadeh, M. 2016). It undergoes oxidation to form ethylene -bis-isothiocynate, ethylene thiuram monosulfide, ethylenethiourea and ethylenediamine (Doneche, B., et al., 1983). This fungicide has several modes of actions that can control many plant diseases. Dithiocarbamates will most likely continue to play an important role as consistent resistance management methods to extend the efficacy of single site fungicides. Some of these fungicide's primary metabolite, ethylene thiourea, is thought to be a reproductive and endocrine disruptor in animals (Thind, T. S., & Hollomon, D. W. (2018).

One of the most significant approaches to protecting the environment from various hazardous components is bioremediation, sometimes known as the green revolution. This approach is eco-friendly, cost-effective, and the most promising technology for removing xenobiotic components by beneficial microbes, green plants, or their enzymes in order to return the natural environment to its original state and prevent pollution (Gajjar, P et al., 2020).

The objective of this study was to isolate and characterize mancozeb degrading bacteria and to determine the degradation potential of these strains in both sterile as well as non-sterile soil. Moreover, the assessment was made for plant growth promoting potential of these bacteria and the strains' ability to bioremediate soil and to enhance plant growth in contaminated soil were also tested.

2. MATERIALS AND METHODS

2.1 Soil sample collection

Ten soil samples were collected in February 2020 using suitable sampling methods and equipment's from the potato producing area in several villages in Gujarat's Banaskantha district for the purpose of this study. Soil samples were taken by four quarters method. This method was repeated 3-4 times, and the final samples were air dried and sieved with 2 mm size containing mesh. Temperature and pH were measured with the help of thermometer and pH strip as well as pH meter (Patil, R. B., & Saler, R. S. 2013).

2.2 Chemicals and Culture media

Mancozeb (Rediniol 75%, powder) was obtained from Palanpur, Banaskantha, Gujarat, and concentrated stock solutions (100 ppm/L) were produced in dimethyl sulfoxide. HiMedia Laboratory in Gujarat provided the methanol and acetonitrile (HPLC grade), hexene, and ethyl acetate. For bacteria isolation, Luria-Bertani medium with tryptone 10.0g/L, yeast extract 5.0g/L, NaCl 10.0g/L, and pH 7.0 was used, and Mineral salt medium (MSM) with (g/L) $(\text{NH}_4)_2\text{SO}_4$ 2.0, K_2HPO_4 1.5, KH_2PO_4 0.5, NaCl 1.0, MgSO_4 0.2, and pH 7.0 was used. All of the media were autoclaved for 30 minutes at 121.3°C (Lu, P et al., 2019).

2.3 Enrichment, selection and identification of Mancozeb degrading strain

Enrichment, selection and identification of mancozeb degrading bacterial strain was done by the method of Ambreen, S., & Yasmin, A. 2020. Purification of well isolated colonies were done on the nutrient agar slant supplemented with 100 ppm mancozeb. Determination of Mancozeb residue was done by HPLC (Ambreen,

S., & Yasmin, A. 2020). The physiological, morphological and biochemical tests of selected bacterial isolates were performed using standard method and taxonomically identified according to Bergey's Manual of Systematic bacteriology (12) and further confirmation was done by 16s rRNA sequence analysis (Malghani, S., et al., 2009).

2.4 Determination of auxiliary characteristics

Indole acetic acid production was determined using nutrient broth. The broth was inoculated with bacterial isolates and incubated overnight at 28°C on rotary shaker. Broth was centrifuged and 2-3 drops of orthophosphoric acid and 4 ml of sollkouski's reagent was added into 2 ml of supernatant. The samples were incubated for 25 minutes at room temperature and optical density was measured at 530 nm. (Alam, S., et al., 2018). Phosphate solubilization activity was determined by using pikovaskaya agar supplemented with 0.5% tricalcium phosphate. Clear zone of solubilization was measured by scale (Akbar, S., & Sultan, S. 2016). Ammonia production was checked by the method of Kifle, M. H., & Laing, M. D. 2016. Cyanogenic component or hydrogen cyanide production was determined by the method of (Akbar, S., & Sultan, S. 2016).

