ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 2/2023
325–334
https://doi.org/10.18388/abp.2020_6563

Regular paper

Isolation and characterization of indigenous bacterial assemblage for biodegradation of persistent herbicides in the soil

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Abstract: Extensive pesticides (herbicides) use is negatively disturbing the environment and humans. Pesticide bioremediation with eco-friendly techniques bears prime importance. This study aimed to isolate and characterize three different herbicides (metribuzin, clodinafop- propargyl, MCPA (2-methyl, 4 chlorophenoxyacetic acids) and Bromoxynil) degrading bacterial strains from agricultural fields of Punjab University, Pakistan. Among the 12 bacterial isolates, 5 were metribuzin degrading, 3 were clodinafop propargyl degrading and, 4 were MCPA and Bromoxynil degrading bacteria. Morphological, microscopic, and molecular characterization revealed that the majority of these bacterial strains were gram-negative and belonged to *Bacillus* and *Pseudomonas* genera. The isolates A6, B3, and C1 were subjected to respective herbicide degradation and the data was confirmed through GC-MS analysis. The effect of herbicide concentrations, pH, and temperature on bacterial growth was determined at OD_{600}. The strain A6 degraded 14.8% metribuzin out of the provided concentration of 50 ppm by following the deamination pathway. While the isolates B3 and C1 degraded 23.2% and 33.9% clodinafop, MCPA and bromoxynil, respectively, at a spiking concentration of 50ppm. The clodinafop, MCPA & Bromoxynil were metabolized into less toxic products i.e., dicarboxylic acids and 2-methyl phenol respectively, and metabolized *via* decarboxylation and dehalogenation mechanism. The present study evaluates the herbicides degrading bacterial strains that could potentially be used for bioremediation of agricultural contaminated sites.

Keywords: *Bacillus*, Bioremediation, GC-MS analysis, herbicides, Pseudomonas

Received: 28 November, 2022; revised: 01 February, 2023; accepted: 28 February, 2023; available on-line: 31 May, 2023

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Acknowledgements of Financia Support: The researchers would like to acknowledge Deanship of Scientific Research, Taif University for funding this work.
Abbreviations: GCMS, Gas Chromatography Mass Spectrometry, MCPA, 2-methyl, 4 chlorophenoxyacetic acids

INTRODUCTION

Different varieties of chemicals used to repel or destroy pests including animals, insects, plants, bacteria, fungi, or other microbes; that are harmful to cultivated crops and animals are known as Pesticides (Alam *et al.,* 2018; Nawab *et al.,* 2015). They are classified according to their chemical formulation and targeted organism, but the latter is more common. Based on target organisms, these are grouped as insecticide, weedicide and fungicide, rodenticide, bactericide, etc. (Richardson *et al.,* 2019).

Pakistan is an agricultural country, and its economy greatly depends upon this sector in terms of labor participation; provision of food to the whole nation, and a primary source of foreign exchange earnings (Khan *et al.,* 2021). The share of agriculture in Gross Domestic Product (GDP) is 19.3% for the FY2020and it is gradually shrinking in the last few decades. Different challenges like global warming, insect/pest attack, and water shortage hinder the overall potential of this sector (GOP 2020; Koondhar *et al.,* 2021). Weeds are unwanted plants that are not grown intentionally at a place and negatively impede human activities. Almost, there are 250 000 species of plants in the world; out of which approximately 8 000 species i.e., 3% are considered weeds. Weeds are problematic because of their rapid growth, long-term survival, and competition with normal plant growth for sunlight, air, water, space, and soil minerals (Storkey *et al.,* 2021).

To control the growth of weeds the most extensively used form of pesticide is herbicide. Herbicides are a chemical used to eradicate or kill unwanted vegetation (weeds) that interferes with normal plant growth and restrain the overall yield of several crops (Clapp 2021; Okieimen *et al.,* 2020). Although the global use of pesticides ensures high production yield, on the other hand, it also produces high levels of environmental contamination because of their excessive use (Bakshi *et al.,* 2020). These deadly pollutants are directly exposing the trophic food web and enter into the ecosystem either by direct application, spillage, and disposal (Khan *et al.,* 2020; Lone *et al.,* 2014).

Physicochemical methods are mostly used as an alternative to chemical pesticides, which are costly because these techniques require the excavation of polluted soil

from the site of contamination and transferring it to another place for treatment. As pesticides can accumulate in food and water supplies, it is need of the time to design environmentally friendly and cost-effective technologies to replace hazardous pesticides.

