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Biodegradation and Kinetic Study of Hazardous Metribuzin Herbicide Using a Novel Soil Bacterial Isolate *Olivibacter oleidegradans* Strain SP01 in Aqueous Solution

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ABSTRACT

In the present work, degradation of the herbicide metribuzin ($C_8H_{14}N_4OS$) has been performed. A novel metribuzin-degrading bacterium, *Olivibacter oleidegradans* strain SP01, was isolated from the metribuzin-contaminated soil by an enrichment technique. To investigate the effect of various parameters on metribuzin degradation, various experiments were performed at an initial concentration in the range of 20-100 $mg.L^{-1}$, a pH of 5-9, and a temperature of 25-40°C. Around 85% of the highest percentage degradation of metribuzin was obtained at a concentration of 20 $mg.L^{-1}$ in 120 h under optimized conditions. The current work for the Metribuzin degradation study fits well with first-order reactions. Also, at higher concentrations, i.e., 100 $mg.L^{-1}$, only 40.3% degradation of metribuzin was observed. The Olivibacter oleidegradans strain SP01 has the potential to be extremely beneficial in the removal of Metribuzin from the environment.

INTRODUCTION

Pesticides are used to kill or prevent the growth of pests that cause damage to crops, shrubs, trees, timber, and other desirable vegetation. Pesticides are classified into various groups based on their application, such as insecticides, herbicides, fungicides, nematicides, rodenticides, micro biocides, and so on. Worldwide, various pesticides of different chemical formulas are widely used for both agricultural and non-agricultural practices. Currently, in Asia, India is the leading pesticide manufacturing and utilizing country and has acquired the 12th rank in the global market (Mathur 1999). India is the world's second-most populous country, and its economy is mainly based on agriculture. Hence, a large number of pesticides are used to increase agricultural yield and meet the food demand of the population. Pesticides enter the environment through different routes: (i) agricultural water runoff; (ii) effluent discharge from pesticide manufacturing industries; (iii) formulating industries; and (iv) chemical spills (Sakkas et al. 2005). Once it enters the water stream, it causes severe threats to human beings and the environment.

Metribuzin is a herbicide of the triazinone class. It inhibits the process of photosynthesis in various broadleaf weeds and grasses (Arsenault & Ivany 2001). Hence, it is widely applied as a pre and post-emergence herbicide in the fields of crops such as sugarcane, tomato, potato, and maize. Furthermore, it is lethal to aquatic plants and has long-term effects on aquatic organisms (Fairchild & Sappington 2002). Inhalation of metribuzin by mammals affects the central nervous system, kidney, thyroid, and liver. The half-life of metribuzin is recorded as between 5 and 50 days (Quesada-Molina et al. 2007). Its water solubility is recorded as 1.05 $g.L^{-1}$ (Yahiaoui et al. 2011). Metribuzin's liquid solubility and weak sorption to soil cause surface and groundwater pollution by its residue. Many researchers have reported on surface and groundwater contamination by metribuzin. Metribuzin also disrupts the endocrine system in higher animals and leads to physiological disorders (Maumbe & Swinton 2003). Due to this, many countries have incorporated metribuzin into their list of endocrine disruptors. Also, metribuzin residue affects the establishment of subsequent crops and disrupts the crop rotation system (Huang et al. 2018). Apart from this, metribuzin residue also affects soil microflora and its activities (Lone et al. 2014). Hence, to tackle environmental pollution by metribuzin, it attracts attention to the need to develop effective eco-friendly bioremediation strategies at point sources to protect water bodies and the environment. So far, different remediation strategies have been used to clean up pesticide pollution (Arora et al. 2012). Microorganisms can convert toxic pollutants into less toxic or inactive compounds (Abatenh et al. 2017). As of today, only a few

