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Degradation of methyl parathion by a soil bacterial isolate: A pot study

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ABSTRACT

The indiscriminate use of chemicals fertilizers and pesticides has not only deteriorated the environment but also the groundwater resources as well. Their intensive consumption causes deleterious effects on plant, animal, and aquatic ecosystems thereby causing serious problems related to health. Since these are one of the factors which are responsible for the contamination of air, water, and terrestrial ecosystems and also the cause of disruption of biogeochemical cycling, it is extremely important to bring down, the toxicity levels that these chemicals impose on the environment, by the use of effective technology, which is cost-effective and safe. It has been suggested that biodegradation becomes an attractive option for the destruction of pesticides since it utilizes a natural process and offers the potential for being cost-effective as well as safe technology. Thus, the present study deals with the isolation and use indigenous bacteria for degradation of an organophosphorus pesticide methyl parathion present in the soil samples which were collected from the pesticide contaminated agricultural field.

KEY WORDS: Biodegradation, bioremediation, gas chromatography, methyl parathion

INTRODUCTION

Environmental pollution has become the most important universal concern, due to the rapid growth of urbanization, industrialization, and use of modern agricultural productivity enhancers (pesticides, fertilizers, etc.). Pesticides usages are rigorous all over to control insect pests, millions of tons of pesticides are produced and used worldwide in close association with agriculture. The continuous and ignorant use of organophosphorus insecticides such as methyl parathion (MP) and methamidophos for the control of wide range of insects throughout the world have led to the potential neurotoxicity to the animal ecosystem. Consequently, this has led to serious concerns for the development of safe and convenient strategies to deal with its widespread dispersal in the atmosphere (Singh and Walker, 2006). Several conventional techniques are used for the detoxification of such toxic compounds, namely, incineration, landfilling, excavation but due to operational difficulties, treatment expenditure, the above-mentioned methods have met serious opposition. Degradation with the help of microbial flora is generally considered to be safe and effective technique for detoxification of such chemicals in a cost-effective way. Many amazing characteristics of microbes such as their small size, ubiquitous distribution, high specificity, surface area, potentially rapid growth rate, and unrivaled enzymatic and nutritional adaptability make them, as one of the most important recycling agents of pesticides in nature. Biodegradation is nothing but a metabolic process that involves the complete breakdown of an organic compound in the context of environmental sciences; it is defined as the use of biological agents to eliminate hazardous substances from the environment (Roe *et al.*, 1998). There are two main approaches proposed for the bioremediation with microorganisms. In the first case, microorganisms can be applied directly to degrade pollutants and wastes in a reactor or *in situ*. In the second case, cell extracts or purified enzymes preparations of microbial origin could be used for decontamination purposes (Chapalamaduguru and Chaudhary, 1999). Direct application of microbes in contaminated sites for the remediation of toxic chemicals is a cheapest way of remediating the toxic compounds when conditions are favorable for the growth of microorganisms, but if the conditions are unfavorable, their growth would be very slow, and sometimes they die. In these conditions, the addition of nutrients plays a vital role in the bioremediation process that enhances the growth of microbes and ultimately the rate of biodegradation (Goldstein *et al.*,

1985). Pot studies are perhaps one of the most appropriate tests to access the potential of microbe, for degradation of pesticides including MP. Since the degradation efficiency of the test strain was analyzed previously in soil microcosm experiments, the study was taken forward from laboratory conditions to the field conditions, and thus the pot study was conducted. Indigenous bacterial strains were isolated from the soil samples which were contaminated with pesticides and screened for their potential in degrading the concerned pesticide.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples were collected from the fields which had a history of pesticide application of at least 10 years. After proper survey, the soil samples were collected from the agricultural field at, Jabalpur, Madhya Pradesh. Samples were taken randomly from a depth of 5 to 10 cm to minimize air contamination. Mandatory soil was taken by means of sterilized spatulas and collected in sterile polythene bags. The soil samples were then brought to the laboratory and maintained at 4°C. The samples were then subjected to microbiological analysis.