Enormous biological techniques have been designed in which toxic organic pollutants are degraded by microbes (Bakshi *et al.,* 2020; Oladipo *et al.,* 2020). Bioremediation is one such technique in which naturally occurring microbes such as bacteria and fungi are used for the breakdown and removal of pollutants (Huang *et al.,* 2018). This method is relatively cheaper in comparison to physicochemical methods. Without excavating the material from the contaminated site, they have the potential to treat the polluted soil and groundwater because of this reason it requires very less energy input and preserves the soil structure which otherwise can either be disturbed by excavation (Pertile *et al.,* 2020). The present study is to focus on environment-friendly techniques for the minimization and elimination of persistent herbicide levels via biodegradation by using locally isolated bacterial strains.

MATERIALS AND METHODS

Soil Sampling

Total 9 soil samples were randomly collected at the depth of 0–12 inches from wheat fields of Punjab University located near the fishponds; where the soil had a previous history of clodinafop and MCPA & Bromoxynil applications but had never been treated with metribuzin over the last few years. The samples were taken to the laboratory, air dried at room temperature, sieved through 2 mm mesh size to remove stones and debris, and mixed thoroughly to make a composite soil sample and stored at 4°C.

Isolation and Screening of Bacterial Isolates

Four grams of soil samples were placed in 250 mL Erlenmeyer flasks containing 100 mL of mineral salt medium (Glucose 5, KH_2PO_4 0.5, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.2, NaCl 0.2 g/L). The stock solutions of three herbicides (metribuzin, clodinafop, and MCPA & Bromoxynil) were prepared in distilled water. Then, 100 µL of each respective herbicide stock solution providing the final concentration of 5ppm was inoculated into the above sample medium. The samples were incubated for one week in the dark at 30°C and 160 rpm shaking. Afterwards, 10 mL of each culture medium was transferred into 90 mL of fresh mineral salt medium fortified with each of the herbicides at 10, 20, and 50 ppm by adding 200 µL, 400 µL and 1 mL of each herbicide stock solution, respectively. The samples were again incubated for 7 days at 30°C at 160 rpm in dark. After 7 days, 200 µL from each subculture was spread on L.B plates fortified with each respective herbicide at a concentration of 20 ppm. The plates were incubated for 4 days at 30°C and different bacterial colonies were observed after 4 days.

Purification of Bacterial Colonies

Morphologically different bacterial colonies were picked and streaked on L.B plates aseptically and incubated at 30°C for 24 hours to obtain pure bacterial colonies. The purified colonies were maintained on L.B slants and stored at 40°C for further use.

Identification of Bacterial Isolates

The isolated bacterial strains were identified by performing morphological analysis, gram staining technique, and 16S rRNA gene sequencing analysis.

Morphological analysis

Bacterial isolates were morphologically analyzed based on color, shape, texture, and size. Morphological analysis was carried out visually.

Gram staining

Bacterial isolates were analyzed microscopically by gram staining technique and their cell shape, size, and arrangement were determined.

Molecular identification of herbicides degrading bacterial strains

Extraction of bacterial genomic DNA. Almost 2–3 bacterial colonies from freshly grown cultures were scratched and mixed well in DNase or RNase-free water or elution buffer in sterilized Eppendorf tubes. Thermo fFisher Scientific catalogue no AM9923. The bacterial suspension was provided heat shock in boiling water for about 10 min. The tubes were then ice-cooled and centrifuged at 12 000 rpm for 10 minutes. The supernatant containing the template DNA was taken carefully; its quality was observed by electrophoresis ion a 1% agarose gel at 100 V and visualized under a UV-light illuminator. (Daihan Scientific Model no. Wuv-L50).

Amplification of 16S rRNA Gene and Sequencing reaction. 1.5 kb DNA fragment of 16S rRNA gene was amplified using the following set of ribotyping universal primers: forward primer 27F (5'-AGAGTTT-GATCCTGGCTCAG-3') and reverse primer 1522R (5'-AAGGAGGTGATCCA(AG)CCGCA-3') (Hasnain *et al.,* 1994; Johnson *et al.,* 1994). Reaction was carried out in 20 µL containing template DNA 5 µL, 10XPCR buffer 2 µL, 25 mM MgCl2 2 µL, 2.5 mM dNTPs 2 µL, Primer-forward 2 µL, Primer-reverse 2 µL, 5 units/µL 1 µL and ddH_2O (nuclease free) 4 µL. The template DNA was initially denatured at94°Cfor 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing of primers at 54°C for 30 seconds, and elongation at 72°C for 1 minute and 40 seconds. The PCR reaction was finally extended for 10 minutes at 72°C. The PCR was then separated on 1% agarose gel observed under a UV light illuminator. Required DNA bands were precisely cut with a sterilized surgical blade and purified from gel and sent to Korea for sequencing analysis. The sequencing analysis was performed by the dideoxy chain termination method (Sanger *et al.,* 1977).