bioremediation techniques have been reported so far by various authors in the literature for metribuzin removal (Tamilselvan et al. 2014, Myresiotis et al. 2012). Zhang et al. (2014) have reported about 73.5% degradation of metribuzin using *Bacillus* sp. (N1) at an initial concentration of 20 $mg.L^{-1}$ in 5 days, at optimum pH 7 and temperature of 30°C. Furthermore, (Gopal et al. 2011) reported about 86% of metribuzin degradation by using *the Burkholderia cepacia* strain within 20 days. Apart from this, Wahla et al. (2018) observed about 98.63% of metribuzin degradation at an initial concentration of 45 $mg.L^{-1}$, optimum pH 7, and temperature of 30 °C with an inoculum density of 5 x 10^5 $CFU.mL^{-1}$ using bacterial consortia such as *Rhodococcus rhodochrous, Bacillus aryabhattai, Bacillus tequilensis*, and *Bacillus safensis*. Also, Tamilselvan et al. (2014) studied metribuzin degradation by bacterial isolates such as *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis,* etc.

For successful bioremediation of metribuzin, efficient inocula, optimization of media components and various environmental factors such as incubation temperature, pH, pesticide concentrations and inoculum size plays a major role (Varjani & Upasani 2017, Annadurai et al. 2008, Jabeen et al. 2015). Hence, the optimization of various parameters for metribuzin degradation is carried out in the present work by using a novel soil isolate. Also, it is evident from the previous literature that an isolated bacterial strain has not been studied earlier for metribuzin degradation. Therefore, enrichment methods have been used for the isolation of bacterial strains from the metribuzin-contaminated soil. A novel bacterial strain is effective and efficient for the removal of metribuzin and showed good removal of metribuzin.

MATERIALS AND METHODS

Materials

Commercial grade metribuzin having an MW of 214.2 $g.mol^{-1}$ (88.55% w/w) was purchased from a local shop, Bhavani Krushi Seva Kendra, Hingangaon Bk., Sangli, Maharashtra, India. All other required chemicals and Agar-agar were purchased from Hi-Media Laboratories Pvt.Ltd., Mumbai. Also, the AR-grade sodium hydroxide (NaOH) and sulfuric acid (HCL) chemicals were obtained from S. D. Fine Chemicals Ltd., Mumbai, India.

Enrichment and Isolation of Metribuzin Degrading Bacteria

For the enrichment, soil samples were collected from the sugarcane vegetated agricultural field at Hingangaon Bk, Sangli, Maharashtra, India, where a large amount of metribuzin was used repeatedly. The sampling farm is situated at a latitude of 17.3914°N, a longitude of 74.3419°E, and an altitude of 669 meters above sea level. At the time of sampling, the sugarcane crop was 4 months old. Soil samples were collected at random depths ranging from 0 to 15 cm below the soil surface. The soil samples were collected in the sterilized polythene bag and immediately stored and maintained at 4 °C temperature (Mishra 2015). In the laboratory, the soil samples were sieved through 2 mm sieves to remove gravel and debris. 1 gm. of soil was mixed into 250 mL Erlenmeyer flasks (A) containing 100 mL of Minimal Salt Medium (MSM) with a metribuzin concentration of 100 $mg.L^{-1}$ and incubated for the next 12 days at 150 rpm. 1 mL of sample from the incubated flask (A) was transferred into a 250-mL Erlenmeyer flask (B) containing fresh 100 mL MSM broth with a 100 $mg.L^{-1}$ concentration of metribuzin and incubated for 72 hours at 150 rpm. 0.1 mL of the sample from the flask (B) was spread on MSM agar plates having a 100 $mg.L^{-1}$ concentration of metribuzin and incubated at 30 °C temperature for 48-72 hrs. After 48-72 h of incubation, morphologically distinct colonies were picked up and restreaks on MSM agar plates until purified colonies were obtained. Ten pure cultures were isolated, grown on nutrient agar slant, and stored in the refrigerators at 4°C until required in subsequent experiments.