2.5 Biodegradation studies

2.5.1 Inoculum preparation and Mancozeb degradation in liquid medium

Each bacterial isolate was grown in a nutrient broth supplemented with the concentration of 50 mg/L of mancozeb. Following growth, the cultures were centrifuged for 5 minutes at 4600x g. These suspensions' colony forming units (cfu/ml) were determined using the dilution plate counting technique. Pesticide biodegradation investigations employed an equal amount of cells concentration (1.6×10^7 cfu/ml). In triplicate, Erlenmeyer flasks (250 ml) containing mineral salt media (100 ml) supplemented with 100 ppm mancozeb were inoculated with bacterial isolates. The flasks were incubated at 30°C with 150 rpm shaking condition, and an uninoculated flask served as a control. 1 ml culture was withdrawn from the broth at regular intervals of two days each for 10 days continues and growth was evaluated as optical density at 600 nm. Residues of mancozeb extraction and determination was done by HPLC (Akbar, S., et al., 2015).

2.5.2 Biodegradation of Mancozeb in soil

Analysis of mancozeb degradation by selected bacterial isolates was conducted in sterilized as well as non-sterilized soil. 100 gm soil samples were spiked with mancozeb to a final concentration of 200 mg/kg by the addition of DMSO-based mancozeb solution. The solution was initially added in 10 gm of soil, then mixed with the remaining soil quantity. The soil samples were inoculated and incubated at 30°C. The test was performed in triplicate and inoculated sterile and non-sterile soils were used as controls. Sample removal, extraction and pesticide residue estimation were carried out as described earlier ((Akbar, S., & Sultan, S. 2016).

2.5.3 Plant – microbe interaction and Mancozeb degradation

Pot experiment with potatoes (*Solanum tuberosum* L.) (Kufri Chipsona 3) were conducted in order to examine the effect of bacterial inoculation on plant growth and pesticide degradation. Tuber of *S. tuberosum* were surface sterilized by treatment with 0.1% HgCl_2 solution for 5 min followed by washing with sterilized glass distilled water. 1.5 kg soil samples were spiked with mancozeb to a concentration of 200 mg/kg. Samples were then inoculated with microbial suspension to give final concentration of 1.6×10^7 cells/g. The test was carried out in triplicate, where, as control, uninoculated spiked and non-spiked soil samples were utilized. Sterilized tubers of *S. tuberosum* were grown in the sample soils and then the soil was moistened with water. It was taken care that the ambient light and temperature reach the pots. The process was daily observed and plants were grown for 3 months. The following parameters of plant were recorded such as percentage of germination, shoot length, root length, leaf length, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight (Akbar, S., & Sultan, S. 2016).

2.5.4 Data analysis

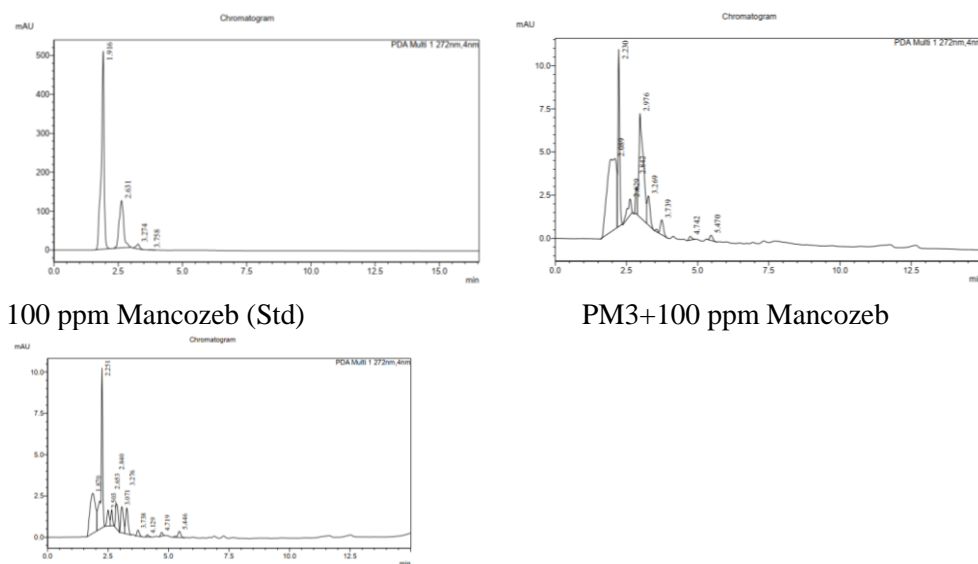
Each data represented were means of three replicates. The data were analyzed statistically by ANOVA.