Isolation of MP Degrading Microbes: Enrichment Technique

Enrichment technique was used for the isolation of MP degrading strain. 1 g of soil sample was taken and added to 50 ml of minimal media (MM) which had the following composition K_2HPO_4-5.8 g/L, KH_2PO_4-4.5 g/L, $(NH_4)_2SO_4$-2 g/L, $MgCl_2$-0.16 g/L, $CaCl_2$-20 mg, $NaMoO_4$ -2 mg, $FeSO_4$-1 mg, $MnCl_2$-1 mg with 1 g glucose/L with 5 ppm MP (final concentration) and kept for incubation on a rotary shaker at 120 rpm for 24 h. After 24 h, 1 ml of sample was taken and inoculated into fresh MM with stepwise increase in MP concentration. After the 4th cycle of enrichment, 1 ml broth culture was serially diluted and plated on nutrient agar plates and kept for incubation at 37°C for 24 h. After 24 h, selected colonies were picked and patched on nutrient agar plates with 50 ppm MP. Selected colonies were transferred on series of nutrient agar plates to obtain axenic culture. Only one strain was found to be potential MP degrader and thus was selected for future studies.

Factors Affecting MP Utilization by the Test Bacterium

Effect of inoculum concentration on in vitro MP utilization

To standardize the suitable inoculum concentration for maximum degradation of MP by the test strain, cell densities, namely, 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9 cfu/ml were added to the medium and incubated for 15 days at 35°C. The degradation of MP was analyzed with the help of gas chromatography–mass spectrometry (GC-MS) (Labana *et al.,* 2005).

Effect of temperature on in vitro MP utilization

To determine the effect of temperature on MP degradation, the cells of test strain (1×10^5) was added to different flasks with 5 ppm MP and incubated at various temperatures such as 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. All flasks were incubated for 15 days. The degradation of MP was analyzed with the help of GC-MS at various time intervals (Labana *et al.,* 2005).

Effect of hydrogen ion concentration on in vitro MP utilization

To determine the optimum pH, MM medium had pH values (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10) supplemented with 5 ppm MP inoculated with cells of test strain (1×10^5) and incubated for 15 days. Degradation of MP was analyzed with the help of GC-MS at various time intervals (Labana, 2005).

Effect of carbon source on in vitro MP utilization

To select the best source of carbon, the carbon present in glucose was replaced by various sources of carbon (cellulose, raffinose, xylose, glucose sucrose) in MM, inoculated with cells of test strain (1×10^5) along with 5 ppm MP. Inoculated flasks were incubated for 15 days at 35°C. Samples were withdrawn at various time intervals and analyzed for degradation of MP with the help of GC-MS (Labana, 2005).

Effect of nitrogen source on in vitro MP utilization

To select the best source of nitrogen, the amount of nitrogen present in $(NH_4)_2SO_2$ was replaced with different sources of nitrogen ($NaNO_3$, $NaNO_2$, KNO_3, NH_4CL, NH_4CO_3) in MM and inoculated with cells of test strain (1×10^5) along with 5 ppm MP. Inoculated flasks were incubated for 15 days at 35°C. Samples were withdrawn at various time intervals and analyzed for degradation of MP with the help of GC-MS (Labana, 2005).

Effect of incubation period on in vitro MP utilization

To determine the effect of incubation period on degradation of MP under optimize condition of temperature and pH, the flask containing MM and cells of test strain (1×10^5) along with 5 ppm MP, were incubated at different time intervals. Samples were withdrawn at various time intervals and analyzed for degradation of MP with the help of GC-MS (Labana and Pandey, 2005; Kaiser, 1996; Richard *et al.*, 2006).

Degradation of MP in Soil: A Pot Study

The experimental setup was as follows: A completely randomized block was designed. Pots measuring 40 cm in length, top and bottom diameters of 40 cm and 15.2 cm, respectively, were filled with 3 kg of soil to which MP was added exogenously. For this, air-dried soil was sieved (2 mm) and aqueous solution of MP was added to the soil to form thick slurry. The final concentration of MP was 60 ppm. Cells of test strain were inoculated in the soil to obtain 1×10^5 cfu/g of soil. Simultaneously, the controls without the microbe were also maintained. The nitrogen and phosphorous sources were applied 1 week after the treatment of MP. The slurry was air dried at 40-45°C for 4 days, and the dried soil was pulverized before being used in the pots. These pots were then placed randomly in natural environmental condition. The moisture level in all the pots (control + experimental) was maintained throughout the period of an experiment by sprinkling tap water periodically whenever necessary. The pots were maintained at room temperature for 30 days (Labana *et al.,* 2005).