Identification of the Bacterial Isolates

For the identification of the novel metribuzin-degrading bacterial isolates, 16S rRNA gene sequence analysis was conducted at a professional molecular biology laboratory (Geneom Biotech Pvt. Ltd., Pune, India), and the universal primers 8F (5' AGAGTTTGATCCTGGCTCAG-3'), 806R (5' GGACTACHVGGGTWTCTAAT-3'), 515F (5' CCATCTCATCCCTGCGTGTCTC-3'), and 13B (5' AGGCCCGGGAACGTATTCAC-3'), were used for the amplification of the 16S rRNA gene (Turner et al. 1999). The gene sequence was deposited in the NCBI GenBank with an accession number of OM527185.1. The blast analysis of nucleotide sequences was performed at the National Center for Biotechnology Information (NCBI) for phylogenic analysis. The 16 S r RNA sequence of the strain Olivibacter shared 99% identity with *Olivibacter oleidegradans* and *Olivibacter jilunii* (Fig 1). A phylogenetic tree has been constructed by using the neighbor-joining technique by MEGA6 software.

Culture Maintenance and Media

The isolated strain was maintained on Minimal Salt Medium (MSM) Agar having a composition of- Minimal Salt Medium containing: 5 g glucose, 3 g K_2HPO_4, 6 g Na_2HPO_4, 0.1 g

$MgSO_4.7\ H_2O$, 2 g NH_4Cl, and 5 g NaCl dissolved in 1 L of DW. A metribuzin biodegradation experiment was performed in MSM broth**.**

Experimental Methodology

Metribuzin Degradation Capacity of the Isolated Strain

To check the degradation capacity of an isolated strain, experiments were performed in the laboratory. A 24-hour old grown culture of *Olivibacter oleidegradans* strain SP01 was added to 100 mL of MSM medium in a 500 mL flask with metribuzin as the sole carbon and energy source and incubated at 30°C., at 150 rpm in a rotary shaker. The uninoculated flask is considered as a control. The experiments lasted 5 days, and samples were collected every 24 hours and centrifuged for 15 minutes at 10000 rpm at 4°C temperature, with the supernatant filtered through a 0.45 mm membrane filter to remove any other particles. The supernatant was analyzed for the residual concentration of metribuzin using a UV-visible spectrophotometer at 293 nm. All the experiments were performed in triplicate. To test the effect of metribuzin concentration, a concentration of (20-100 $mg.L^{-1}$) and an operating pH of (5-9) were maintained using HCl and NaOH. Further, the effect of temperature on metribuzin degradation was studied in the range of 25-40°C) respectively.

Analytical Methods

In the present study, to obtain cell-free medium, a sample was centrifuged at 10,000 rpm for 15 min., and the supernatant was filtered using a membrane filter (0.45 mm) to remove any other particles (Anwar et al. 2009). The absorption was measured using a UV-visible spectrophotometer at 293 nm. The concentration of Metribuzin was calculated by analyzing the absorbance of the metribuzin solution.

RESULTS AND DISCUSSION

Isolation and Screening of Metribuzin Degrading Bacteria

Initially, the isolation of metribuzin-degrading bacteria was carried out by the enrichment method, and morphologically different ten pure cultures were isolated by streak plate methods. To check the metribuzin resistance capacity of these isolates, pure cultures were streaked on MSM agar plates having various metribuzin concentrations of 20, 40, 60, 100, 200, 250, 300, 400, and 500 $mg.L^{-1}$. The resistance capacity of isolates was studied using MSM agar plates with metribuzin as the sole 'C' and energy source. Furthermore, for the identification of metribuzin-degrading bacteria, isolated strains were cultured individually in MSM broth with a 20 $mg.L^{-1}$ concentration of metribuzin as the sole 'C' and energy source, and after one week of incubation, the residual concentration of the metribuzin was measured and the degradation percentage was calculated. The isolate (B2), later identified as *Olivibacter oleidegradans* strain SP01, tolerated metribuzin up to 400 ppm and had a higher efficiency of metribuzin degradation than all other isolates, so it was chosen for further study.

Microorganism used, Molecular Sequence and Evolutionary Studies

The degrading bacteria is isolated from a metribuzin-contaminated farm field using an enrichment technique. The identification of the isolates was done using 16S rRNA gene sequencing. The identified nucleotide sequence is very close to that of the Olivibacter genus; hence, the strain was designated as *Olivibacter oleidegradans* strain SP01 sp.