Biodegradation of Herbicides by Soil Bacteria

Preparation of samples for GC-MS

L.B. broth (250 mL) was taken in each 1 litter flask and amended with each of the herbicide 50 ppm by adding 2 mL of each respective herbicide stock solution. The herbicides (metribuzin, clodinafop, and Bromoxynil+MCPA) fortified media were then inoculated with bacterial isolates A6, B3, and C1, respectively, aseptically and incubated for 4 days at 30°C and 160 rpm in dark. The controls contained the same herbicide concentration without bacterial culture. After 4 days, 50 mL of each sample was transferred in sterilized 50 mL falcon tubes. Samples were centrifuged at 12 000 rpm for 10 minutes. The respective supernatants were collected carefully, transferred into new sterile falcon

tubes, labeled, and underwent the liquid-liquid extraction technique. For this 50 mL of each sample was placed in a glass separating funnel with equal volume (50 mL) of dichloromethane. The samples with dichloromethane were vortexed well to thoroughly mix both solvents and allowed for separation until the two distinct layers were formed. The components moved from the media phase to the dichloromethane layer. The upper layer consisting of the medium was discarded and the lower dichloromethane layer containing the components was collected from each sample carefully without any mixing with the above layer. Dichloromethane was evaporated and the final volume of each sample was adjusted with n-hexane. Final samples were then transferred in screw cap glass sterile vials and stored at 40°C until preceded for gas chromatography-mass spectrometry (GC-MS) analysis. Statistical analysis was performed to determine Pearson's correlation test between herbicide response factors and the peak areas of herbicide residues in the sample by using SPSS 16.0.

Effect of different parameters on herbicides degradation and bacterial growth

Effect of herbicides concentrations on herbicides degradation. L.B. broth (50 mL) was taken in each of the 250 mL flasks amended with different concentrations (20, 50, 80, and 100 ppm) of each of the three herbicides and inoculated with respective bacterial isolates A6, B3, and C1 under aseptic conditions. The flasks were incubated at 300°C and 160 rpm in a shaker incubator. After every 24, 48, 72, and 96 hours, the OD_{600} of each treatment was measured with a mass spectrophotometer and biodegradation in terms of bacterial growth (OD_{600}) was noted. It is recommended to monitor the bacterial growth at 600 nm.

Effect of pH on herbicides degradation. L.B. broth (50 mL) was taken in each of the 250 mL flasks with pH values maintained at 5.0, 6.0, 7.0, and 8.0 and fortified with herbicides at a concentration of 20 ppm. The media were inoculated with respective isolates A6, B3, and C1 and incubated in dark at 300°C and 160 rpm in a shaker incubator. The OD_{600} was measured every 24, 48, 72, and 96 hours with a mass spectrophotometer.

Effect of temperature on herbicides degradation. L.B. broth (50 mL) was taken in each of 250 mL Erlenmeyer flasks with each of the three herbicides at a concentration of 20 ppm and inoculated with respective bacterial isolates. These culture media were incubated at different temperatures i.e., 30, 35, and 400°C and 160 rpm in a shaker incubator. The OD_{600} was measured every 24, 48, 72, and 96 hours and the biodegradation in terms of bacterial growth (OD_{600}) was noted.

Statistical Analysis

Statistical analysis was performed to determine the effect of different parameters on herbicide degradation with the help of a three-way Analysis of Variance (ANOVA) by using SPSS 16.0 (Wahla *et al.,* 2019; Pacciani *et al.,* 2020).

RESULTS

Isolation and Screening of Herbicides Degrading Bacteria

Bacteria were isolated by inoculating an aliquot of soil sample into the mineral salt medium amended with three different herbicides and incubated for 7 days at 30°C and 160 rpm in dark. 10mL of this cell suspension was transferred into a fresh mineral salt medium containing each of the three herbicides at 10, 20 and 50 ppm and incubated again under the same conditions as above. Morphologically different bacterial colonies were purified and stored at 4°C. A total of 14 herbicides degrading bacterial colonies were isolated from soil samples. Among these, 6 colonies (A1–A6) were found effective in degrading metribuzin; 3 colonies (B1–B3) degraded clodinafop and 5 of these bacterial colonies (C1–C6) metabolized MCPA & Bromoxynil as shown in Fig. 1 (A–C).

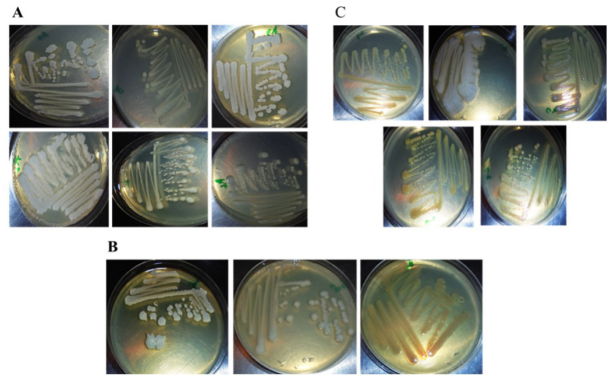

Figure 1. Morphological characteristics herbicide degrading bacteria (A, Metribuzin; B, Clodinafop; C, Bromoxynil)

Identification of Herbicides Degrading Bacteria

Morphological characteristics of bacterial colonies

The morphological characters (color, shape and texture) of herbicide-degrading bacteria were observed visually. The bacterial isolates were also gram stained and their properties were observed under a light microscope.