Extraction

1 g soil was withdrawn at various time intervals (0, 5, 10, 15, 20, 25, 30 days). 1 g soil was suspended in 10 ml of 5% NaOH, vortexed for 10 min and centrifuged at 1500 rpm for 10 min. Supernatant was acidified to pH 2.0 with HCL and extracted with double the volume of ethyl acetate. The aqueous phase was extracted again with ethyl acetate; both the extracts were pooled together and passed through anhydrous sodium sulfate to remove the traces of water. The filtrate was dried at room temperature and the residue was finally dissolved in 1 ml of acetone and analyzed through GC-MS (Labana *et al.,* 2005).

RESULTS AND DISCUSSION

A total 10 strains were isolated from the soil samples supplemented with MP. Out of these, six strains, namely, C1, C2, C3, C6, C8, and C10 which appeared in higher concentration were further screened for their ability to tolerate maximum concentration of MP. Thus, C1 was most potent strain, finally selected for further evaluation. Thus, on the basis of detailed biochemical test and 16s sequencing carried out with the help of a private company named "EUROFINS" Bengaluru, India, the bacterial isolate was identified as *Achromobacter xylosoxidans*.

Optimization of physiochemical conditions for maximizing the rate of MP degradation has given a considerable insight into the regulatory aspect of biodegradation. The ability of various bacteria for degradation of MP under different physiochemical conditions is not a general property but differs greatly under different growth conditions (Hanne *et al.*, 1993; Kulkarni and Chaudhari, 2006). It also varies significantly not only at generic levels but also at strains level (Rani and Lalithakumari, 1994; Silva *et al.*, 1999; Singh *et al.*, 2003; Jain *et al.*, 2005; Whitfield, 2005; Karpouzas and Singh, 2006; Ghosh *et al.*, 2010; Abdel-Rahman *et al.*, 2011). Thus, it cannot be generalized for all the bacterial strains and requires extensive standardization. The present study was conducted to determine the optimum conditions for rapid biodegradation of MP. It is evident from the data recorded in Figure 1 that the rate of degradation varied significantly with various concentrations of inoculum (1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9). Maximum degradation was recorded in case of *A. xylosoxidans* when applied at 1×10^5 cfu/ml. It was observed that the maximum percentage of pesticides degradation after 15 days was 89.23%. It is evident from the data recorded in Figure 2 that the rate of MP degradation varied significantly with temperature. It was observed that degradation was gradually increased with temperature and reached its maximal at 35°C ± 2°C as evident by maximum MP degradation, but beyond this temperature, degradation was gradually declined. It was also observed that the isolated strain *A. xylosoxidans* #C1 is a thermo-tolerant organism which was able to degrade MP at 35-50°C ± 2. Several other investigators have also reported similar observations with the bacterial

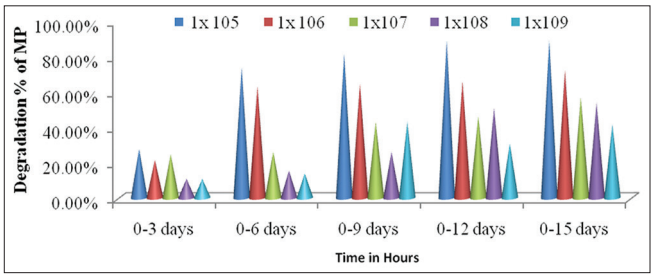

Figure 1: Effect of inoculum concentration on *in vitro* methyl parathion utilization by the test rain

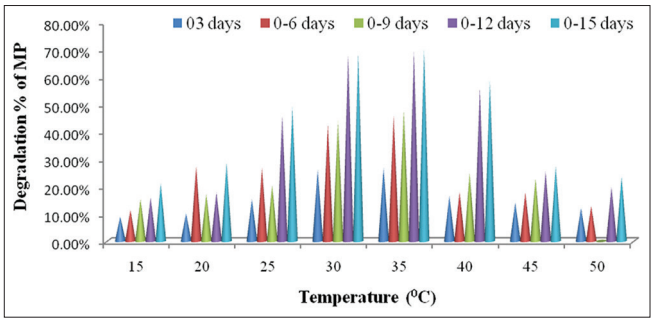

Figure 2: Effect of temperature on *in vitro* methyl parathion utilization by the test strain