The sequence of Olivibacter was submitted to the NCBI GeneBank, and they have given it the accession number OM 527185.1. Fig. 1 shows the phylogenetic relationship of Olivibacter sp. with species of the identical genus of the GenBank database. The accession numbers of other species are presented in parentheses**.** In the phylogenetic branch, the homology relationship results showed that Olivibacter sp. has a maximum of 99% similarity with other species such as the *Olivibacter oleidegradans* strain and the *Olivibacter jilunii* strain. Many authors have reported the functions of Olivibacter. Szabo et al. (2011) reported the oil (hydrocarbon) degradative properties of the *Olivibacter oleidegradans* strain. Also, Chen et al. (2013) isolated *Olivibacter jilunii* from DDT-contaminated soil. However, to our knowledge, no study has been performed to date on the degradation of metribuzin using the Olivibacter strain.

Pesticide Degradation Kinetics

Bacteria utilize pesticides as the sole carbon and energy source for their growth and hence degradation of pesticide occurs. The rate constant for the degradation process was calculated by using the following equation.

$$\ln\left[\frac{C_0}{C}\right] = k't \qquad \ldots(1)$$

Where C represents the concentration of pesticide in $mol.L^{-1}$ and is the initial concentration. k' represents a rate constant (hr^{-1}) and degradation time is denoted as t. As shown in Fig. 2, the plot of ln v/s time (t) is a straight line passing through the origin. This confirms that the degradation of metribuzin by bacteria is showing a first-order reaction kinetics.