Molecular characterization of herbicides degrading bacteria

The 16SrRNA gene of 12 herbicides degrading bacterial strains was amplified by PCR. The amplified products of the 1.5 kb fragment were purified and sequenced for further conformation (Fig. 2).

Determination of Herbicides Degradation by GC-MS Analysis

Herbicides degrading bacterial isolates were inoculated in 250 mL LB broth supplemented with 50 ppm of each herbicide to assess their biodegradation potetial. Af-

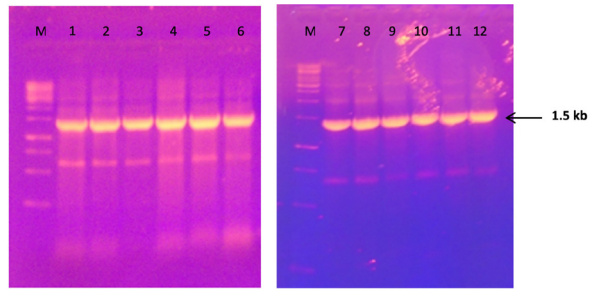

Figure 2. Amplification of 16S rRNA gene of bacterial strains. (M, DNA Marker; 10 kb 1–5 (Metribuzin degrading strains), 6–8 (Clodinafop degrading strains), 9–12, (MCPA & Bromoxynil degrading strains)

Table 1. Percentage degradation of experimental herbicides through peak areas and response factor

Sample name	Peak area of a sample	Response factor of standard	Degradation %
Metribuzin	148519263	3482815.62	14.8%
Clodinafop propargyl	5753002	149808.32	23.2%
MCPA & Bromoxynil	20651611	624497.74	33.9%

Pearson's correlation at α=0.01(2-tailed test) was used to identify the relationship between the response factor and % degradation for studied herbicides.

Table 2. Pearson correlations through SPSS

		Response factor	Degradation %
response factor	Pearson Correlation	1	–.748**
	Sig. (2-tailed)		.462
	N	3	3
% degradation	Pearson Correlation	-.748**	1
	Sig. (2-tailed)	.462	
	N	3	3

**Correlation is significant at the 0.01 level of significance (2-tailed)

Table 3. Variation in metribuzin concentration and optical density as a result of herbicide degradation by bacterial strain A6

Time period	Metribuzin concentration			
	20 ppm	50 ppm	80 ppm	100 ppm
Day 1	0.882	0.813	0.885	0.818
Day 2	1.804	1.78	1.77	1.705
Day 3	1.722	1.774	1.716	1.753
Day 4	1.712	1.701	1.708	1.675

Table 4. Variation in Clodinafop concentration and optical density as a result of herbicide degradation by bacterium B3

Time period	Clodinafop propargyl concentration			
	20 ppm	50 ppm	80 ppm	100 ppm
Day 1	0.405	0.447	0.491	0.147
Day 2	1.708	1.69	1.665	1.705
Day 3	1.726	1.792	1.767	1.806
Day 4	1.667	1.635	1.71	1.725

ter four days, the bacterial samples were further analyzed through gas chromatography-mass spectroscopy (GC-MS) to study possible degradative products after herbicide degradation. The results showed that metribuzin was metabolized into deaminated metribuzin by *Bacillus* sp. A6. Clodinafop propargyl, MCPA, and Bromoxynil degraded into corresponding dicarboxylic acid and 2-methyl phenol by strain and C1, respectively. Pearson correlation between different herbicide response factors and the peak areas of herbicide residues in each sample was identified using SPSS 16.0 (Table 1 and 2).

Table 2 shows the resulting matrix of Pearson's correlation test which revealed that the co-efficient r is equal to –0.748 which is statistically significant at $p<0.01$. It confirmed a strong negative correlation between the response factor and percentage degradation of all herbicides. The more the response factor; the less will be the percentage degradation.

Effect of Different Parameters on Biodegradation of Herbicide and Bacterial Growth

Effect of herbicides concentration

The herbicide biodegradation at different concentrations (20, 50, 80, and 100 ppm) was determined by measuring the optical density (OD) at 600nm (Table 3, 4, 5 and Figs 3, 4, and 5).