strains studied by them (Singh and Walker, 2006; Tognetti *et al.*, 2007). It is clear from the data recorded in Figure 3 that the pH 6.5 supported maximum degradation by the test strain after all days of incubation (9-15). However, it was observed that 7.5 and 8.0 pH supported higher degradation at the initial stage which failed to support at a later stage. Similarly, the data recorded in Figure 4, shows that different types of carbon sources had a significant effect on the MP degradation by *A. xylosoxidans* #C1. Better MP degradation was recorded in medium supplemented with glucose (60.50%) after more or less at all the incubation. It was maximum after 15 days of incubation. It was followed by sucrose. Raffinose also supported the degradation of MP up to some extent. Several earlier workers have also reported glucose as one of the best carbon sources for rapid degradation of MP (Whitfield, 2005; Sánchez-Barragán *et al.*, 2007). Data recorded Figure 5 clearly indicates that different types of N sources had a significant effect on degradation of MP by the test strain. Maximum MP degradation was recorded in NH_4Cl after all the incubation and increased gradually. KNO_3 and $NaNO_2$ which supported MP utilization after 3 and 6 days of utilization respectively failed to support a later stage, i.e., 9-15 days of incubation where NH_4CO_3 induced better utilization of MP by the test strain. Similar observations have also reported by other investigators (Yadav *et al.*, 2003; Sánchez-Barragán, 2007; Gavrilescu, 2005; Hussain *et al.*, 2009; Díaz, 2004; Dua *et al.*, 2002; Jokanovic and Prostran, 2009). Data recorded in Figure 6, clearly indicates biodegradation of MP varied significantly with the incubation period. It was gradually increased with incubation period and reached its maximal after 27 days of incubation. The rate of degradation was declined after 27 days of incubation. Therefore, on the basis of above discussion, it may be concluded the 35°C, 6.5 pH and 27 days of incubation are the best suitable condition for rapid degradation of MP by the test strain. After optimizing the growth conditions for maximum degradation of MP, pot studies were initiated. Pot studies are perhaps one of the most appropriate tests to access the potential of microbe for degradation of pesticides including MP. The pots were filled with 3 kg soil per pot with 60 ppm MP. Samples were withdrawn at various time intervals. After the extraction procedure, the samples were analyzed with the help of GC-MS. It is clear from the data recorded in Figures 7 and 8 and in Table 1 that the test bacterium (C1) was able to degrade the target

Table 1: Degradation % of MP in soil (0-30 days) by *A. xylosoxidans* #C1

Incubation in days	Amount detected in sample (ppm)	% degradation
0-5	0.016	11.11
0-10	0.010	44.44
0-15	0.007	61.11
0-20	0.004	77.77
0-25	0.002	88.88
0-30	0.001	94.44
SD	0.0056	31.3592
SEM	0.0025	14.0242

SD: Standard deviation, SEM: Standard error of mean, MP: Methyl parathion, *A. xylosoxidans: Achromobacter xylosoxidans*

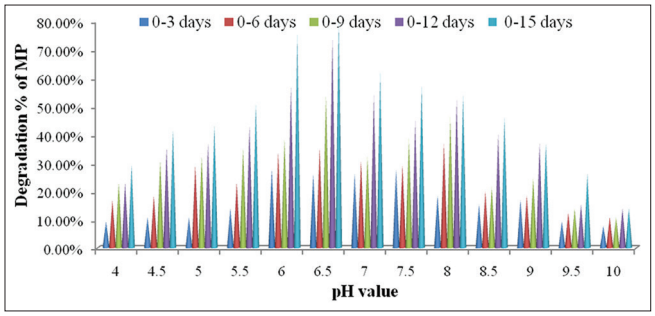

Figure 3: Effect of hydrogen ion concentration on *in vitro* methyl parathion utilization by the test strain

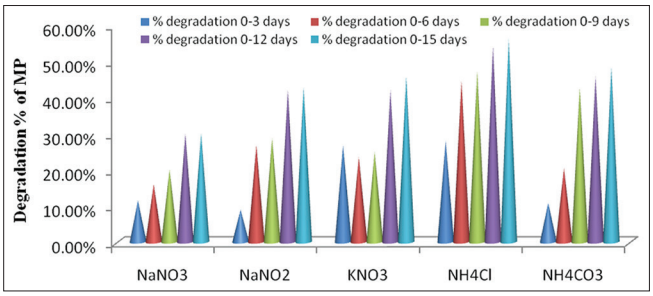

Figure 5: Effect of nitrogen sources on *in vitro* methyl parathion utilization by the test strain