3. RESULTS

3.1 Isolation and characterization of Mancozeb degrading strains

Several promising morphologically different colonies were isolated and purified from the enrichment culture. Purified isolates were grown in mineral salt medium supplemented with 100 ppm Mancozeb as a sole source of carbon. Estimation of mancozeb degradation potential by HPLC demonstrated that the isolates were able to

degrade 14 – 70% of supplemented Mancozeb within a time frame of 7 days. Maximum Mancozeb degradation was exhibited by bacterial isolates PM3 and PM10 that degrade 40% and 14% of the applied Mancozeb respectively. Based on biochemical characterization and 16S rRNA sequence analysis, these isolates were identified as *Pseudomonas sp.* and *Rhizobium sp.* The 16S rDNA sequences of strains PM3 and PM10 exhibited closest homology (%) with *Pseudomonas aeruginosa* and *Rhizobium pusense* respectively.



PM10+100 ppm Mancozeb
Figure 2 HPLC Chromatograph of Mancozeb pesticide

3.2 Bacterial growth and biodegradation of Mancozeb in liquid medium

The bacterial strains were able to utilize mancozeb as the sole source of carbon and exhibited effective growth up to a period 5 days of incubation without undergoing an initial lag phase. Increase in growth was very slow between 5th and 7th day, and thereafter the decline phase was initiated (as per HPLC graph I have to mention decrease of mancozeb concentration (Fig.2). mancozeb degradation by bacterial strains was exhibited as a decrease in mancozeb concentration that was proportional to increase in bacterial growth and a time dependent loss of Mancozeb was observed in bacterial cultures. *P. aeruginosa* PM3 degraded 97.49% of Mancozeb while *Rhizobium pusense* PM10 degraded 98.99% of mancozeb. In the control flasks, mancozeb degradation as a byproduct of abiotic losses was significant (5%) at the end of 10 days. HPLC also revealed that PM3 and PM10 were able to mineralize mancozeb.

3.3 Determination of auxiliary characteristics

The plant growth promoting activities of bacterial strains, both in the absence as well as the presence of mancozeb were determined (Table 1). The bacterial strains under study exhibited a substantial production of indole acetic acid following 24 hr. of incubation. A concentration dependent increase in production of IAA was observed: PM3 and PM10 produced 13.08 and 13.12 $\mu\text{g mL}^{-1}$ of IAA at 50 $\mu\text{g mL}^{-1}$, 17.5 and 17.8 $\mu\text{g mL}^{-1}$ and 21.3 and 21.7 $\mu\text{g mL}^{-1}$ of tryptophan respectively. Both the strains also showed phosphate solubilization activity by producing a clear zone surrounding the colony. Furthermore, bacterial strains also showed positive for ammonia production (Table 1).

Table 1 Plant growth promoting activities of mancozeb degrading strains in presence and absence of mancozeb

Name of bacterial isolates	Phosphate solubilization (zone size in mm)	Indole production (different concentration (µg/ml)	Acetic Acid (µg/ml tryptophan)	Ammonia production	Nitrate reduction	HCN production	
		50	100	150			
PM3	17 mm	13.08	17.5	21.3	++	_-ve	++++
PM10	14 mm	13.12	17.8	21.7	++++	-ve	-ve

• 3.4 Growth experiment with potato (*S. tuberosum*)

The study has been conducted for influence of bacterial presence on plant growth and pesticide using plant growth experiments. Mancozeb addition to soil affected a reduction in certain plant parameters such as % germination (75%), shoot fresh length (20cm), shoot fresh weight (39.4gm), and root fresh weight (15.4 gm), root dry weight (0.8 gm). plants grown in mancozeb supplemented soil with mancozeb degrading bacterial strains exhibited significant enhancement in growth in terms of height and weight. An increase of 23 cm and 22.5 cm in shoot length and 27 cm and 28 cm in root length was observed in case of PM3 and PM10. Leaf length was enhanced up to 12 cm. the increase in shoot weight increase was studied in terms of fresh and dry shoot weight; an increase of 43.9 gm and 57.2 gm in fresh shoot weight and 21.1 and 20.99 gram in shoot dry weight was observed in plants inoculated with PM3 and PM10 respectively. Root weight was also enhanced significantly; PM3 and PM10 enhanced root fresh weight by 18.8 gm and 18.7 gram and root dry weight 1.2 gm and 1.1 gm respectively.