The metribuzin-degrading bacterium A6 showed the highest growth rate on the second and third days (48–72 h) at concentrations of 20 ppm and 50 ppm. The isolate was able to degrade metribuzin at concentrations of 20 and 50 ppm. Strain A6 showed a moderate growth pattern at (80–100 ppm) concentrations of herbicide. Whereas clodinafop degrading bacteria B3 showed almost similar growth rates at all the concentrations but the maximum clodinafop propargyl biodegradation was observed on 2nd and 3rd day with peak metabolism at the concentrations ranging from 50–100 ppm on the 3rd day. The MCPA & Bromoxynil degradation behav-

Table 5. Variation in MCPA & Bromoxynil concentration and optical density as a result of herbicide degradation by bacterial isolate C1

Time period	MCPA & Bromoxynil concentration			
	20 ppm	50 ppm	80 ppm	100 ppm
Day 1	0.902	0.81	0.757	0.842
Day 2	1.286	1.093	1.259	1.11
Day 3	1.137	1.473	1.52	1.393
Day 4	1.348	1.728	1.378	1.305

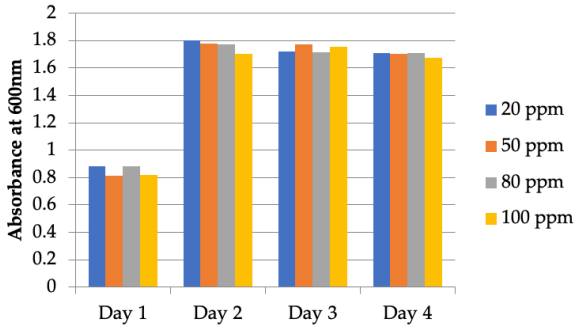

Figure 3. Growth rates of metribuzin-degrading bacteria at varying herbicide concentrations

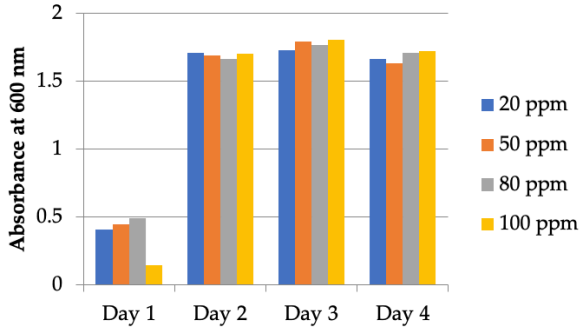

Figure 4. Growth rates of Clodinafop degrading bacteria at varying herbicide concentrations

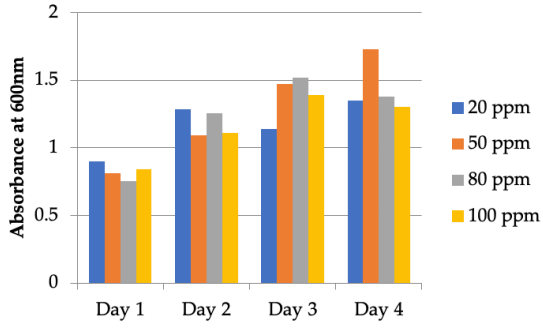

Figure 5. Growth rates of MCPA & Bromoxynil degrading bacteria at varying herbicide concentrations

ior by strain C1 was a little bit different than the two herbicides i.e., the maximum degradation of MCPA & Bromoxynil was noticed on the fourth day (96 hours) at 50 ppm.