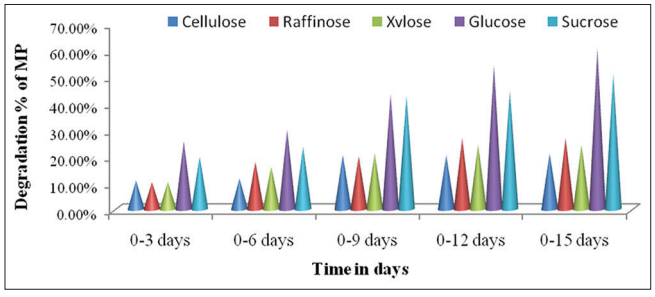

Figure 4: Effect of carbon sources on *in vitro* methyl parathion utilization by the test strain

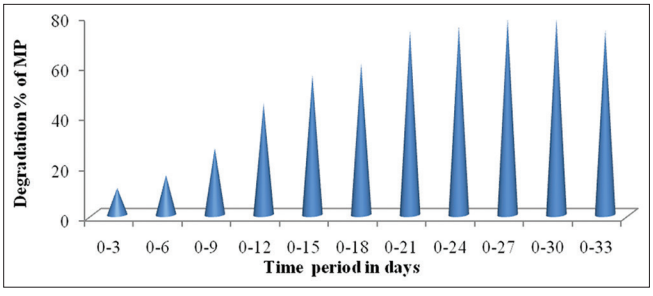

Figure 6: Effect of incubation period on *in vitro* methyl parathion utilization by test strain

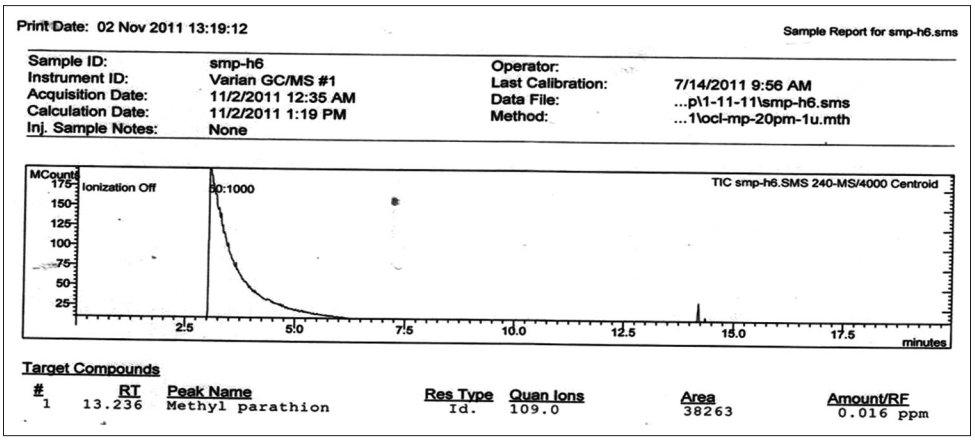

Figure 7: Detection of methyl parathion (ppm) on the 5th day of incubation in soil (pot study)