Table 2 – Measurement of growth parameters of *S. tuberosum* in different microbiologically active soils supplemented with mancozeb concentration was 200 mg/kg soil.

Growth parameters	Control	Mancozeb Potato	+ Mancozeb + Potato tuber + PM3	Mancozeb + Potato tuber + PM10
% germination	75%	58.33%	100%	91.67%
Shoot length (cm)	20 cm	19 cm	23.00 cm	22.5 cm
Root length (cm)	25 cm	23.5 cm	27 cm	28 cm
Leaf area (cm ²)	311.8	378.2	397.7	392.4
Shoot fresh weight (g)	39.4	21.0	43.9	57.2
Root fresh weight (g)	35.0	30.0	18.8	18.7
Shoot dry weight (g)	19.3	18.5	21.1	20.99
Root dry weight (g)	0.8	0.76	1.2	1.1
Chlorophyll content (index)	21.63	25.6	32.6	33.8

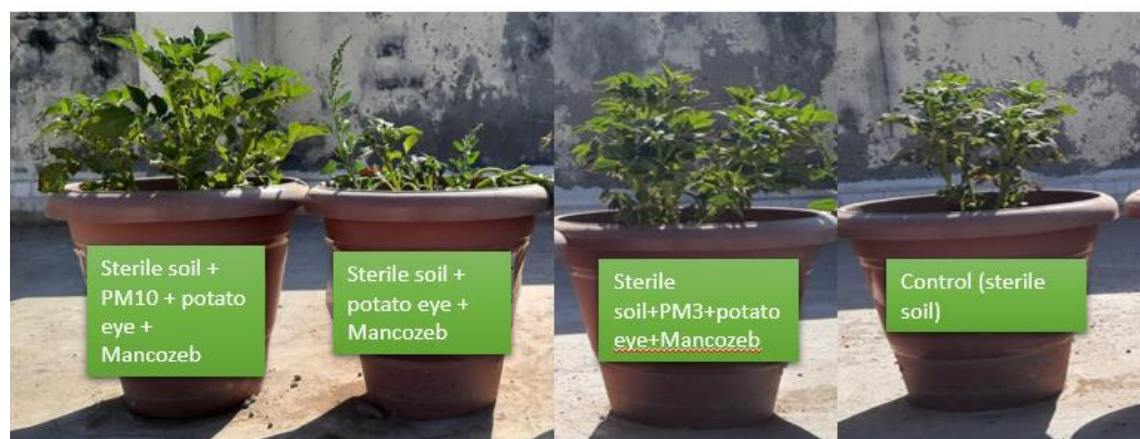


Figure 3 Pot study

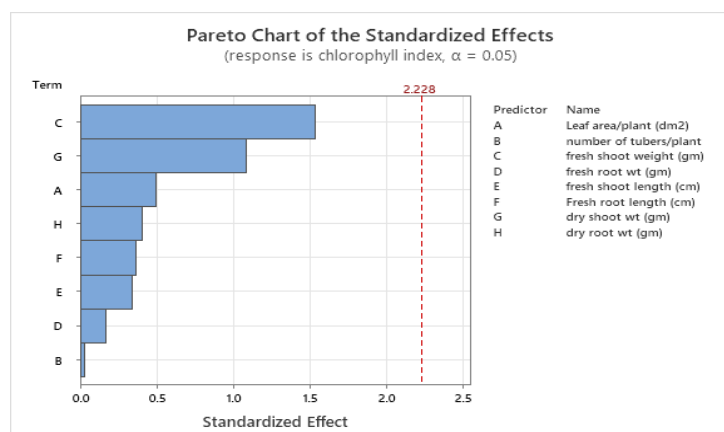


Figure 4 Pareto chart illustrating the effect of various factors on higher chlorophyll index

In the factorial design of 2^8 variables with constant pH 7.0 and temperature 30°C, the response was measured regarding chlorophyll index. Pareto plot mainly offers the relative impact of the eight variables, i.e., leaf area, number of tubers/plants, fresh shoot weight, fresh root weight, fresh root length, dry shoot weight, and dry root weight, on the chlorophyll index by showing the relative frequency of individual and cumulative impact of the input variables. The Pareto chart in Figure 4 shows that fresh root weight has the highest impact on the chlorophyll index, and a number of tubers/plants has the most negligible impact. Moreover, the remaining variables show the impact on the chlorophyll index in decreasing order.