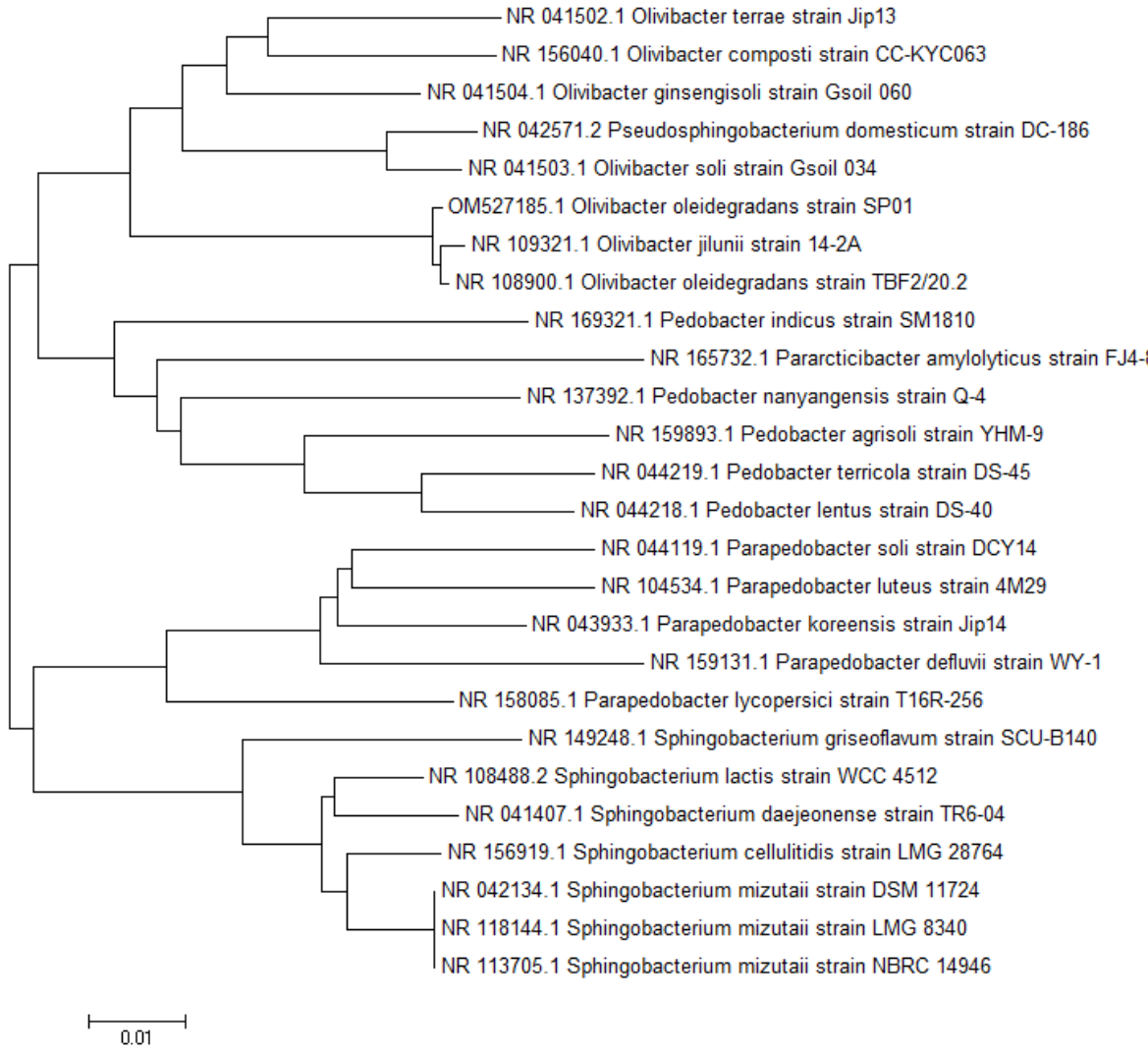

Fig.1: Phylogenetic tree constructed by using neighbor-joining analysis of 16S rRNA sequences from 25 bacteria. GeneBank accession numbers are given with species names.

Effect of Initial Concentration

To study the effect of various concentrations, experiments were performed at initial concentrations such as 20, 40, 60, and 100 mg.L^{-1} at constant pH 7, inoculation of 4%, and a temperature of 30°C. Table 1 shows that metribuzin degradation decreased from 80.38% to 40.3% with an increase in the concentration of Metribuzin from 20 mg.L^{-1} to 100 mg.L^{-1}. Furthermore, the rate constants were observed in decreasing order from 14.1 10^{-3} hr^{-1} to 4.10 10^{-3} hr^{-1}.

A maximum metribuzin degradation rate of 80.38% was observed at the 20 mg.L^{-1} concentration. Fig. 2 shows that at lower metribuzin concentrations, i.e., 20 mg.L^{-1}, the metribuzin degradation rate was higher. Toxicity to bacteria occurs at higher concentrations, which may affect the organism's growth. Hence, at higher concentrations, very little metribuzin degradation was observed. According to the literature, similar findings were made for the organic

Table 1: Metribuzin degradation and kinetic rate constant with respect to various concentrations. (pH-7, Temp. 30°C, Inoculations 4%, capacity-100 mL).

Concentration (mg.L^{-1})	Degradation (%)	Rate constant (k'×10^3 hr^{-1})	R^2
20	80.38	14.1	0.98
40	68.26	9.20	0.98
60	64.16	7.80	0.97
100	40.3	4.10	0.98