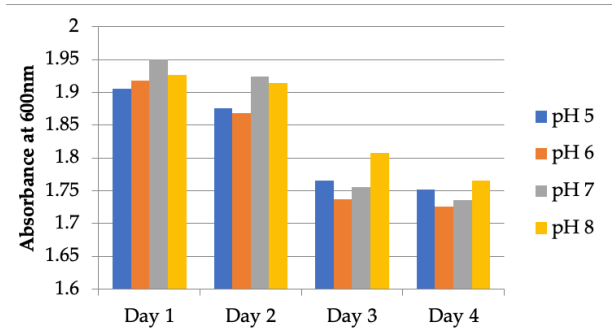

Figure 6. Growth rates of Metribuzin degrading bacteria at varying pH values

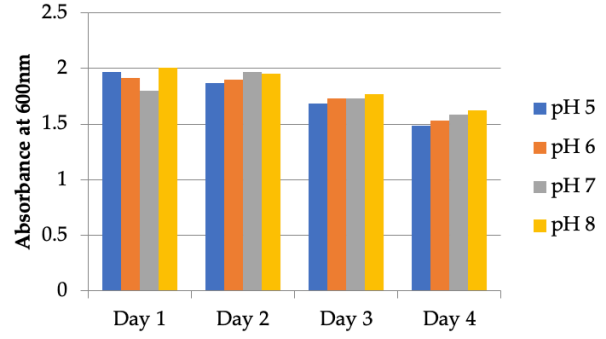

Figure 7. Growth rates of Clodinafop degrading bacteria at varying pH values

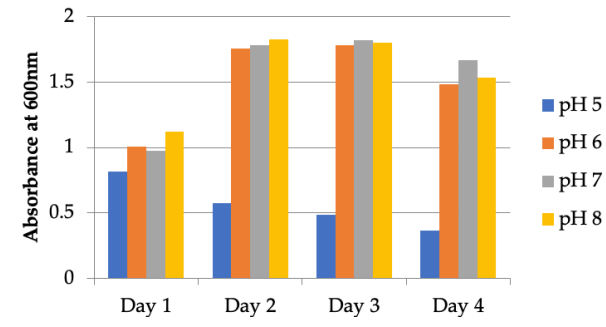

Figure 8. Growth rates of MCPA & Bromoxynil degrading bacteria at varying pH values

Effect of pH

The biodegradation of herbicides at different pH levels (5.0, 6.0, 7.0, and 8.0) was also studied (Tables 6, 7, 8 and Figs 6, 7, and 8). Results revealed that maximum degradation activities in terms of bacterial growth were

Table 6. Variation in pH and optical density as a result of metribuzin herbicide degradation by bacteria A6

Time period	pH			
	5.0	6.0	7.0	8.0
Day 1	1.905	1.918	1.95	1.926
Day 2	1.876	1.868	1.924	1.914
Day 3	1.765	1.737	1.756	1.807
Day 4	1.752	1.726	1.736	1.765

Table 7. Variation in pH and optical density as a result of clodinafop herbicide degradation by bacteria B3

Time period	pH			
	5.0	6.0	7.0	8.0
Day 1	1.968	1.918	1.799	2.008
Day 2	1.869	1.903	1.968	1.952
Day 3	1.683	1.729	1.732	1.768
Day 4	1.488	1.532	1.582	1.624

Table 8. Variation in pH and optical density as a result of MCPA & Bromoxynil herbicide degradation by bacteria C1

Time period	pH			
	5.0	6.0	7.0	8.0
Day 1	0.813	1.006	0.976	1.124
Day 2	0.573	1.756	1.784	1.825
Day 3	0.484	1.785	1.82	1.799
Day 4	0.367	1.483	1.667	1.533

Table 9. Variation in temperature and optical density as a result of metribuzin herbicide degradation by bacteria A6

Time period	Temperature		
	30°C	35°C	40°C
Day 1	1.05	1.55	0.871
Day 2	1.835	1.939	1.664
Day 3	1.877	1.879	1.461
Day 4	1.613	1.747	1.271

Table 10. Variation in temperature and optical density as a result of clodinafop herbicide degradation by bacteria B3

Time period	Temperature		
	30°C	35°C	40°C
Day 1	0.739	1.676	0.693
Day 2	1.221	1.957	1.348
Day 3	1.67	2.068	1.426
Day 4	1.528	1.854	1.232

observed at the end of day 1 and day 2 at pH 7.0–8.0 and minimum at pH 5.0. There was more biotic degradation in alkaline ranges of pH than in acidic.

Effect of Temperatures

The biodegradation of herbicides at different temperatures (30°C, 35°C and 40°C) was determined and the results are summarized in (Tables 9, 10, 11, and Figs 9, 10, 11). The results depicted that the optimized temperature for bacterial growth was observed at a temperature range of 35–37°C. The metribuzin degrading bacterium A6 showed maximum growth on day 2nd at 35°C and 40°C, while at 30°C maximum growth was recorded on the 3rd day. On day 4th a slight decline in bacterial growth rate was noticed at all temperatures. Almost similar trends were observed for clodinafop degrading

Table 11. Variation in temperature and optical density as a result of MCPA & Bromoxynil herbicide degradation by bacteria C1

Time period	Temperature		
	30°C	35°C	40°C
Day 1	0.523	0.819	0.386
Day 2	0.978	1.085	0.767
Day 3	1.382	1.516	1.258
Day 4	1.23	1.372	0.963

Table 12. Tests of between-subjects effects

Dependent variable: bacterial growth

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Herb	2.244	2	1.122	460.575	.000
Conc	.086	3	.029	11.824	.000
Time	22.610	3	7.537	3.094E3	.000
Herb* Conc	.054	6	.009	3.718	.002
Herb* Time	3.522	6	.587	240.957	.000
Conc* Time	.261	9	.029	11.917	.000
Herb* Conc* Time	.607	18	.034	13.832	.000**
Error	.234	96	.002		
Total	298.956	144			
Corrected Total	29.618	143			