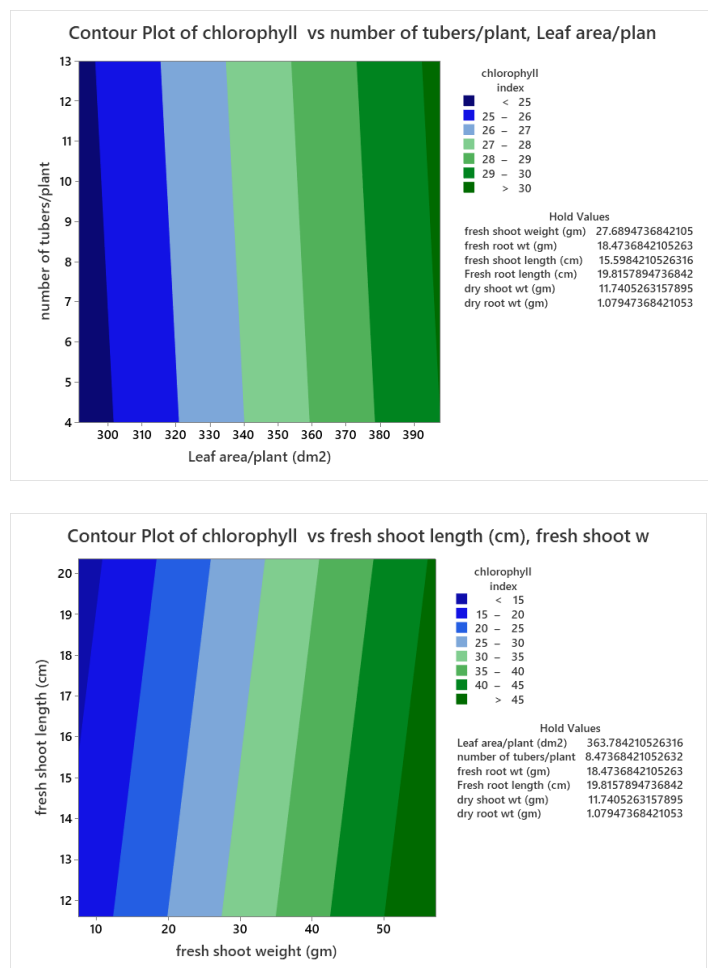


Figure 5 Contour plot of selected variables

The darker green region in the figure 5 shows higher quality of chlorophyll production. This higher response values seems to form the upper to the lower of graph. Figure showed that leaf area of the potato plant was 363.78 dm² and 8.473 was the number of tubers produced after treatment of mancozeb and selected bacterial isolates.

Regression Equation

Chlorophyll Index = 2.9 + 0.052 Leaf area/plant (dm²) + 0.03 number of tubers/plants + 0.661 fresh shoot weight (gm) – 0.058 fresh root wt (gm) – 0.46 fresh shoot length (cm) + 0.279 Fresh root length (cm) – 0.989 dry shoot wt (gm) + 2.09 dry root wt (gm)

Coefficients

Term	Coef	SE Coef	T-value	P-value	VIF
Constant	2.9	44.5	0.07	0.949	
Leaf area/plant (dm ²)	0.052	0.106	0.49	0.633	1.59
Number of tubers/plants	0.03	1.07	0.03	0.977	1.26

Fresh shoot weight (gm)	0.661	0.430	1.54	0.155	5.38
Fresh root weight (gm)	-0.058	0.352	-0.17	0.872	2.31
Fresh shoot length (cm)	-0.46	1.35	-0.34	0.741	1.97
Fresh root length (cm)	0.279	0.761	0.37	0.721	2.72
Dry shoot weight (gm)	-0.989	0.915	-1.08	0.305	6.16
Dry root weight (gm)	2.09	5.11	0.41	0.691	2.51

Analysis of Variance (ANOVA)