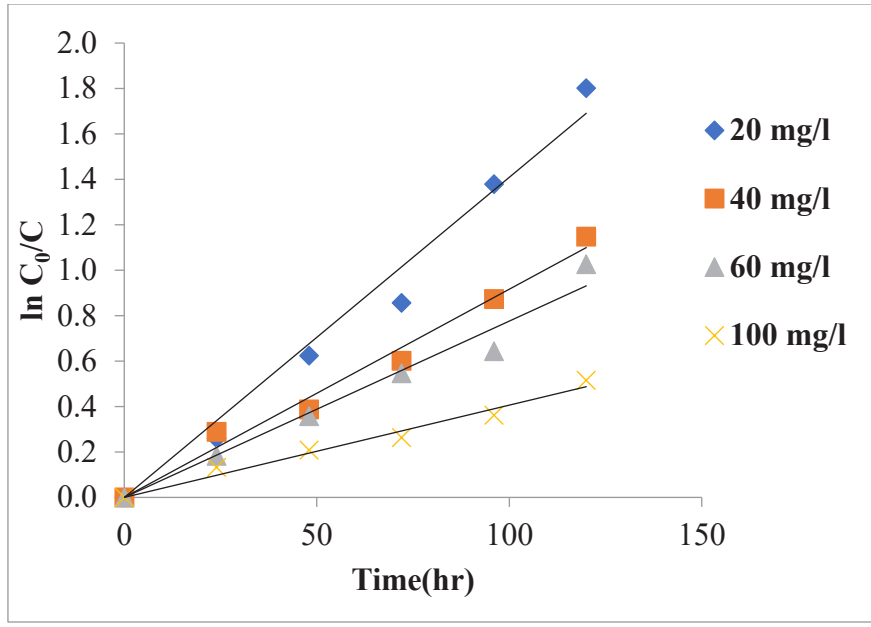

Fig. 2: Effect of various concentrations on metribuzin degradation (pH -7, Temp. 30°C, Inoculations 4%, capacity-100 mL).

pollutants degradation study. Zhang et al. (2014) have stated that the biodegradation rate of metribuzin decreased with an increase in its concentration when Bacillus sp. was used. Also, Wahla et al. (2018) have reported the highest degradation of metribuzin at lower concentrations by using a bacterial consortium.

Effect of Initial pH

Several experiments were carried out at pH ranges of (5, 6, 7, 8, & 9) to investigate the effect of pH on Metribuzin degradation. The obtained results were represented in Fig. 3 It shows that maximum degradation rates of 83.38 % were observed at pH 7, i.e., at neutral pH. It is concluded from Table 2 that at acidic conditions, i.e., at pH 5, and basic conditions, i.e., at pH 8 and 9, the decreased rate constant was observed.

The obtained results indicate that bacteria are adaptable to neutral pH and that pH has a direct impact on bacterial growth. Another reason is that pH also affects the metabolic activities of bacteria, and hence, maximum degradation was observed at an optimum neutral pH. Zhang et al. (2014) observed maximum degradation of metribuzin at pH 7 by using *Bacillus* sp. In addition, Phugare & Jadhav (2014) reported a similar pH trend for the biodegradation study of Acetamiprid using a Rhodococcus sp.

Effect of Temperature

In the present study, to examine the effect of temperature on metribuzin degradation, various experiments were performed at temperatures in the range of 25 to 40°C. The percent degradation with respect to its temperature has been represented in Table 3. It can be concluded from Fig. 4 that maximum degradation of 84.46% was observed at 35°C. The observed percent degradation at 25°C and 45°C was 42% and 64%, respectively. It can be concluded that temperature plays an important role in bacterial degradation activity.

Table 2: Percentage of degradation and kinetic rate constant with respect to various pH (concentration of 20 mg.L^{-1}, Temp. 30°C, Inoculations 4%, capacity-100 mL).

pH	Degradation (%)	Rate constant (k' $10^3 hr^{-1}$)	R^2
5	31	3.30	0.98
6	58	7.50	1
7	80.38	14.1	0.98
8	33	3.60	0.99
9	23	2.20	0.98

Table 3: Percentage of degradation and kinetic rate constant at various Temperatures. (concentration of 20 mg.L^{-1}, pH-7, Inoculations 4%, capacity-100 mL).

Temperatures (°C)	Degradation (%)	Rate constant (k' 10^3 hr^{-1})	R^2
25	42	4.80	0.99
30	80.38	14.1	0.98
35	84.46	14.3	0.98
40	64	8.50	1