**Three-way interaction is significant at 0.05 level of significance

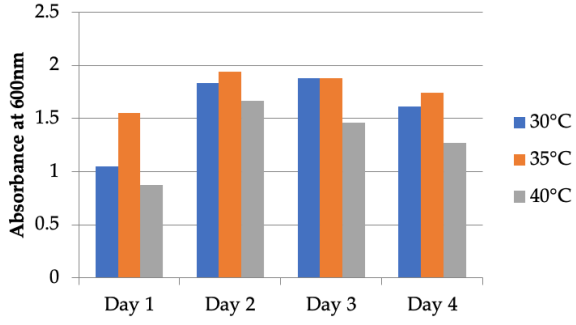

Figure 9. Growth rates of Metribuzin degrading bacteria at varying temperatures

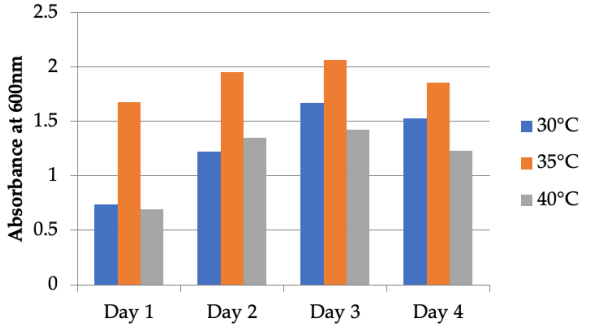

Figure 10. Growth rates of Clodinafop degrading bacteria at varying temperatures

bacterium B3 and Bromoxynil + MCPA degrading bacterium C1, with the maximum optical densities recorded

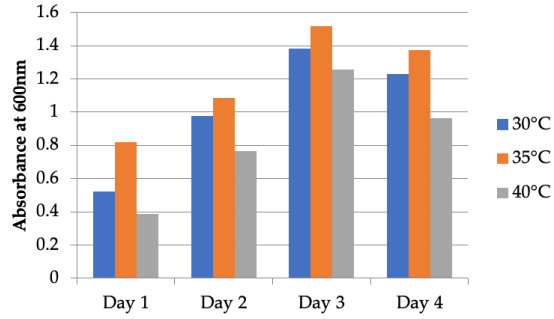

Figure 11. Growth rates of MCPA & Bromoxynil degrading bacteria at varying temperatures

at 35°C on 3rd day following a decline in growth on 4th day.

Statistical Analysis of the Effect of Different Parameters on Bacterial Growth

A three-way ANOVA with α=0.05 (level of significance) was used to determine the statistically significant effect of herbicides, concentrations, and time intervals on bacterial growth. Table 12 illustrates that there is a significant effect of the three-way interaction of herbicides, concentration, and time on bacterial growth at $p<0.05$. Similarly, the effect of herbicides, pH, and time intervals on the bacterial growth also showed significant interaction at a 95% significance level i.e. $p<0.05$ as shown in Table 13. The three-way interaction of herbicides, temperature, and time intervals on bacterial growth was also statically significant at $p<0.05$ (Table 14).

Table 13. Test of between-subject effects on three-way analysis of variance

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Herb	8.731	2	4.366	1.077E3	.000
pH	3.630	3	1.210	298.548	.000
Time	.980	3	.327	80.555	.000
Herb* pH	6.219	6	1.036	255.708	.000
Herb* Time	2.193	6	.365	90.175	.000
pH* Time	.891	9	.099	24.433	.000
Herb* pH* Time	1.340	18	.074	18.366	.000**
Error	.389	96	.004		
Total	408.869	144			
Corrected Total	24.477	143			

**Three-way interaction is significant at 0.05 level of significance

Table 14. Tests of between-subjects effects by three-way analysis of variance

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	20.124[a]	35	.575	421.673	.000
Intercept	195.606	1	195.606	1.435E5	.000
Herb	5.843	2	2.921	2.143E3	.000
Temp	4.782	2	2.391	1.753E3	.000
Time	7.105	3	2.368	1.737E3	.000
Herb* Temp	.801	4	.200	146.823	.000
Herb* Time	.812	6	.135	99.236	.000
Temp* Time	.558	6	.093	68.262	.000
Herb* Temp* Time	.223	12	.019	13.644	.000**
Error	.098	72	.001		
Total	215.828	108			
Corrected Total	20.222	107			

**Three-way interaction is significant at 0.05 level of significance

DISCUSSION

The use of chemical pesticides to reduce the pest attack on crops and to improve the yield has become routine in recent times. These hazardous chemicals are responsible for soil and groundwater contamination as they have a long persistence time and can percolate through the soil to the groundwater table and pose a serious threat to human health. There is an increasing need to identify different bioremediation approaches to degrade and detoxify these toxic pesticides. Several studies have demonstrated the use of bacteria, which can effectively degrade herbicides (Huang *et al.,* 2018). Because of its effectiveness and low cost, bioremediation appeared as the most favorable alternative treatment for the removal of agrochemicals (Arora, 2018).