Source	DF	Adj SS	Adj MS	F-value	P-value
Regression	8	386.71	48.338	0.43	0.880
Leaf area/plant (dm ²)	1	27.46	27.459	0.24	0.633
Number of tubers/plants	1	0.10	0.097	0.00	0.977
Fresh shoot weight (gm)	1	267.40	267.402	2.36	0.155
Fresh root weight (gm)	1	3.11	3.107	0.03	0.872
Fresh shoot length (cm)	1	13.07	13.068	0.12	0.741
Fresh root length (cm)	1	15.26	15.259	0.13	0.721
Dry shoot weight (gm)	1	132.34	132.338	1.17	0.305
Dry root weight (gm)	1	18.90	18.898	0.17	0.691
Error	10	1131.83	113.183		
Total	18	1518.53			

Multiple Response Prediction

Variables	Setting
Leaf area/plant (dm ²)	397.7
Number of tubers/plants	13
Fresh shoot weight (gm)	57.2
Fresh root weight (gm)	2.2
Fresh shoot length (cm)	11.6
Fresh root length (cm)	28
Dry shoot weight (gm)	1.8
Dry root weight (gm)	2.9

4. DISCUSSION

Mancozeb is a very popular fungicide that is used extensively for the purpose of fungus disease control in vegetables and fruits. However, it is well known that mancozeb is toxic for mammals and can lead to contamination of soil and water resources which makes its removal from the environment an extremely urgent issue. Bioremediation is the process that utilizes the degradation potential of microbes to provide a cost effective and reliable approach for pesticide abatement. To this purpose mancozeb degrading bacterial strains were isolated from contaminated agriculture soil samples. Two promising mancozeb degrading isolates were identified were *Pseudomonas aeruginosa* PM3 and *Rhizobium pusense* PM10 which were found to degrade 97.49% and 98.99% of mancozeb (100 mg/L), respectively, within 10 days of time period.

For analyzing mancozeb degradation, an inoculum size of 1.6×10^7 cfug-1 was used. Mancozeb degradation was observed to be higher in uninoculated non-sterilized soils as compared to sterilized soil. This finding demonstrates that mancozeb degradation is mediated by soil microflora and that indigenous microbes are able

to degrade mancozeb in soil. Mancozeb degradation was high in inoculated non sterile soils as compared to inoculated sterile soils indicating the bioremediation potential of mancozeb degrading strains. An increase in mancozeb degradation was observed in non-sterile soils supplemented with 100 ppm mancozeb degrading strains *P. aeruginosa* (PM3) and *R. pusense* (PM10). The observed increase in Mancozeb utilization can be attributable to the fact that inoculating soil with degrading bacteria increases its catabolic potential. Furthermore, the capacity of the indigenous bacteria to employ the applied pesticide component most likely played a synergistic effect in the bioremediation. Previous research has shown that amplification of potential degrading bacterial strains increases the rate of pesticide depletion in contaminated soils. *Burkholderia* sp. FDS-1, for example, increased fenitrothion utilization when inoculated in FT-contaminated soil with local microbiota (Hong Q et al., 2007). Similarly, *Serratia marcescens* decomposed deltamethrin more quickly in non-sterile soils than in sterile soils (Cycoń, M. et al., 2014).

The existence of significant phosphate solubilization, indole acetic acid production, ammonia utilization by mancozeb degrading strains was found during the determination of plant growth promoting properties. According to literature *P. aeruginosa* strain, isolated from tomato plants have a multiple plant growth promoting attributes and high in vitro and in vivo inhibition of growth and pathogenicity of phytopathogens as well as it serves as a potential biocontrol agent (Ghadamgahi, F. et al., 2022). On the basis of recent study *R. pusense* strain showed all PGPR traits as well as siderophore production and enhance the growth of maize (Amezquita-Aviles, C. F et al., 2022).

In conclusion, the mancozeb degrading bacterial strain isolated from this study shown great mancozeb degrading capacity and was capable of bioremediating soil with mancozeb concentrations as high as 200 mgkh⁻¹. These bacterial isolates were discovered to not only have pesticide degrading abilities, which reduce the toxic effects of these pesticides on plants, but also to have a variety of other traits such as the production of phytohormones such as indole acetic acid, which promotes cell growth, phosphate solubilization, which aids in root uptake, and nitrogen fixation for plant uptake. Combining excellent biodegradation capability with a variety of biological characteristics. These isolates have the potential to become attractive applicants for bioremediation strategy development.

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