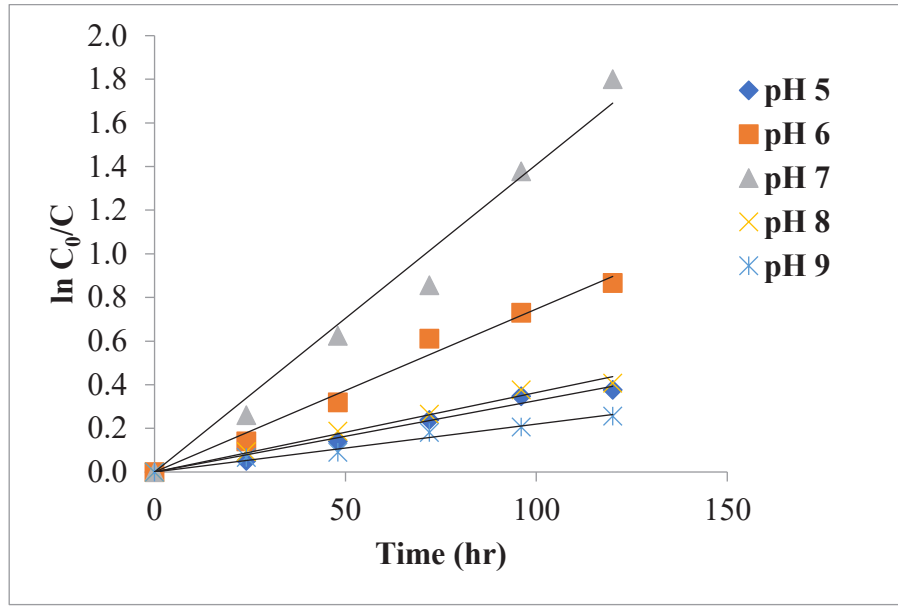

Fig. 3: Metribuzin degradation with respect to various pH (concentration 20 mg.L^{-1}, Temp. 30°C, Inoculations 4%, capacity-100 mL).

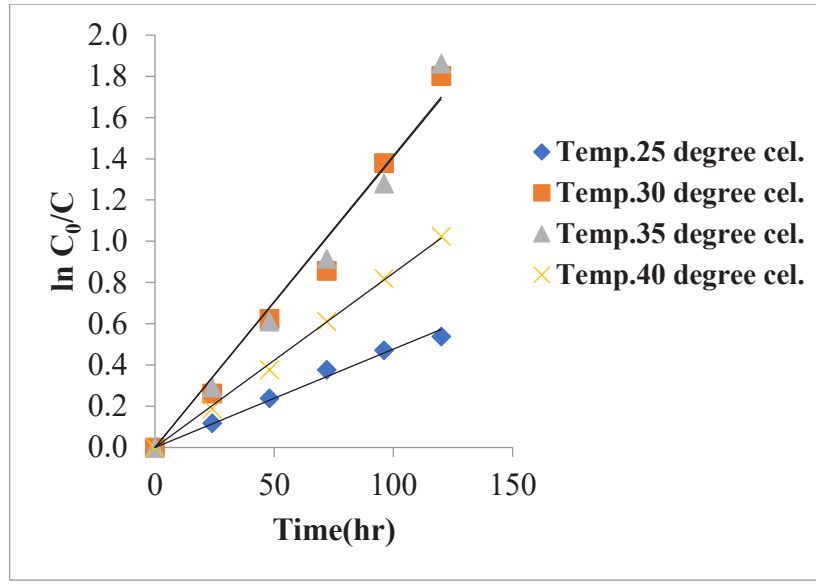

Fig. 4: Metribuzin degradation with respect to various temperatures (initial concentration 20 mg.L^{-1}, pH 7, Inoculations 4%, capacity-100 mL).

Higher or lower temperatures may affect the metabolic and enzymatic activity of the bacteria, resulting in significantly less degradation when compared to the optimum temperature of 35°C. Also, lower or extreme temperatures inhibit the growth rate of the bacteria, and hence, the degradation rate was observed to be lower than the optimum temperature. Also, Wahla et al. (2018) reported the highest degradation of metribuzin at 35°C temperature by using bacterial consortium. Furthermore, Phugare & Jadhav (2014) also reported a similar trend of the temperature for the biodegradation study of Acetamiprid by using a Rhodococcus sp.

CONCLUSION

Metribuzin biodegradation was accomplished in the current study under a variety of operating conditions, including initial concentration, pH, and temperature. Lower concentration favors the degradation of metribuzin. The maximum

degradation of metribuzin was observed at a neutral pH of 7 and a temperature of 35°C. Metribuzin-degrading *Olivibacter oleidegradans* strain SP01 is successfully identified in the present study. *Olivibacter oleidegradans* strain SP01 is highly efficient for the removal of metribuzin and can be used for the bioremediation of this herbicide. In addition, the kinetic parameters of metribuzin degradation were also studied. A 1st order fitted well for the biodegradation study of metribuzin by using *Olivibacter oleidegradans* strain SP01.

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