In the current study, three herbicides (metribuzin, clodinafop propargyl, 2-methyl, 4-chlorophenoxyacetic acid and Bromoxynil) degrading bacterial strains were isolated and characterized. All the herbicide-degrading bacterial isolates were rod=shaped and belonged to the *Bacillus* genera. In a previous study conducted by Sehrawat and others (Sehrawat *et al.,* 2021), it was examined that pesticides in the environment are mostly mineralized and detoxified by bacterial genera like "*Bacillus* and *Pseudomonas*" because these bacteria possess a variety of enzymes that are helpful in the degradation of toxic compounds.

Out of fourteen bacterial isolates, six were able to metabolize metribuzin. The bacterial isolate A6 was further used to study the metabolism of metribuzin herbicide and results were confirmed by GC-MS analysis. Bacterial isolate metabolized the metribuzin into the deaminated metribuzin. The results showed that the bacteria adopted the deamination pathway for sensor metribuzin degradation. The metribuzin herbicide was broken down into degradative products by the release of an amino group from the parental herbicide compound and converting it into a less toxic product i.e., deaminometribuzin. The bacterial strain A6 degraded 14.8% metribuzin out of the provided concentration of 50 ppm. In a similar study Wahla and others (Wahla *et al*., 2019) investigated the biodegradation of two pesticides metribuzin and profenofos by three bacterial isolates "*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*" and determined the effect of pH, temperature, and various carbon and nitrogen sources on degradation. The strains exhibited excellent growth at pH 6, 30°C in minimal salt broth amended with 25 mg/L pesticides containing dextrose as carbon and malt extract as nitrogen source. *Bacillus subtilis* was found active in degrading the pesticides after that *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

In this study, three Bacillus sp. were found efficient in the metabolism of clodinafop propargyl and the bacterium B3 was further selected for biodegradation study through GC-MS analysis. The bacterial strain B3 degraded 23.2% clodinafop propargyl out of the provided concentration of 50 ppm. The isolate B3 degraded the clodinafop into corresponding acids and adopted a dehalogenation pathway for the breakdown of herbicide. The bacterium released the chloride ions from the compound, due to which the pyridyl ring underwent further degradation and broken down into the corresponding dicarboxylic acids which is an allosteric activator of acetyl co-enzyme-A decarboxylase (Singh *et al.,* 2013). *Aeromonas* sp. isolated from the crop field degraded 81.3% of clodinafop propargyl with a concentration of 80 ppm as the bacteria consumed it as a sole carbon and nitrogen source (Dong *et al.,* 2017). The formation of 4-(4-chloro-2-fluorophenoxy) phenol as a main degradative metabolite was reported. In another study conducted by Singh and others (Singh *et al*., 2013) the degradation efficiency of clodinafop by *Pseudomonas* sp. was determined which consumed about 87.14% of herbicide out of the initial concentration of 80 ppm. The breakdown of clodinafop propargyl released chloride ion and confirmed the breakdown of CF into 4-(4-chloro-2-fluorophenoxy) phenol and clodinafop acid. The production of phenol as the metabolic product reveals the presence of esterase activity (Yuan *et al.,* 2015).

Were found to metabolize the MCPA & Bromoxynil. The strain C1 was efficient in degrading MCPA through the decarboxylation mechanism and forming 2-methyl phenol as a major degradative metabolite. (Mierzejewska *et al*., 2016) reported three bacterial strains *Xanthomonas maltophilia*, *Pseudomonas* sp., and *Rhodococcus globerulus* that degraded 99% MCPA in non-contaminated soil and up to 61% in the contaminated soil by *Xanthomonas maltophilia.*

CONCLUSIONS

Bioremediation has become a popular environment-friendly approach for the decontamination of a heavy buildup of toxic recalcitrant compounds from the environment. Microbial degradation plays a pivotal role to endorse a clean and sustainable environment. In this study three bacterial isolates namely, A6, B3, and C1 were found efficient for degrading metribuzin, clodinafop, and Bromoxynil & MCPA herbicides respectively, and their respective degradation capacities recorded as 14.8% for metribuzin, 23.2% for clodinafop and 33.9% MCPA & Bromoxynil. The optimum temperature and pH for the biodegradation of herbicides was 35°C and pH ranging from 7.0–8.0. These bacterial strains were found efficient in the metabolism of herbicides and can be utilized for the bioremediation of polluted sites in the future.

Declarations

Supplementary Materials. The following supporting information can be downloaded: Table S1: Morphological characterization of herbicides degrading bacteria at https://ojs.ptbioch.edu.pl/index.php/abp/

Ethical approval. This article does not contain any studies with animals performed by any of the authors**.**

Data availability. All major data generated and analyzed in this study are included in this manuscript and its supplementary information files.

Conflict of interest. The authors declare no conflict of interest.

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