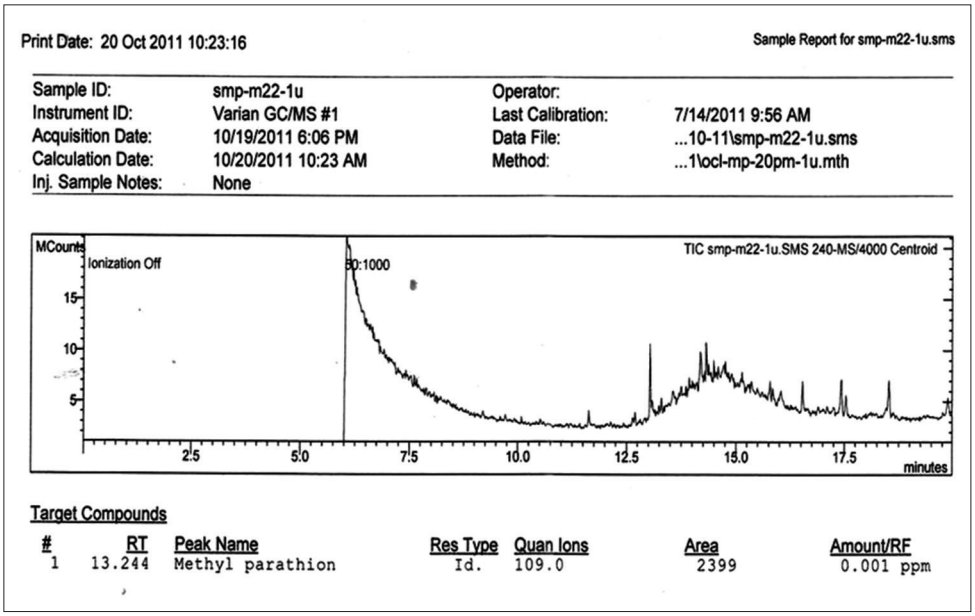

Figure 8: Detection of methyl parathion (ppm) on the 30th day of incubation in soil (pot study)

pesticide up to 94.44% after 30 days of incubation. Similar results were also obtained by Petersen *et al.* (1998), Sharma *et al.* (2003), Gopinath and Tarnjis (2008). Labana *et al*. (2005) have reported pot, and field studies on bioremediation of p-nitrophenol (PNP) contaminated soil using *Arthrobacter protophormiae* RKJ100. Bioremediation of PNP was first carried out in pots using immobilized and free cells of the RKJ100 to ascertain the role of a suitable carrier material. They reported that the strain enhanced degradation on immobilization and the rate of PNP depletion decreased with increasing depth of soil. PNP was totally depleted in 5 days by immobilized cells whereas free cells were able to deplete 75% of PNP in the same period. Pakala *et al.* (2006) reported biodegradation of MP and PNP by a gram negative *Serratia* sp. strain DS001. The bacteria were capable of utilizing MP as a carbon source. They reported for the first time the existence of PNP hydroxylase component "A" typically found in gram-positive bacteria, in a gram-negative strain of the genus *Serratia* sp. Liu *et al.* (2007) reported biodegradation of MP by *Acinotobacter radioresistens* USTB-04. An initial concentration of 130 mg/L was completely biodegraded in 2 h in the presence of cell-free extract with a protein concentration of 148.0 mg/L which was increased with the increase of pH from 5 to 8. Shen *et al.* (2010) reported degradation of MP by a *Stenotrophomonas* sp. SMSP-1. This strain could hydrolyze MP to PNP. Naqvi *et al.* (2011) reported similar observation in case of biodegradation of carbaryl in soil. Greeshma and Vasudevan (2013) reported the toxicity that these chemicals impose on the environment and the bioremediation of pesticides in agricultural soil. Hassan and Ahmed (2014) reported that different factors such as pesticide structure, pesticide concentration, soil types, soil moisture, pH, temperature,

salinity, organic matter, microbial flora, and microbial biomass have a significant effect on the degradative capacity of an organism. Kavita and Geeta (2014) reported the bioremediation and biodegradation of pesticide from contaminated soil and water. Similarly, Mahuddin and Fakhruddin (2014) reported degradation of diazinon - An organo-phosphorous pesticide by a bacterial isolate. Verma and Jaiswal (2014) reported the pesticide consequence and their microbial degradation in soil.

Thus, the present study is a model study that could be used for decontamination of sites contaminated with MP.

CONCLUSION

1. A total 10 strains were isolated from the soil samples supplemented with MP. Out of these, six strains, namely, C1, C2, C3, C6, C8, and C10 which appeared in higher concentration were further screened for their ability to tolerate maximum concentration of MP. Thus, C1 was most potent strain, finally selected for further evaluation
2. Different factors were optimized for maximum degradation of MP by the test strain (C1), such as inoculum concentration, temperature, pH, different carbon sources, different nitrogen sources, and incubation period. It was recorded that maximum degradation was recorded when cells of bacterium C1 were applied at 1×10^5 cfu/ml, at temperature 35°C, at 6.5 pH.
3. It was recorded that utilization of MP was maximum when glucose was used as a carbon source and when NH_4Cl was used as a nitrogen source.
4. It was seen that better degradation of MP was reported after 27 days of incubation.
5. When pot studies were conducted it was reported that, the test bacterium was able to degrade the target pesticide up to 94.44% after 30 days of incubation.

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